MOLECULAR MECHANISMS UNDERLYING FAST-ACTING ANTIDEPRESSANT EFFICACY

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Dedicated to my mom and dad,
Linda and George Szabla,
And to all of my family and friends
For their unconditional love and support

EXPLORING MECHANISMS OF FAST-ACTING ANTIDEPRESSANT ACTION

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EXPLORING MECHANISMS OF FAST-ACTING ANTIDEPRESSANT ACTION

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Major Depressive Disorder is a devastating mental illness with a profound disease burden, particularly in the United States. Major Depressive Disorder is a heterogeneous disorder that is characterized by dysregulated mood and/or anhedonia with intense feelings of despair and sadness, agitation, self-deprecation, and suicidal ideation. Antidepressants, such as selective serotonin reuptake inhibitors, are the most common form of treatment for Major Depressive Disorder, however the precise mechanism by which these drugs work is largely unknown. Moreover, the time they take to reach clinical effect can take weeks to months, and some patients never truly respond, leaving a critical need for more rapidly acting antidepressants with sustained efficacy. In the laboratory, we have explored aspects of the neurotrophic hypothesis of

depression and have made progress toward understanding the role of brain-derived neurotrophic factor in animal models. We have also made progress in understanding the role of brain-derived neurotrophic factor in cellular and molecular mechanisms which underlie fast-acting antidepressant efficacy. First, we examined whether ketamine, a novel fast acting antidepressant, functioned in a dose dependent manner to elicit its antidepressant effects. We found that only low, nonpsychomimetic doses of ketamine produce antidepressant effects, whereas high, psychomimetic doses did not produce antidepressant responses. We also demonstrated that only low dose ketamine triggered robust increases in BDNF translation, which our lab has previously shown to be required for ketamine's fast acting antidepressant effects. Next we examined the role of calcineurin in relation to our model of ketamine action, and we uncovered a parallel L-type calcium channel mediated calcium signaling pathway that dephosphorylates eukaryotic elongation factor 2 and competes with the previously identified *n*-methyl-*d*-aspartate receptor dependent signaling that activates eukaryotic elongation factor 2 kinase function. The balance between these two calcium signaling pathways determines the degree of eukaryotic elongation factor 2 phosphorylation and the extent of BDNF protein translation, which in turn gauges the efficacy of ketamine-mediated rapid antidepressant responses in preclinical mouse models. Finally, we investigated the molecular mechanisms underlying scopolamine's fast acting antidepressant effect and discovered that scopolamine mediated antidepressant effects require brain-derived neurotrophic factor transcription.

TABLE OF CONTENTS

Dedication……ii
Acknowledgements……..v
Abstract……...vi
Table of contents……viii
Prior publications……..x
List of Figures…….....xi
List of abbreviations …….xiii

Chapter 1: Introduction……1
BDNF synthesis……1
BDNF signaling……3
The impact of BDNF on synaptic plasticity……5
BDNF and Major Depressive Disorder ……...8
BDNF and the antidepressant response…….....13
BDNF and fast acting antidepressant efficacy……...15
eEF2 kinase and ketamine action……...16
Conclusion…….....20

Chapter 2: Dose dependent divergence of ketamine-elicited neuronal signaling and fast-acting antidepressant efficacy……24
Introduction…….....24
Methods……...26
Results…….....30
Discussion……...35

Chapter 3: L-type calcium channel-mediated activation of calcineurin is required for ketamine's rapid antidepressant effects……46
Introduction ……...46
Methods……...48
Results…….53
Calcineurin dephosphorylates eEF2 in neurons……..53
Calcineurin is required for ketamine's rapid antidepressant response……....54
L-type calcium channels are the primary source of calcium that drives calcineurin activity……..56
L-type channels impact ketamine's rapid antidepressant response…….69
Discussion……....61

Chapter 4: The molecular mechanisms underlying the fast acting antidepressant effect of scopolamine……74
Introduction……74
Methods……...76
Results……82
Scopolamine produces a rapid antidepressant effect in mice ……..82
Scopolamine significantly increases phosphorylated S421 MeCP2……..82

M1 selective AchR antagonists increase phosphorylation of S421 MeCP2…..83
Inhibition of calcineurin triggers scopolamine mediated increases in phosphorylated MeCP2……...85
Discussion……...87

Chapter 5: Conclusions and future directions……..100

References…….109

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LIST OF FIGURES

Figure 1-1. An overview of BDNF signaling through TrkB receptors……22

Figure 1-2. Synaptic mechanism underlying ketamine's action……...23

Figure 2-1. Ketamine's effect on locomotor activity and PPI behavior is dose dependent…...39

Figure 2-2. Ketamine's impact on acute and long-lasting antidepressant responses is dose dependent……..41

Figure 2-3. Ketamine induces acute changes in protein expression in a dose dependent manner 30 minutes after administration……....42

Figure 2-4. Ketamine dosage does not diferentially impact protein expression at 2 hours.…...44

Figure 3-1. Inhibition by calcineurin blocks ketamine-mediated reductions in p-eEF2 levels..64

Figure 3-2. Inhibition of calcineurin blocks ketamine-mediated antidepressant responses and ketamine-mediated changes in p-eEF2 and BDNF protein *in vivo*…….....66

Figure 3-3. BAPTA but not EGTA blocks ketamine-mediated decreases in p-eEF2 levels..…68

Figure 3-4. Inhibition of L-type Ca^{2+} channels blocks ketamine-mediated antidepressant behavior and ketamine-mediated changes in p-eEF2 and BDNF levels in vivo….70

Figure S3-1. FK506 and nimodipine do not alter locomotor behavior……72

Figure S3-2. Model……...73

Figure 4-1 Scopolamine produces an antidepressant effect in mice……...92

Figure 4-2. Scopolamine produces an increase in phosphorylated S421 MeCP2…….93

Figure 4-3. The effect of CaMKII inhibition on scopolamine mediated increased in pS421 MeCP2…….94

Figure 4-4. M1 muscarinic acetylcholine receptor blockade increases pS421 MeCP2 levels and inhibition of calcineurin activity triggers scopoloamine-mediated increases in

pS421 MeCP2…….95

Figure S4-1. Scopolamine does not alter levels of pMeCP2 in the prefrontal cortex or the nucleus accumbens……..…..97

Figure S4-2. Inhibition ofM2, M3, andM4 acetylcholine receptor subtypes do not alter pMeCP levels ……..98

Figure S4-3. Working Model……99

LIST OF ABBREVIATIONS

NGF – nerve growth factor

BDNF – brain-derived neurotrophic factor

NT – neurotrophin

IGF – insulin-like growth factor

FGF – fibroblast growth factor

VEGF – vascular endothelial growth factor

DNA – deoxyribonucleic acid

CREB – cyclic AMP response element binding protein

MeCP2 – methylated CpG binding protein

$P75^{NTR}$ – low affinity neurotrophin receptor

LTD – long –term depression

TrkB – tropomysin- related kinase B

LTP – long –term potentiation

Shc – Src homology 2-containing protein

PLCγ - phospholipase Cγ

RAS – Rat sarcoma

ERK – extracellular signal –regulated kinase

Grb2 – growth factor receptor-bound protein 2

SOS – son of sevenless

Gab1 – grb2- associated binding protein 1

DAG – diacylgycerol

IP3 – inositol triphosphate

CamK – calmodulin kinase

NMDA – *n*- methyl-*d*- aspartate

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

cAMP – cyclic adenosine monophosphate

mRNA – messenger ribonucleic acid

CA1 – *cornu ammonus 1*

MDD – major depressive disorder

SSRI – selective serotonin-reuptake inhibitor

SNRI – selective serotonin/norepinephrine –reuptake inhibitor

NAc – nucleus accumbens

BLA – basolateral amygdala

FST – forced swim test

LH – learned helplessness test

TST – tail suspension test

SPT – sucrose preference test

VTA – ventral tegmental area

ECT – electroconvulsive therapy

MAOI – monoamine oxidase inhibitor

AMPAkines – alpha-amino-3-hydoxy-5 –methyl-4isoxazolepropionic acid

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

eEF2 – eukaryotic elongation factor 2

CaMKII – calcium/calmodulin dependent protein kinase II

mEPSCs – miniature excitatory postsynaptic currents

mGluR – metabotropic glutamate receptor

AKT – protein kinase B

eEF2-P – phosphorylated eukaryotic elongation factor 2

eEF2K – eukaryotic elongation factor 2 kinase

CaMKIII – calcium calmodulin-dependent kinase III

mTOR – mammalian target of rapamycin

PPI – prepulse inhibition

NSF – novelty suppressed feeding

RIPA buffer – radioimmunoprecipitation assay buffer

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

HC – hippocampus

PFC – prefrontal cortex

mTORC1 – mammalian target of rapamycin complex 1

mTORC2 – mammalian target of rapamycin complex 2

KI – knock-in

veh – vehicle

ket 3 – ketamine 3mg/kg

ket 20 – ketamine 20mg/kg

ket 50 – ketamine 50mg/kg

P-eEF2 – phosphorylated eEF2

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

ARAC – cytosine arabinoside

DIV – days in vitro

PBS – phosphate buffered saline

TTX – tetrodotoxin

BCA – bicinchoninic acid

SSC – sodium sodium citrate

ANOVA – analysis of variance

PP2A – protein phosphatase 2A

OA – okadaic acid

PP1 – protein phosphatase 1

ket – ketamine

ket/TTX – ketamine/tetrodotoxin

cycA – cyclosporin A

nim – nimodipine

AF-DX116 – 11-[[2-[(Diethylamino)methyl] -1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one

4 DAMP – 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide

ASCF – artificial cerebral spinal fluid

fEPSP – field excitatory post-synaptic potential

PPR – paired pulse ratio

NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

AMPA – α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

RT-PCR – real time polymerase chain reaction

Q-PCR – quantitative polymerase chain reaction

pMECP2 – phosphorylated S421methylated CpG binding protein

VEH – vehicle

SCA – scopolamine

BDNF KO – inducible brain-derived neurotrophic factor knockout

CTL – control

ActD – actinomycin D

DG – dentate gyrus

CHAPTER I

INTRODUCTION

Neurotrophic factors are critical regulators of neuronal survival, development, function, and synaptic plasticity (Lewin and Barde, 1996). Of the neurotrophic factors, the neurotrophin family, comprising nerve growth factor (NGF) (Levi-Montalcini, 1966), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) and neurotrophin- 4 (NT-4) (Hohn et al., 1990), is the best characterized (Huang and Reichardt, 2001). Other factors, such as members of the insulin –like growth factor (IGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) also regulate growth, survival, and neuronal plasticity (Duman and Monteggia, 2006). Of these, however, BDNF has been the best described in terms of its role in adult synaptic plasticity (Kossel et al., 2001; Levine et al., 1995; Lohof et al., 1993) as well as its putative role in disease pathology or treatment of various neuropsychiatric diseases (Duman and Monteggia, 2006).

BDNF synthesis

The gene structure of neurotrophins, including BDNF, are complex, and have recently been reviewed for rodent (Aid et al., 2007; Liu et al., 2006) and human (Liu et al., 2005). In brief, the rodent *Bdnf* gene contains nine promoters, each of which precedes an exon (I-IX), that, through alternative splicing events, results in a large number of BDNF transcripts that all contain exon IX, which encodes the BDNF protein (Aid et al., 2007). Interestingly, the transcription start sites are differentially activated by neuronal activity and DNA methylation status, which consequently leads to the regulation of the transcripts. Neuronal depolarization

enhances expression of exons I, IV, V, VII, VIII, and IX, whereas gene demethylation increases the transcription of exons I, IV, V, VIII, and IX (Aid et al., 2007). Exon IV is known as the "activity –dependent" exon of BDNF, because it responds to neuronal activity, and it is a known target for activity- and calcium-sensitive transcription factors, such as cAMP response element binding protein (CREB), upstream stimulatory factors, calcium responsive transcription factors, and methylated CpG binding protein (MeCP2) (Chen et al., 2003a; Chen et al., 2003b; Martinowich et al., 2003; Tabuchi et al., 2002; Tao et al., 1998). Despite the fact that the regulation of these transcripts is important and may affect cellular localization, among other functions, how transcript regulation relates to neuropsychiatric disorders or its treatment is currently unknown.

At the protein level, BDNF is synthesized as pre-pro-BDNF in the endoplasmic reticulum, which is then converted into a pro-BDNF precursor protein by removal of the signal peptide (Lessmann et al., 2003). It has been suggested that the physiological significance of this precursor protein is that pro-BDNF, which gets folded (Heymach et al., 1996) and sorted (Lee et al., 2001) into the secretory pathway in the Golgi, is necessary for the correct folding of mature BDNF (Carvalho et al., 2008). Pro-BDNF is cleaved to generate the mature form of BDNF, but whether this cleavage occurs intracellularly or after its secretion remains controversial (Leal et al., 2014). Recent work suggests that mature BDNF is released in its precursor form (pro-BDNF) (Mizoguchi et al., 2011; Nagappan et al., 2009; Pang et al., 2004; Woo et al., 2005; Yang et al., 2014; Yang et al., 2009) and the molecule is further processed into the mature form extracellularly via the action metalloproteases and plasmin (Waterhouse and Xu, 2009). Studies performed with primary hippocampal neuron cultures using epitope tagged BDNF suggest that BDNF is mainly released in the precursor form and the formation of mature BDNF is proposed

to depend on the activity of the enzymes that cleave pro-BDNF (Nagappan et al., 2009). Electrical stimulation at different frequencies reveal that extracellular accumulation of the mature form of BDNF following high frequency stimulation depends on the co-release of tissue plasminogen activator and plasmin, which may not occur at low frequencies (Nagappan et al., 2009) These findings suggest that secreted pro-BDNF may undergo activity-dependent conversion into mature BDNF.

In contrast with the evidence described above, studies performed in adult brain tissue suggest that pro-BDNF is rapidly converted intracellularly into mature BDNF for later storage and release by excitatory input (Matsumoto et al., 2008). In agreement with this data, a recent study reports the presence of BDNF and its cleaved pro-peptide in large dense core vesicles located at presynaptic terminals of excitatory neurons in the hippocampus of the adult brain, suggesting that cleavage occurs inside the secretory granule (Dieni et al., 2012). It is thus important to understand whether BDNF is secreted in its precursor form (pro-BDNF) or its mature form in the adult brain versus cultured neurons, since pro-BDNF and mature BDNF have very different functions in the mammalian nervous system (for a review, see (Teng et al., 2010)).

BDNF signaling

Recent work suggests that pro-BDNF and mature BDNF activate distinct signaling pathways (Matsumoto et al., 2008; Woo et al., 2005; Yang et al., 2009). Pro-BDNF signals through the low affinity neurotrophin receptor $p75^{NTR}$ where it induces neuronal apoptosis (Lessmann et al., 2003; Teng et al., 2005) as well as long term depression (LTD) (Leal et al., 2014; Woo et al., 2005; Yang et al., 2014); whereas mature BDNF signals through its high affinity tropomyosin-related kinase B (TrkB) receptor to induce neuroprotective effects (Almeida

et al., 2005) and facilitate long-term potentiation (LTP) (Chen et al., 1999; Duman and Monteggia, 2006; Figurov et al., 1996; Kang et al., 1997; Korte et al., 1998; Messaoudi et al., 2002; Minichiello et al., 1999). The binding of BDNF to TrkB promotes its dimerization and autophosphorylation on specific tyrosine residues located within the intracellular kinase activation domain (Autry and Monteggia, 2012; Carvalho et al., 2008), which triggers the activation of intracellular signaling cascades**,** as well as the potentiation of *n*-methyl-*d*-aspartate (NMDA) receptor currents (Levine et al., 1998). The activation of BDNF-TrkB has been shown to regulate at least three signal transduction pathways (Autry and Monteggia, 2012). The phosphorylation of tyrosine residues located outside the kinase activation domain of the TrkB receptor mediates its interaction with either Src homology 2-containing protein (Shc) or phospholipase Cγ (PLCγ). Shc recruitment to active TrkB receptors leads to the activation of the Ras/extracellular signal-regulated kinase (ERK) pathway through the recruitment of growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS) (Figure 1-1). The binding of Shc to TrkB receptors also activates the phosphostidylinositol 3-kinase (PI3K) pathway, either through PI3K's interaction with Ras or through the recruitment of the adaptor protein grb2-associated binding protein 1(Gab1) (Figure 1-1). Additionally, phosphorylation of TrkB on the Tyr785 residue recruits PLCγ to the receptor. PLCγ subsequently hydrolyzes inositol-1,4,5-triphosphate to diacylglycerol (DAG) and inositol triphosphate (IP3) which facilitates the release of calcium from intracellular stores (Figure 1-1). Each of these signaling pathways confer a unique function of BDNF on cells (for review, see (Mattson, 2008; Yoshii and Constantine-Paton, 2010)). In brief, the impact of BDNF on cell survival is mediated by the PI3K signaling pathway by controlling downstream gene transcription. The ERK pathway confers the important role of BDNF in cell growth and differentiation by regulating downstream

transcription, as well as translation; whereas the effect of BDNF on rapid synaptic and ion channel effects are regulated by the PLCγ-mediated release of calcium stores which enhances calmodulin kinase (CamK) activity. All three pathways converge on the transcription factor, CREB, which up-regulates gene expression (Figure 1-1).

The impact of BDNF on synaptic plasticity

BDNF acts as an indicator for proper axonal growth throughout development (Yoshii and Constantine-Paton, 2010). During development, secreted BDNF stimulates neurite outgrowth by binding to TrkB receptors and facilitating TrkB receptor internalization and signaling to the cell nucleus, thereby altering gene expression. (Yoshii and Constantine-Paton, 2010). The proper development and survival of dopaminergic, cholinergic, GABAergic, and serotonergic neurons also requires BDNF (Pillai, 2008).

BDNF additionally functions critically in the mature brain in synaptic plasticity and learning and memory processes (Poo, 2001). BDNF and TrkB localize at both pre- and post-synaptic sites, where BDNF is released in an activity dependent manner (Waterhouse and Xu, 2009). Presynaptic BDNF signaling facilitates neurotransmitter release, whereas postsynaptic BDNF signaling enhances ion channel function, including the *n*-methyl-*d*-aspartate (NMDA) receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, transient receptor potential cation channels, as well as sodium and potassium channels (for a review, see (Rose et al., 2004)). BDNF functions at both excitatory and inhibitory synapses in the mature brain (Kovalchuk et al., 2004) and experimental evidence indicates that BDNF modulates both spontaneous and evoked neuronal activity (Schuman, 1999; Tyler et al., 2006)

BDNF has thus emerged as a major regulator of activity –dependent plasticity in the mammalian central nervous system. In particular, much attention has been given to the role of BDNF in the regulation of long-term potentiation (LTP), which is a sustained enhancement of synaptic strength between neurons that is initiated by synchronous depolarization. This phenomenon is considered to be the electrophysiological correlate of associational learning and memory processes. Sustained TrkB activation is thought to support LTP as a result of dendritic protein translation or transcription of BDNF (Kang and Schuman, 1996). A positive transcriptional feedback loop has been suggested to occur through TrkB-mediated CREB activation to facilitate the binding of CREB to cAMP response element sites in the BDNF promotor to enhance gene expression (Lu et al., 2008). Previous work shows that blocking the dendritic localization of BDNF mRNA reduces BDNF protein levels in the dendrites and leads to a selective impairment of LTP (An et al., 2008). Application of BDNF also enhances LTP in the neonatal hippocampus, where endogenous BDNF levels are low (Figurov et al., 1996). The pairing of a weak burst of synaptic stimulation with a brief dendritic BDNF application further causes immediate and robust induction of LTP (Kovalchuk et al., 2002). Moreover, although acute application of BDNF enhances synaptic transmission (Kang and Schuman, 1995), other studies show that BDNF alone does not potentiate basal synaptic transmission at cornu ammonus 1 (CA1) synapses (Figurov et al., 1996; Kovalchuk et al., 2002; Patterson et al., 1996; Tanaka et al., 1997). Together, these results suggest a critical role for dendritically synthesized BDNF in synaptic plasticity and indicate that BDNF plays a modulatory rather than an instructive role in hippocampal LTP.

In contrast with the above observations, other studies demonstrate no evidence for dendritically synthesized BDNF. A recent study using high resolution imaging and deep

sequencing techniques to evaluate the distribution of endogenous BDNF mRNA in hippocampal neurons shows that BDNF mRNA is present in the soma but is rarely detected in the dendrites (Will et al., 2013). In agreement with these findings, high resolution fluorescence microscopy studies demonstrate that endogenous BDNF protein is preferentially located at presynaptic structures in cultured hippocampal neurons and rarely if ever detected in dendritic compartments (Andreska et al., 2014). This is also consistent with the detection of BDNF dense core vesicles in the Schaffer collaterals but not in the CA1 pyramidal cell dendrites (Dieni et al., 2012). These results raise questions regarding the ability of endogenous BDNF to localize to dendrites and the potential of being translated locally. While activity driven local synthesis of BDNF controls the plasticity of dendrites in hippocampal neurons (Verpelli et al., 2010), further research is needed to address whether LTP inducing stimuli are capable of inducing the local synthesis of BDNF *in vivo*. Despite these controversies, BDNF facilitates LTP by converting early LTP to late LTP through potentiating the threshold of activation required for late LTP in acute hippocampal slices (Nagappan and Lu, 2005).

Given the essential role for BDNF in LTP facilitation, it is not unexpected that experimental loss of BDNF signaling through genetic models or pharmacological manipulation leads to impaired LTP (Monteggia et al., 2004; Patterson et al., 1996) and decreases in learning and memory in behavioral paradigms (Lu et al., 2008). Additional studies on loss of BDNF signaling in the adult brain have led to the discovery of many more roles for BDNF in the modulation of behavior other than learning and memory. In addition to its important role in learning and memory (for review, see (Cowansage et al., 2010)), more recent work has revealed BDNF's pivotal role in cognition, as well as mood-related disorders. For this reason, BDNF is widely studied in relation to neuropsychiatric diseases, including, but not limited to, major

depressive disorder (MDD), bipolar disorder, and schizophrenia, addictive disorders, such as drug/alcohol addiction and eating disorders, and Rett syndrome.

BDNF and Major Depressive Disorder (MDD)

Major depressive disorder is the leading cause of disability in the United States. Current estimates suggest that the 12 –month prevalence of MDD is approximately 6.7% of the US adult population with a larger population being affected by milder forms of the disorder (Kessler et al., 2005). Intriguingly, women are almost twice as likely as men to experience MDD within a given year. The clinical presentation of MDD consists of a continuum of neuropsychiatric symptoms, including anxiety, loss of pleasure, feelings of inappropriate guilt, appetite changes, sleep disturbances, cognitive problems, feelings of helplessness or hopelessness, and suicidal ideation (Shelton, 2007). Pharmacological therapies for the treatment of MDD include selective serotonin- reuptake inhibitors (SSRIs), selective serotonin/norepinephrine -reuptake inhibitors (SNRIs), tricyclic antidepressants, and monoamine oxidase inhibitors. These drugs target monoamine neurotransmitter systems to increase signaling at the synapse, indicating an essential role for monoaminergic systems in mood-related behavior. However, given that these drugs have rapid synaptic effects but are simultaneously associated with a delayed onset of clinical efficacy, it has been suggested that molecular targets downstream of serotonergic or noradrenergic signaling are responsible for antidepressant efficacy.

MDD is a heterogeneous disorder which has a clear genetic component. Interestingly, studies show that environmental effects, such as stress, can also contribute to disease onset and/or trigger major depressive episodes (Shelton, 2007). Intriguingly, these studies demonstrate that BDNF expression is decreased by stress, suggesting that BDNF may be the molecular

substrate of stress, which is an important risk factor for MDD (Martinowich et al., 2007). Furthermore, antidepressant treatment increases BDNF levels in the hippocampus, which is opposite to that of stress and/or depression (Castren and Rantamaki, 2010). Because BDNF expression is decreased by stress and MDD, increased by antidepressants, and normalized in patients with MDD treated with an antidepressant, BDNF has been proposed to be a biomarker for depression, as well as a potential target for the treatment of MDD.

BDNF expression in human patients with MDD. Human patients with MDD show anatomical reductions in hippocampal volume (Bremner et al., 2000). Interestingly, BDNF and TrkB expression are decreased in the hippocampus in postmortem tissue taken from patients with MDD and completed suicides (Castren and Rantamaki, 2010; Castren et al., 2007; Zhou et al., 2011). BDNF is also decreased in serum taken from patients with MDD (Castren and Rantamaki, 2010; Castren et al., 2007; Zhou et al., 2011). Thus, the structural changes in the hippocampus related to MDD may be attributed, to a certain extent, to the observed reductions in BDNF and TrkB expression (Yu and Chen, 2011). The prefrontal cortex, which is another region of the brain essential to emotional processing, has also been examined in regards to pathological hallmarks associated with MDD. The prefrontal cortex also shows decreased volume in patients with MDD, and this decrease in volume is correlated with decreased BDNF and TrkB expression (Castren, 2004; Dwivedi et al., 2003; Pandey et al., 2008). Collectively, these findings indicate that MDD affects BDNF expression in limbic regions.

In contrast to the reductions in BDNF expression in the hippocampus and prefrontal cortex that appears to be triggered by MDD; BDNF expression has been found to be enhanced in other areas of the brain in MDD. Studies have revealed that BDNF protein is increased in the nucleus

accumbens (NAc) region in patients with MDD (Krishnan et al., 2007). Functional imaging studies additionally consistently report abnormal amygdala activity in MDD (Rubinow et al., 2014). However, neuroanatomical correlates are less clear: imaging studies have produced mixed results on amygdala volume (Depping et al., 2014; Frodl et al., 2002; Tebartz van Elst et al., 2000). Interestingly, postmortem studies of the basolateral amygdala (BLA) indicate that depressed subjects have a larger lateral nucleus than controls and a greater number of total BLA neurovascular cells than controls (Rubinow et al., 2014). While it is unclear whether BDNF is involved in this abnormality in human patients, preclinical studies indicate that BDNF expression is increased in the amygdala in response to stress, a significant risk factor for MDD (Yu and Chen, 2011). Taken together, these data suggest an emergent circuity in MDD, in which BDNF signaling is increased in the amygdala and the nucleus accumbens and, in contrast, decreased in the hippocampus and the prefrontal cortex.

BDNF expression is increased by antidepressant treatment in human patients with MDD. Postmortem tissue studies reveal that BDNF levels are increased in the hippocampus and the prefrontal cortex after long-term antidepressant treatment (Frodl et al., 2008). Long-term antidepressant treatment has been additionally correlated with increased hippocampal and prefrontal cortical volumes and enhanced clinical efficacy (Frodl et al., 2008). Further work shows that serum levels of BDNF are normalized by long-term antidepressant treatment in patients suffering from MDD (Duman and Monteggia, 2006), a finding that has been validated by meta-analysis of multiple studies (Sen et al., 2008). Despite these findings, studies of BDNF expression in postmortem tissue and on changes in brain volume upon antidepressant treatment are correlative. Moreover, the origin and function of serum-derived BDNF remain unclear.

Therefore, scientists have turned to animal models to establish a causal link between BDNF expression and depression-like behavior.

BDNF expression in animal models. To assess whether BDNF is required for depression-like behavior, multiple animal models of deficient BDNF signaling have been generated. Constitutive BDNF homozygous knockout mice have been generated. These mice show severely impaired coordination and a short lifespan (Ernfors et al., 1994). Due to the severe impairments associated with BDNF constitutive knockouts, BDNF heterozygous mice were next studied for baseline depression-like behavior. BDNF heterozygous mice did not show depressive phenotypes in the forced swim test (FST), learned helplessness paradigm (LH), tail suspension test (TST), or sucrose preference test (SPT) (Chourbaji et al., 2004; Ibarguen-Vargas et al., 2009; MacQueen et al., 2001; Saarelainen et al., 2003). However, baseline behavior in BDNF heterozygous mice may be difficult to interpret, because constitutive reduction of BDNF over the course of development may lead to developmental compensation. Additionally, it may be that 50% expression of BDNF is sufficient for normal behavioral phenotypes. To address this potential confound, conditional and inducible BDNF knockout mice have therefore been generated in order to remove BDNF in a regionally and temporally dependent manner. In mice in which BDNF is ablated from forebrain neurons, postnatally, there are no overt changes in depression like behavior in male mice (Monteggia et al., 2004), however female mice lacking BDNF may display behavioral alterations in some assays, suggesting a gender difference in vulnerability to depressive-like behavior. This vulnerability to depression-like behavior described for female conditional and inducible BDNF knockout mice is not applicable to antidepressant –like responses. All lines of conditional or inducible BDNF knockout mice, both

male and female, consistently display an inability to respond to antidepressant treatment, suggesting a necessary role for BDNF in the expression of behavioral antidepressant responses (Autry et al., 2011; Hu and Russek, 2008; Malberg and Blendy, 2005; Monteggia et al., 2004; Monteggia et al., 2007; Tardito et al., 2006). A potential caveat of this type of deletion, however, is that behavioral effects cannot be attributed to specific neural circuits.

To more accurately target particular brain regions, viral-mediated deletion techniques have been used to remove BDNF from spatially restricted brain regions. These studies have enhanced our understanding as to how BDNF functions in specific neural circuitry. Moreover, these studies have exposed conflicting data as to the functional contribution of particular brain regions in depression-related behavior. Localized deletion of BDNF in the CA1 or the dentate gyrus subregions of the hippocampus does not alter baseline depression-like behavior, however expression of BDNF in the dentate gyrus is required for antidepressant efficacy (Adachi et al., 2008). In contrast to the CA1 and the dentate gyrus subregions of the hippocampus, region specific deletion of BDNF in the mesolimbic dopamine neurons of the ventral tegmental area (VTA) produces an antidepressant response (Berton et al., 2006; Krishnan et al., 2007). The discrepancies in localized BDNF deletion on behavior are often explained by the notion that the deletion of BDNF and its precursor pro-BDNF results in divergent behavioral phenotypes in different brain areas (Martinowich et al., 2007). Alternatively, these discrepancies are accounted for as an example of the complex circuitry associated with mood disorders (Castren et al., 2007). Detailed experimental assessments on these theories, however, have yet to be undertaken. In the future, it will be important to define how the region-specific contribution of BDNF and regional brain circuitry influences depression –like behavior and antidepressant efficacy.

BDNF and the antidepressant response

Although BDNF alone may not be sufficient to explain depression –related behaviors, BDNF is essential for antidepressant efficacy. In animals models, similar to findings in human tissue, the upregulation of BDNF is observed in the hippocampus in response to different classes of antidepressants, including electroconvulsive therapy (ECT), selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), and tricyclic antidepressants (Altar et al., 2003; Balu et al., 2008; Nibuya et al., 1995). It is notable that the most clinically effective antidepressants, ECT and MAOIs, have the greatest effect on the induction of BDNF expression (Nibuya et al., 1995). BDNF induction is also dependent on chronic chemical antidepressant treatment, which is consistent with the time course for the therapeutic action of antidepressants. Interestingly, the administration of other classes of psychotropic drugs, such as opiates, benzodiazepines, antipsychotics, and psychostimulants, does not increase BDNF in the hippocampus, thus demonstrating the pharmacological specificity of antidepressant action on BDNF(Duman and Monteggia, 2006). It is also notable that other treatments that are known to have antidepressant efficacy also increase BDNF expression in the hippocampus. These include the administration of alpha-amino -3 hydroxy-5 methyl-4 isoxazolepropionic acid (AMPA)kines (Lauterborn et al., 2003) and *n*-methyl-*d*-aspartate (NMDA) receptor antagonists (Autry et al., 2011; Nosyreva et al., 2013), as well as transcranial magnetic stimulation (Muller et al., 2000).

The upregulation of BDNF by antidepressant treatment has been confirmed by a number of studies (Altar et al., 2004; Altar et al., 2003; Coppell et al., 2003; De Foubert et al., 2004; Dias et al., 2003; Garza et al., 2004; Holoubek et al., 2004; Lauterborn et al., 2003; Marvanova et al., 2001; Muller et al., 2000; Nibuya et al., 1995; Nibuya et al., 1996; Russo-Neustadt et al., 1999;

Russo-Neustadt et al., 2004; Smith et al., 1997; Vinet et al., 2004). However there have been some inconsistent reports with some classes of antidepressants. Electroconvulsive therapy and monoamine oxidase inhibitor antidepressants consistently increase BDNF expression in the hippocampus of rodents (Altar et al., 2004; Altar et al., 2003; Coppell et al., 2003; Dias et al., 2003; Garza et al., 2004; Nibuya et al., 1995; Russo-Neustadt et al., 1999; Smith et al., 1997). Additionally, multiple studies report that infusion of BDNF into the midbrain, ventricles, or regions of the hippocampus increases antidepressant–like behavior (Hoshaw et al., 2005; Hu and Russek, 2008; Shirayama et al., 2002). In most studies, the administration of SSRIs (Coppell et al., 2003; Holoubek et al., 2004; Nibuya et al., 1995; Nibuya et al., 1996) and SNRIs (Dias et al., 2003; Nibuya et al., 1995; Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2004; Vinet et al., 2004) increase the expression of BDNF. However, some studies do not report increases in hippocampal BDNF upon chronic antidepressant administration (Altar et al., 2003; Coppell et al., 2003; Miro et al., 2002). This discrepancy could be due to the paradigm used, including the dose of the drug, the time of treatment, or the behavior assessed (Coppell et al., 2003; De Foubert et al., 2004; Russo-Neustadt et al., 2004). BDNF expression is activity dependent and regulated by a variety of environmental stimuli (Duman and Monteggia, 2006). It is thus critical that environmental conditions are closely monitored when conducting in vivo drug studies on the impact of BDNF expression.

The survival and function of neurons in the central nervous system are dependent on a perpetually growing list of neurotrophic factors, and the impact of antidepressants on some of these factors has been investigated. Although many growth factors, such as nerve growth factor (NGF) and insulin growth factor (IGF), also increase after antidepressant treatment and cause behavioral effects similar to those observed after BDNF infusion (for review, see (Duman and

Monteggia, 2006)), the majority of data show that animal models lacking BDNF are unable to respond to antidepressants (Adachi et al., 2008; Monteggia et al., 2004; Monteggia et al., 2007; Saarelainen et al., 2003), suggesting that BDNF is required for antidepressant efficacy. Additionally, overexpression of a dominant negative TrkB leads to a loss of antidepressant efficacy (Saarelainen et al., 2003), thus indicating that TrkB activation by BDNF is required for antidepressants to elicit behavioral effects. Antidepressant drugs have interestingly been shown to rapidly activate the TrkB receptor as well as the PLCγ signaling pathway (Rantamaki et al., 2007), suggesting that the TrkB –PLCγ pathway may be a common mechanism for antidepressant action. Collectively, these data imply that BDNF activates TrkB and downstream signaling and that both are necessary and sufficient for antidepressant responses.

BDNF and fast acting antidepressant efficacy.

Traditional antidepressants (i.e. serotonin reuptake inhibitors) take weeks to months to reach clinical efficacy. This delay in therapeutic onset is a major drawback to current MDD therapies, leaving a need for faster acting antidepressants particularly for patients at risk for suicide. Thus, one of the most exciting findings in the field of depression is that ketamine, a noncompetitive *n*-methyl-*d*-aspartate receptor (NMDAR) antagonist, produces rapid and long-lasting antidepressant effects in depressed patients previously unresponsive to traditional treatments (Berman et al., 2000; Price et al., 2009; Zarate et al., 2006). A single, low, non-psychomimetic intravenous infusion of ketamine (0.5mg/kg) has been shown to alleviate symptoms of depression within 2 hours of administration with effects lasting up to two weeks in patients with MDD and bipolar disorder. However, the cellular and molecular mechanism by which ketamine exerts this behavioral effect remains unclear. Recent studies show that ketamine

elicits a fast- acting antidepressant response in mice following chronic unpredictable stress (Autry et al., 2011; Autry and Monteggia, 2012). Ketamine also produces fast –acting behavioral effects in naïve mice (Autry et al., 2011; Autry and Monteggia, 2012). The antidepressant-like effects of ketamine are dependent on BDNF and the subsequent activation of the high affinity BDNF receptor, TrkB, as ketamine –mediated behavioral effects are lost in inducible BDNF knockout mice as well as in conditional TrkB knockout mice. The fast acting antidepressant effects of ketamine require protein translation, not transcription, as ketamine application results in a rapid increase in dendritic BDNF protein levels that are important for the behavioral effect. As previously stated above, BDNF is a well characterized molecule which has been linked to the action of traditional antidepressants. BDNF expression is increased in the hippocampus by antidepressant treatment (Chen et al., 2001) and BDNF deletion in the hippocampus impairs behavioral responses elicited by the administration of classical antidepressants (Adachi et al., 2008; Monteggia et al., 2004). These observations implicate BDNF-dependent signaling as a common pathway where classical antidepressant action and the fast-acting antidepressant effects of ketamine merge. Interestingly, a single dose of ketamine can produce an elevation in BDNF protein in a short time period (~ 30 min), whereas classical antidepressants require repeated administration to reach the same endpoint (Duman and Monteggia, 2006). BDNF may then act on several downstream signaling pathways that impact synaptic plasticity and neuronal activity (Autry and Monteggia, 2012; Duman and Monteggia, 2006).

eEF2 kinase and ketamine action

The rather immediate action of ketamine on BDNF translation is consistent with recent work which suggests a causal link between blockade of resting NMDA receptor activation and rapid increases in local dendritic protein translation in vitro (Sutton et al., 2006; Sutton et al., 2004). Ketamine causes a decrease in the phosphorylation of eukaryotic elongation factor 2 (eEF2) in vivo, which when phosphorylated normally impedes translation, suggesting a translational de-repression of BDNF mRNA in the presence of ketamine (Autry et al., 2011; Nosyreva and Kavalali, 2010). Inhibitors of eEF2 kinase, which prevent eEF2 phosphorylation, also produce fast – acting antidepressant effects in animals and elicit enhanced BDNF protein levels (Autry et al., 2011). These findings suggest a behavioral and clinically relevant correlate of dendritic translational de-repression through blockade of NMDA receptors at rest (Autry et al., 2011). Moreover, these data demonstrate that a potential target for antidepressant action is eEF2-kinase dependent regulation of BDNF transmission (Autry et al., 2011; Nosyreva et al., 2013).

A major aspect of NMDA receptor activity and eEF2 kinase regulation is its specificity towards distinct forms of neurotransmission. Two forms of neurotransmission occur within the brain: evoked transmission and spontaneous transmission. Evoked neurotransmission occurs when a neurotransmitter is released in response to a presynaptic action potential, which then acts on postsynaptic receptors to mediate specific effects on intracellular signaling cascades. In contrast, spontaneous neurotransmission occurs as a result of the low probability that a synaptic vesicle will fuse with the presynaptic membrane in the absence of an action potential. Recent work has suggested that spontaneous neurotransmitter release activates postsynaptic receptors which are distinct from evoked neurotransmission (Kavalali et al., 2011). Further, NMDA receptor antagonists, such as ketamine, augment dendritic protein synthesis by the blockade of

spontaneous but not evoked neurotransmission (Nosyreva et al., 2013). Spontaneous NMDA receptor activation triggers eEF2 kinase to phosphorylate eEF2 and releases this protein from the translational machinery, thereby effectively halting protein synthesis. Acute blockade of NMDA receptors by ketamine inhibits this tonic eEF2 kinase activity, leading to a dephosphorylation of eEF2 and an increase in the translation of target transcripts (Figure 1-2), including BDNF (Autry et al., 2011; Nosyreva et al., 2013; Sutton et al., 2007).

eEF2 kinase regulates dendritic protein translation. eEF2 kinase is a major molecular substrate mediating the rapid antidepressant effect of ketamine. eEF2 kinase contains three functional domains: an N-terminal calmodulin –binding domain (residues 51-96), an α –kinase catalytic domain immediately downstream of the N terminal domain (residues 100-350), and a C-terminal eEF2 binding domain (residues 521-725) (Pigott et al., 2012). eEF2 kinase is the only known target for eEF2 at position threonine 56. Thus eEF2 kinase specifically phosphorylates eEF2 to suppress the activity of eEF2. Active eEF2 is required for the elongation step of protein translation. Active eEF2 catalyzes the translocation of peptidyl-tRNA from the A-site to the P-site on the eukaryotic ribosome to allow for the addition of a new amino acid to the growing polypeptide strand. eEF2 kinase induced phosphorylation of eEF2 causes eEF2 to release from the ribosome, thereby stopping translational elongation of most proteins, including BDNF (Ryazanov et al., 1991; Ryazanov et al., 1988). Intriguingly, phosphorylated eEF2 paradoxically increases the translational elongation rate of certain mRNAs, yet suppresses the majority, indicating many levels of eEF2K regulation (Scheetz et al., 2000).

The ultimate question is how synaptic activity regulates eEF2 kinase function and ultimately protein translation. eEF2 kinase and eEF2 are both located in the postsynaptic

compartment of dendrites together with many other proteins necessary for protein translation, initiation, and elongation (Marin et al., 1997; Park et al., 2008). The presence of the translational machinery at the synapse enables local protein synthesis following synaptic activity and mediates synapse specific LTP and LTD (Asaki et al., 2003). During synaptic neurotransmission, a major source of dendritic calcium influx is facilitated by the activation of NMDA receptors. Calcium entry from NMDA receptors has been well characterized as a key activator of numerous calcium calmodulin dependent substrates, including calcineurin, CaMKII, and nitric oxide synthase. Consistently, earlier studies have shown that direct NMDA receptor activation activates eEF2 kinase function leading to eEF2 phosphorylation and inhibition of protein translation elongation (Scheetz et al., 2000).

Synaptic activation of NMDA receptors increases eEF2 kinase activity and suppresses protein translation (Marin et al., 1997). Calcium influx following NMDA receptor activation and NMDA receptor miniature excitatory post synaptic currents (mEPSCs) also cause an increase in the phosphorylation of eEF2 (Barrera et al., 2008; Marin et al., 1997; Scheetz and Constantine-Paton, 1996; Sutton et al., 2007). Blocking NMDA receptor mediated miniature neurotransmission thus reduces eEF2 phosphorylation and produces increases in local protein translation (Sutton et al., 2007). Intriguingly, eEF2K can be activated in dendrites by metabotropic glutamate receptor (mGluR) 1/5 signaling (Verpelli et al., 2010). eEF2 kinase is physically associated to type 1 mGluRs, including mGluR1 and mGluR5, by its interaction with the scaffolding protein, Homer (Park et al., 2008). Dissociation of the Homer/eEF2 complex activates eEF2 in response to calcium influx.

It is notable that constitutive eEF2 kinase knockout mice are viable and fertile (Ryazanov, 2002). Behavioral characterization of constitutive eEF2 kinase knockout mice do not reveal any

alterations in locomotor activity, anxiety-like behavior, or social interaction compared to littermate controls (Nosyreva et al., 2013). Western blot analysis show a negligible level of eEF2 phosphorylation within the cortex and hippocampus in eEF2 kinase knockouts compared to controls. A single low-dose of ketamine that triggers an antidepressant response and an increase in BDNF in littermate control mice fails to increased BDNF protein levels in the hippocampus of eEF2 kinase knockout mice. Furthermore, ketamine does not trigger an antidepressant response in the eEF2 kinase knockout mice in the FST or NSF paradigms (Nosyreva et al., 2013). These data provide additional support that ketamine mediated fast-acting antidepressant effects require eEF2 kinase. Consistent with previous work (Autry et al., 2011), a 30 – minute ketamine application to slices prepared from control mice produce a robust potentiation and a significant increase in the slope of the input/output curve (Nosyreva et al., 2013). In contrast, the same treatment failed to induce potentiation in slices prepared from eEF2 kinase knockout mice. These results show that the absence of eEF2 kinase, a key biochemical transducer of resting NMDA receptor activity, eliminates synaptic potentiation, as well as the antidepressant effects observed after ketamine treatment. These data also support the view that targeting eEF2 kinase function for treatment advance may result in limited side-effects. These examples, moreover, highlight the complexity of how synaptic activity can regulate eEF2 function and impact dendritic translation of BDNF. Future studies utilizing a combination of presynaptic and postsynaptic approaches are necessary to delineate the mechanisms controlling the eEF2 kinase – eEF2 – BDNF pathway and its ultimate role in mediating rapid antidepressant responses.

Conclusion

While there is a great need for faster acting antidepressants, there is also a need for these drugs to have sustained efficacy. However, it is unclear whether other fast –acting antidepressants use a mechanism similar to ketamine to produce their effect. A striking example of a fast-acting antidepressant with a sustained effect is scopolamine. Scopolamine is a muscarinic acetylcholine receptor antagonist that produces rapid antidepressant effects in depressed patients within 24 -72 hours after administration (Furey and Drevets, 2006; Furey et al., 2010; Furey et al., 2012; Gillin et al., 1991) with effects lasting up to 2 weeks. The fact that scopolamine blocks muscarinic acetylcholine receptors, which are G_q- protein coupled receptors, instead of the ionotropic NMDA receptors that are blocked by ketamine, indicates that the molecular trigger for the antidepressant effect is most likely distinct from that of ketamine. However, one might predict a downstream point of convergence between ketamine and scopolamine, with that possibly being BDNF. There is clearly a need for a better understanding of the synaptic and biochemical mechanisms underlying the action of ketamine and scopolamine, including their potentially converging targets as well as their differences, as this information may uncover additional synaptic proteins that can be used to elicit a rapid antidepressant effects with sustained efficacy (Kavalali and Monteggia, 2012).

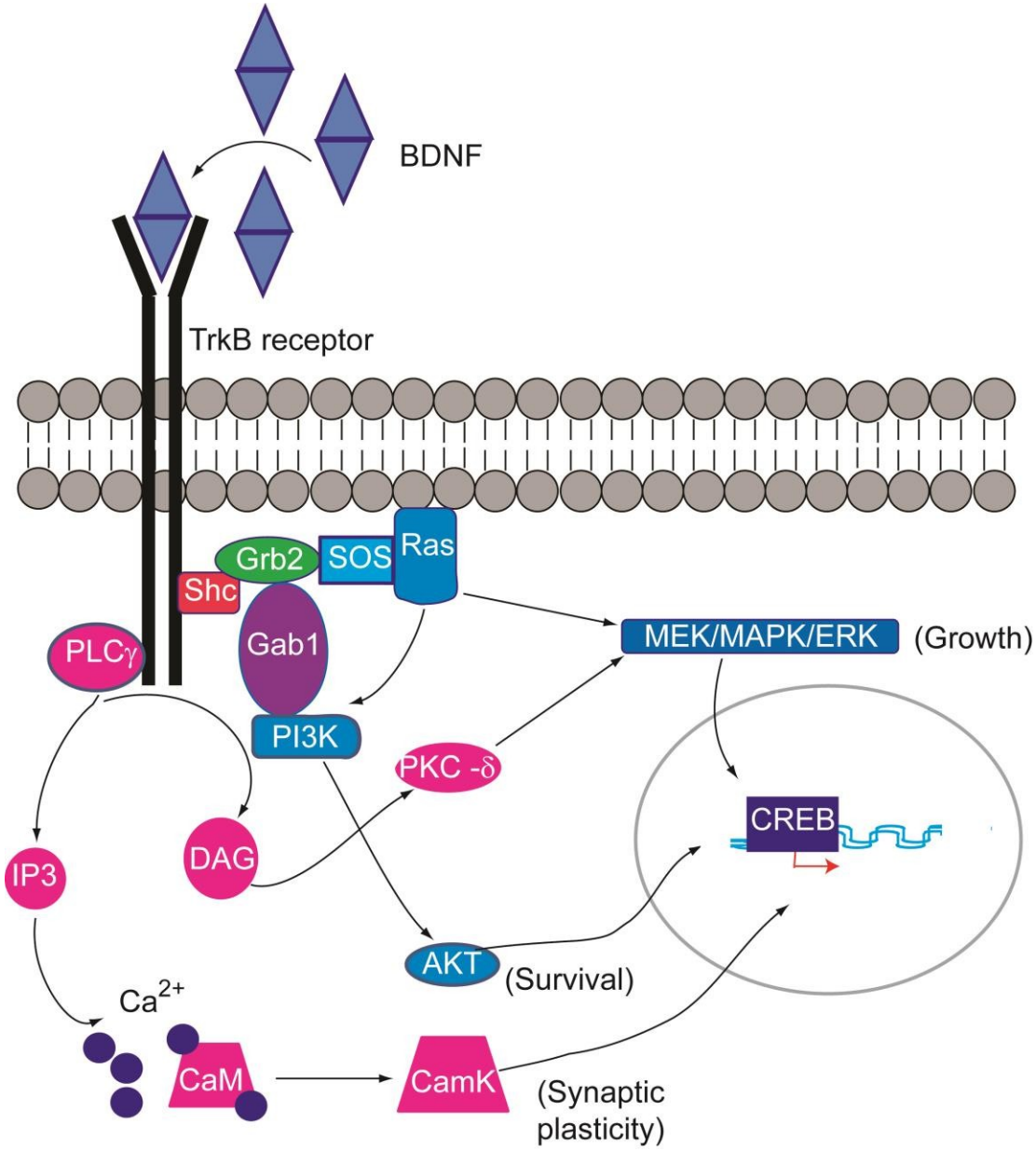
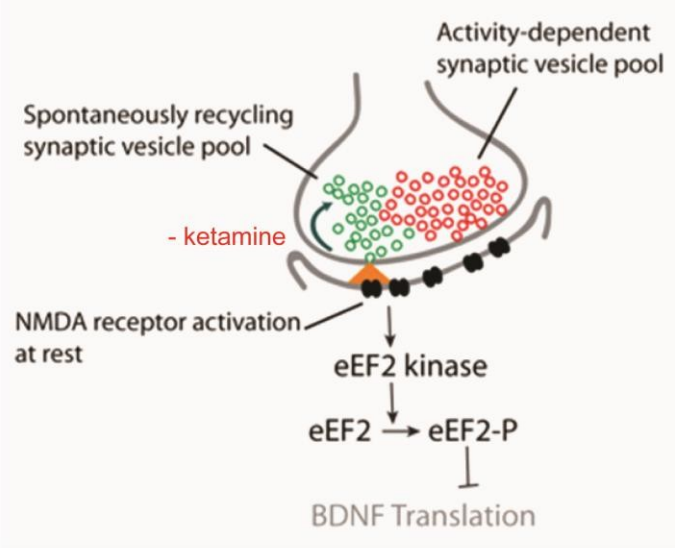


Figure 1-1 An Overview of BDNF signaling through TrkB receptors. Upon binding to BDNF the TrkB receptor becomes phosphorylated. Phosphorylation at various sites leads to the activation of downstream signal transduction pathways. The PI3K pathway, activated by RAS and the adaptor protein Gab1, activates protein kinase B (AKT), leading to cell survival. The MEK/MAPK/ERK pathway, triggered by Shc and adaptor proteins Grb2 and SOS, leads to cell growth and differentiation, whereas the PLCγ pathway activates the inositol triphosphate (IP3) receptor to release calcium from intracellular stores, leading to enhanced calmodulin kinase (CamK) activity and increased synaptic plasticity. All three pathways converge on the transcription factor, CREB, which up-regulates gene expression. Figure modified from (Autry and Monteggia, 2012).



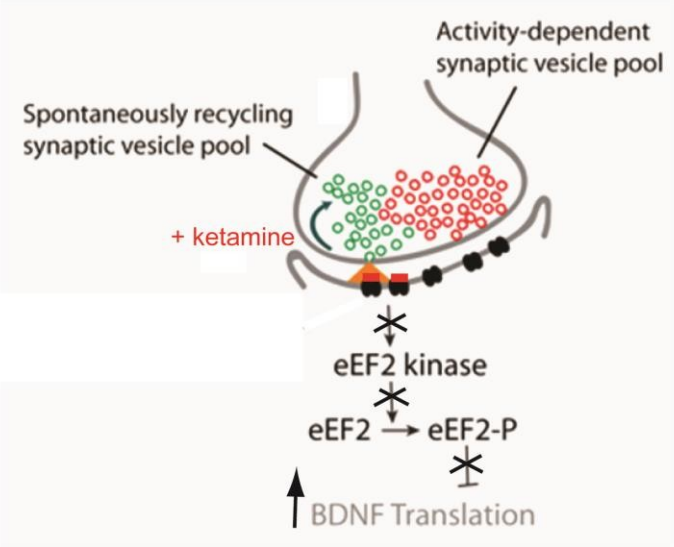


Figure 1-2 Synaptic mechanism underlying ketamine action. Top: Under resting conditions, spontaneous glutamate release and NMDA receptor activation leads to the activation of eEF2 kinase triggering eEF2 phosphorylation and the suppression of BDNF translation. Bottom: Ketamine- mediated blockade of tonic NMDA receptor activity at rest inhibits activation of eEF2 kinase resulting in a gradual loss of eEF2 phosphorylation and a de-suppression of BDNF translation. Figure adapted from (Monteggia and Kavalali, 2013).

CHAPTER II

DOSE DEPENDENT DIVERGENCE OF KETAMINE-ELICITED NEURONAL SIGNALING AND FAST-ACTING ANTIDEPRESSANT EFFICACY

Introduction

Drugs targeting brain glutamatergic signaling pathways have been increasingly recognized as potential antidepressant drug candidates (Alt et al., 2006; Skolnick et al., 2009; Vollenweider and Kometer, 2010). Recent clinical trials consistently demonstrate that a single sub-psychomimetic dose of ketamine, a noncompetitive ionotropic glutamatergic *n*-methyl-*d*-aspartate (NMDA) receptor antagonist, produces rapid antidepressant responses within 2 hours in individuals with treatment-resistant major depressive disorder (MDD) (Maeng and Zarate, 2007; Valentine et al., 2011; Zarate et al., 2006). This rapid antidepressant effect is in contrast to the lag typically associated with traditional antidepressants, such as serotonin reuptake inhibitors, which require weeks or months to reach clinical efficacy. Clinical trials have relied on a low dose of ketamine that does not produce psychomimetic effects that are observed with higher doses of ketamine (Berman et al., 2000; Krystal, 2007; Maeng and Zarate, 2007; Price et al., 2009; Zarate et al., 2006). Ongoing preclinical studies have been investigating the cellular and molecular mechanisms underlying ketamine's rapid antidepressant effects with conflicting results on dosage. Our lab has recently shown that tonic NMDA suppression by low sub-psychomimetic doses of ketamine (3 mg/kg, i.p) in mice produces rapid and long lasting antidepressant- like effects through the inhibition of eukaryotic elongation factor 2 kinase (eEF2K), also known as calcium calmodulin dependent kinase III (CaMKIII), resulting in reduced eEF2 phosphorylation and increased BDNF protein translation (Autry et al., 2011). A

previous study, however, demonstrated that ketamine (10 mg/kg, i.p) produces fast acting antidepressant effects by rapidly activating the mammalian target of rapamycin (mTOR) in the prefrontal cortex of rats (Li et al., 2010). In contrast, others investigating ketamine's antidepressant effects have suggested that ketamine induces antidepressant-like responses preclinically only at relatively high sub-anesthetic doses (50 mg/kg i.p)(Lindholm et al., 2012; Popik et al., 2008), whereas anesthetic doses of ketamine (80 mg/kg i.p) do not produce antidepressant like effects (Li et al., 2010).

To reconcile the disparities in these studies of ketamine action, we administered a range of ketamine dosages (3, 20 and 50 mg/kg) to investigate animal behavior and found differential effects on locomotor activity, prepulse inhibition (PPI), forced swim, and novelty suppressed feeding behavior. We show that high ketamine (20 or 50 mg/kg) increases total locomotor activity, whereas low ketamine (3 mg/kg) does not. We also show that high ketamine (20mg/kg and 50 mg/kg) produces deficits in PPI behavior. In contrast, low ketamine (3 mg/kg) does not alter PPI behavior. Moreover, we find that only low ketamine (3mg/kg) produces significant antidepressant –like effects in both the FST and the NSF tests, whereas high ketamine doses do not induce an antidepressant response in these tests. Furthermore, we demonstrate that low dose ketamine (3 mg/kg) results in reduced eEF2 phosphorylation and increased BDNF protein 30 minutes after ketamine injection. Whereas, we show that high ketamine dosage (20mg/kg and 50mg/kg) produces no change in eEF2 phosphorylation or BDNF. Together our data provide compelling evidence that ketamine specifically activates eEF2 at low but not at high doses thereby suggesting that ketamine may activate divergent signaling pathways in a dose dependent manner, which may be responsible for ketamine's differential impact on behavior, including antidepressant efficacy.

Materials and Methods

Mice

C57BL/6 male mice aged 6-8 weeks old were habituated to the animal vivarium for one week prior to behavioral testing or use in biochemical analysis. Mice were kept on a 12/12 hour light dark cycle with *ad libitum* access to food and water. For behavioral testing, separate cohorts of mice were used in each experimental task such that animals were not retested in a behavioral paradigm. All experiments were done blind. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Drugs

Mice received a single intraperitoneal (i.p.) injection of ketamine (Fort Dodge Animal Health), at 3 mg/kg, 20 mg/kg, or 50 mg/kg in 0.9% saline, or 0.9% saline vehicle.

Locomotor activity

Mice were placed individually in a standard mouse cage with fresh bedding. Locomotor activity was recorded for 120 minutes under red light by horizontal photocell beams coupled to computer acquisition software (San Diego Instruments).

Forced swim test

The forced swim test was performed as previously described (Adachi et al., 2008; Autry et al., 2011). Briefly, mice were placed in a 4-L Pyrex glass beaker containing 3L of water 24 ± 1ºC for

six minutes. All test sessions were recorded by a video camera positioned on the side of the beakers. The videotapes were scored by an observer blind to group assignment during the last four minutes of the six minute trial.

Novelty suppressed feeding

The novelty suppressed feeding paradigm was performed as described previously (Autry et al., 2011). In brief, group housed animals were food deprived for 24 hours prior to testing. For the test, a single pellet of the mouse's regular food chow was placed in the center of a 42 x 42 cm open field arena at 40 lux. Individual mice were placed in the corner of this arena and allowed to explore for up to 10 minutes. The trial ended when the mouse chewed part of the chow. To control for appetite, animals were then individually housed in temporary home cages. The amount of food consumed within the home cage was defined as weight of chow consumed in a 5 minute period.

Prepulse Inhibition.

C57BL/6 mice were i.p. injected with either vehicle or ketamine at 3 mg/kg, 20 mg/kg, or 50 mg/kg 2 h prior to testing. Prepulse inhibition was performed as previously described (Chan et al., 2008) utilizing SR-LAB test chambers (San Diego Instruments; San Diego, CA). Following a 5- min acclimation period, mice received five trial types repeated for three times: no-stimulus, startle signal (SS), 74-dB PP + SS, 78-dB PP + SS, or 82-dB PP + SS. These stimuli were repeated in randomized fashion. Peak response amplitude (V_{max}) was the dependent measure analyzed. Data is presented as a percentage of the startle response.

Protein quantification and Immunoblotting.

C57BL/6 mice were administered vehicle or ketamine at 3.0 mg/kg, 20 mg/kg, or 50 mg/kg i.p. Anterior hippocampal and prefrontal cortical slices (2/mouse, ~1 mm thick) were dissected at 30 minutes, 2 hours, and 24 hours post-injection and rapidly frozen. Tissue was homogenized in a radioimmunopreciation assay (RIPA) buffer containing: Tris 50 mM, Igepal 1.0%, SDS 0.1%, sodium deoxycholate 0.5%, EDTA 4.0 mM, NaCL 150 mM, pH 7.4. Protease inhibitor cocktail (Roche) and phosphatase inhibitors (sodium pyrophosphate 10 mM, sodium fluoride 50 mM, and sodium orthovanadate 2.0 mM) were added immediately prior to use. Total protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce). Samples were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with anti-phospho-T56 eEF2 antibody (peEF2; 1:2000; Cell Signaling), anti- total eEF2 (teEF2; 1:2000; Cell Signaling), anti-BDNF (1:1000; ABCAM), and anti-GAPDH antibody (1:50,000; Cell Signaling), as previously described (Autry et al., 2011). Immunoreactive bands were visualized by enhanced chemoluminescence and captured on autoradiography film (Light Labs). Digital images were produced by densitometric scans of the autoradiographs on a ScanJet 4300C (Hewlett Packard, Palo Alto, CA) and quantified using Image J software. P-eEF2 and BDNF intensities were taken as a ratio of total eEF2 and GAPDH values, respectively. Significant differences between treatment and control for protein levels were determined using a one way analysis of variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons.

Statisitics

Data was analyzed by Analysis of Variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons unless otherwise specified. Data are presented as mean ± SEM and significance was set at $p < 0.05$.

Results

To explore the dose dependent effect of ketamine on behavior, we administered vehicle or ketamine (3, 20, or 50 mg/kg) i.p. to mice and immediately assessed locomotor activity for a period of 120 min. Mice that received low dose ketamine at 3 mg/kg showed an acute increase in locomotor activity compared to vehicle treated mice for the first 20 minutes post injection; locomotor activity returned to baseline 25 minutes post injection, as has previously been described (Autry et al., 2011) (Figure 2-1a). Similarly, increased locomotor activity was observed in mice treated with the higher ketamine dose (20 mg/kg) for 30 minutes post injection (Figure 2-1a). In contrast, in mice that received highest ketamine dose (50 mg/kg), a dramatic decrease in locomotor activity was observed for 15 minutes post injection (Figure 2-1a). At 35 minutes post injection, an increase in locomotor activity was then detected in these mice for 65 minutes post injection (Figure 2-1a). Locomotor activity then returned to baseline 70 minutes post injection time (Figure 2-1a). Analysis of the total number of beam breaks over the 120 minute period (Figure 2-1a, inset) indicates that both high dose ketamine treatments (20 mg/kg and 50 mg/kg) produced significant increases in total locomotor activity in comparison to both vehicle and low dose ketamine (3 mg/kg).

Pre-pulse inhibition (PPI) is considered a measure of sensorimotor gating; previous work has shown that ketamine disrupts PPI behavior (Cilia et al., 2007). To investigate the dose dependent effects of ketamine in the PPI task, we administered vehicle or ketamine (3 mg/kg, 20 mg/kg, or 50 mg/kg) and examined mice for behavior two hours later. This time was chosen based on our locomotor activity data; assessing dose dependent ketamine–mediated PPI behavior prior to 2 hours would likely be confounded by locomotor effects. Mice treated with the lowest dose ketamine (3 mg/kg) showed PPI behavior that was comparable to vehicle-treated mice at all

decibel intensities tested, whereas mice treated with the higher dosages of ketamine (20 or 50 mg/kg) demonstrated PPI behavior that was significantly reduced at 74, 78, and 82 decibel intensities compared to vehicle (Figure 2-1b). Importantly, in this assay, we found that ketamine dose did not affect startle amplitude to pulse alone (Figure 2-1c).

To examine whether there is a dose dependence of ketamine on antidepressant responses, we utilized the forced swim test (FST) and novelty suppressed feeding (NSF) tests 2 hours after vehicle or ketamine (3 mg/kg, 20 mg/kg, or 50 mg/kg) administration (Figure 2-2 a & b). Again based on our locomotor activity data; assessment of ketamine–mediated antidepressant efficacy prior to 2 hours would likely be confounded by locomotor effects. In the FST, the lowest dose of ketamine (3.0 mg/kg) produced a significant decrease in immobility time compared to vehicle, suggestive of an antidepressant response (Figure 2-2a). In contrast, higher dosages of ketamine (20 mg/kg and 50 mg/kg) had no significant effect on immobility (Figure 2-2a), indicative of an absence of an antidepressant response. Similarly, in the NSF test, ketamine at the lowest dose (3 mg/kg) produced a significant decrease in the latency to feed in comparison to vehicle, again predictive of an antidepressant response (Figure 2-2b). Whereas the higher doses of ketamine (20 mg/kg and 50 mg/kg) produced no effect on the latency to feed, suggesting a lack of an antidepressant effect at high dose ketamine (Figure 2-2b). To control for appetite between groups, the amount of food consumed in the home cage was measured immediately after the NSF trial and no significant changes were observed between groups (Figure 2-2d).

We next investigated whether this dose dependent effect of ketamine on antidepressant responses was observed at 24 hours after drug treatment. In the FST, we found that mice injected with the lowest dose ketamine (3.0 mg/kg) and tested 24 hours later showed reduced immobility in comparison to vehicle treated mice (Figure 2-2c), indicative of a long–lasting antidepressant

effect. In contrast, higher doses of ketamine (20 mg/kg and 50 mg/kg) did not alter immobility time in the FST 24 hours after drug administration, again suggesting that higher doses of ketamine are unable to induce antidepressant responses (Figure 2-2c).

To evaluate the dose dependent effects of ketamine on a proposed mechanism of fast acting antidepressant efficacy, we measured phosphorylated eEF2 and total eEF2 protein levels in the hippocampus (Hc) and prefrontal cortex (PFC) by Western blot analysis 30 minutes after mice were treated with a vehicle or ketamine (3, 20 or 50 mg/kg) injection. As previously reported (Autry et al., 2011; Gideons et al., 2014), ketamine (3 mg/kg) significantly reduced phosphorylated eEF2 compared to total eEF2 in the hippocampus (Figure 2-3 a), whereas in the prefrontal cortex we observed only a trend for a decrease in phosphorylated eEF2 (Figure 2-3c), consistent with previous work (Autry et al., 2011). In contrast, in mice treated with higher doses of ketamine (20 or 50 mg/kg), no significant changes were observed in phosphorylated eEF2 in comparison to total eEF2 in the hippocampus and the prefrontal cortex (Figure 2-3 a &c). The inhibition of phosphorylated eEF2 was previously demonstrated to desupress protein translation and significantly upregulate BDNF protein necessary for the fast acting antidepressant response of ketamine (Autry et al., 2011). Therefore, we examined the protein levels of mature BDNF in the hippocampus and prefrontal cortex in these same mice that were treated with the various doses of ketamine. As previously shown (Autry et al., 2011; Gideons et al., 2014), the lowest dose of ketamine (3 mg/kg) significantly upregulated BDNF protein in the hippocampus and prefrontal cortex (Figure 2-3 b&d). In mice treated with higher doses of ketamine (20 or 50 mg/kg), no change in BDNF was detected in either the hippocampus or prefrontal cortex, thus demonstrating the dose dependent effects on this molecular signaling pathway.

Another purported mechanism of ketamine's fast acting antidepressant response involves phosphorylation of mTOR (Li et al., 2010). mTOR activation can occur at either sites Ser2448 or Ser2481. mTOR is phosphorylated differentially when associated with mTORC1 and mTORC2 complexes. mTORC1 contains mTOR phosphorylated predominantly on S2448, whereas mTORC2 contains mTOR phosphorylated predominantly on S2481 (Copp et al., 2009). Thus, we examined phosphorylated mTOR at these sites relative to total mTOR by Western blot analysis in the hippocampus and prefrontal cortex in the same mice used to examine ketamine's dose dependent effects on phosphorylated eEF2 and BDNF. The dose of ketamine did not impact levels of phosphorylated mTOR at either position Ser2448 or position Ser2481 relative to vehicle in either the hippocampus or prefrontal cortex 30 minutes after ketamine injection (data not shown), which is consistent with our previous work for ketamine at 3mg/kg (Autry et al., 2011).

While previous work has demonstrated the significant decrease in phosphorylated eEF2 and the upregulation of BDNF as an initial trigger for the antidepressant effects of low dose ketamine (Autry et al., 2011), we examined whether these effects were present 2 hours after ketamine administration and whether there would be a dose dependent effect of the drug at this time point. We measured phosphorylated eEF2 compared to total eEF2, as well as mature BDNF protein levels, in the hippocampus and prefrontal cortex by Western blot analysis 2 hours after vehicle or ketamine treatment (3, 20 or 50 mg/kg). Two hours following ketamine administration, the lowest dose of ketamine (3 mg/kg) had no effect on phosphorylated eEF2 compared to total eEF2 in the hippocampus or prefrontal cortex (Figure 4 a, c) or on mature BDNF in these brain regions (Figure 4 b, d). In animals treated with higher doses of ketamine (20 or 50 mg/kg) we also did not see any significant change in phosphorylated eEF2 or BDNF in

the hippocampus or prefrontal cortex at this time point (Figure 2-4 a-d). No significant changes were observed in phosphorylated mTORC at position S2448 or position S2441 in the hippocampus or the prefrontal cortex at 2 hours (data not shown).

Discussion

The present study provides novel insight into the dose dependent effects of ketamine in modulating fast-acting antidepressant responses. We show that higher doses of ketamine (20 and 50 mg/kg) mediate effects on total locomotor activity and prepulse inhibition but do not trigger fast-acting antidepressant responses. In contrast, low dose ketamine (3mg/kg) does not alter total locomotor activity or prepulse inhibition, but, rather, triggers rapid antidepressant responses in the forced swim test and novelty suppressed feeding paradigm. We demonstrate that low dose ketamine (3 mg/kg) rapidly decreases eEF2 phosphorylation, with corresponding increases in BDNF protein levels; a putative mechanism for rapid antidepressant responses that are not observed with high dose ketamine treatment. These results support our hypothesis that low dose ketamine activates a distinct signaling pathway involved in rapid antidepressant action that is not observed with high dose ketamine.

The most salient finding in our study is that our results are consistent with clinical data. Clinically, low dose ketamine produces a fast acting antidepressant response in treatment resistant depressed patients without observable psychomimetic effects, whereas high, anesthetic doses of ketamine have not been proven to induce antidepressant responses in similar patient populations (Berman et al., 2000; Zarate et al., 2006). In our study, low but not high dose ketamine induces rapid antidepressant responses 2 hours after drug injection in the FST and NSF paradigms with no alterations in locomotor activity or PPI. We found that low dose ketamine significantly decreased phosphorylation of eEF2 and increased BDNF protein levels 30 minutes after administration consistent with our recent study which proposed activation of this signaling cascade for rapid antidepressant responses (Autry et al., 2011). We also found that high dose ketamine (20 and 50 mg/kg) did not alter phosphorylation of eEF2 or BDNF levels. This data is

consistent with previous work which showed that hippocampal BDNF levels of mice pretreated with vehicle or ketamine (50 mg/kg) were indistinguishable at 1 hour or 7 days after administration (Lindholm et al., 2012), again suggesting that the theoretical mechanism for rapid antidepressant responses is not observed with high dose ketamine treatment.

Another proposed mechanism of ketamine's fast acting antidepressant response involves phosphorylation of mTOR. While we did not observe alterations in phosphorylation of mTOR relative to total mTOR in either the hippocampus or prefrontal cortex 30 minutes or 2 hours following any of the doses of ketamine administered (data not shown), this data is consistent with our previous work in which we showed that ketamine (3 mg/kg) does not induce phosphorylation of mTOR 30 minutes after administration (Autry et al., 2011). In contrast to our findings, however, another group has demonstrated that ketamine (10 mg/kg) induces mTOR activation one hour after ketamine administration in synaptoneurosomes isolated from the PFC(Li et al., 2010). The discrepancies between these two studies may be due to differences in ketamine dosage, the utilization of cellular extracts versus synaptoneurosomes, and the time assayed. Given the dose dependent aspect of our study, our data suggests that rapid dephosphorylation of eEF2 triggers rapid induction of BDNF protein expression and that activation of the mTOR pathway is downstream of the fast acting effects of low dose ketamine. Indeed, previous work has demonstrated that BDNF is upstream of mTOR and that BDNF is a potent activator of mTOR (Slipczuk et al., 2009). Therefore, it may be conceivable that rapid increases in BDNF protein translation may lead to downstream effects on mTOR at later time points

A critical role for BDNF in the fast-acting antidepressant effects of ketamine has recently been demonstrated in inducible BDNF knockout mice (Autry et al., 2011), as well as in BDNF

Val66Met knock in (KI) mice and BDNF Met66Met KI mice (Liu et al., 2012). In contrast, another study has reported that BDNF heterozygous mice still respond to ketamine mediated antidepressant action (Lindholm et al., 2012). This latter study injected mice with 50 mg/kg ketamine, a dose close to the anesthetic dose of the drug, and tested the mice 45 minutes later in the FST. A potential caveat of this particular study is that the reduction in immobility demonstrated by this group in the FST at 45 min in BDNF heterozygotes may be confounded by hyperactivity, as in our hands we observed a significant increase in locomotor activity 45 minutes after a 50 mg/kg ketamine injection. Moreover, the analysis of behavior in BDNF heterozygous mice may be difficult to interpret, because constitutive reduction of BDNF over the course of development may lead to developmental compensation. It is interesting that classical antidepressants targeted to the monoaminergic system also require BDNF, in particular BDNF expressed in the hippocampus, to exert an antidepressant effect (Adachi et al., 2008; Monteggia et al., 2004; Monteggia et al., 2007). These data would suggest that BDNF may be a common convergence point for antidepressant action, thus supporting the neurotrophic hypothesis of depression (Duman and Monteggia, 2006).

In contrast to our work, a previous study did not find that ketamine's effects were dose dependent (Moghaddam et al., 1997). This study used microdialysis to assay the effect of ketamine on glutamate efflux and found that ketamine did not dose dependently increase glutamate release in the prefrontal cortex (Moghaddam et al., 1997). However, results from this work should be interpreted with caution as the stress of saline injection was noted to produce an immediate increase in glutamate efflux in some animals, similar to that observed before injection of 50 mg/kg ketamine (Moghaddam and Bolinao, 1994) Moreover, the average extracellular

glutamate concentration was reported to be 0.8 pmol/µL, which is not physiological. Most studies report average extracellular glutamate concentrations from different brain regions to range between 1-30 µM, with the majority of studies finding levels between 1 and 5 µM (Moussawi et al., 2011).

While the dose dependent effects of ketamine on behavior are interesting, the link to a dose dependent effect on signaling is surprising. Previous biochemical studies have indicated that NMDA receptor antagonists may increase the release of endogenous excitatory amino acids, glutamate and aspartate (Liu and Moghaddam, 1995), which may then activate glutamatergic neurotransmission at non-NMDA receptors. Our data indicates that higher doses of ketamine (20 and 50 mg/kg) do not invoke BDNF signaling, whereas low dose ketamine (3 mg/kg) invokes BDNF signaling through the inactivation of eEF2 kinase and subsequent dephosphorylation of eEF2. Although the cellular mechanisms underlying high dose ketamine remains unclear, taken together with our behavioral data, which demonstrates that high ketamine mediates increases in locomotor activity and deficits in PPI behavior, our data is consistent with the hypothesis that there is a subset of NMDA receptors, possibly on GABAergic interneurons, that when activated lead to disinhibition of glutamate signaling (Lisman et al., 2008). Further characterization of these actions of NMDA receptor blockade and the signaling pathways that are regulated by high ketamine dosage will provide novel insight into the mechanism of ketamine's action at psychomimetic doses.

In summary, these data demonstrate a dose dependency to ketamine mediated antidepressant responses and to the specificity of the intracellular signaling mechanism involved in triggering an antidepressant response. Our results support a model in which low dose

ketamine specifically inactivates eEF2 kinase leading to a decrease in the phosphorylation of eEF2 and a subsequent rapid increase in BDNF protein which is necessary for rapid antidepressant responses. These data highlight the importance of dose consideration in the use of ketamine in eliciting an 'antidepressant response' in preclinical models as well as in the mechanistic understanding of rapid antidepressant action.

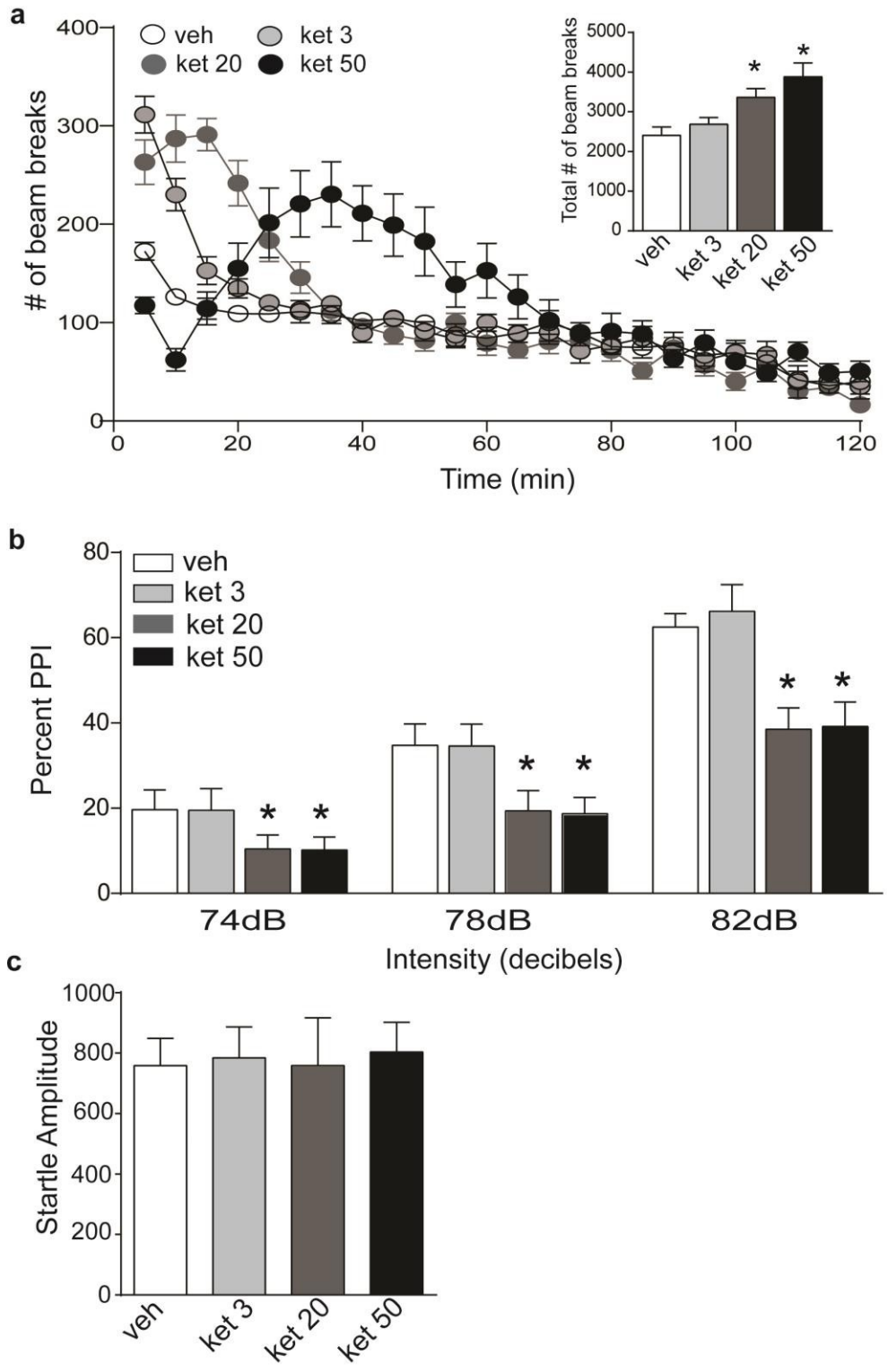


Figure 2-1. Ketamine's effect on locomotor behavior and PPI behavior is dose dependent. **a,** C57BL/6 mice were intraperitoneally injected with either vehicle or ketamine at 3.0 mg/kg, 20 mg/kg, or 50 mg/kg and immediately assessed for locomotor activity by recording the number of

photo beam breaks over 120 min in either 5 min increments or the entire 120 min period (inset). Analysis of variance (ANOVA) $F_{3,31} = 15.45$, $P = 0.0325$ for treatments. Tukey's post hoc test reveals significant increases in locomotor activity at the high dose of 20 and 50 mg/kg of ketamine, but not by the low 3.0 mg/kg of ketamine. Significance was set at *P<0.05. n=10/group. **b,** Pre-pulse inhibition (PPI) behavior was assessed 2 hrs after administration of either vehicle or ketamine at 3, 20, and 50 mg/kg. Percentage inhibition of the startle response was measured at different intensities (74, 78, and 82 dB). ANOVA $F_{15,144} = 164.2$, $P < 0.0001$ for treatments. Tukey's post test showed that at 74, 78, and 82 dB, mice treated with high ketamine doses (20 and 50 mg/kg), but not low doses (3 mg/kg), display significant decreases in PPI behavior in comparison to the vehicle treated group. Significance was set at *P<0.05. n=10/group. **c,** Startle amplitude for vehicle and ketamine treated groups. Significance was set at *P<0.05, n= 10 per group. The values represent mean ± SEM in all figures.

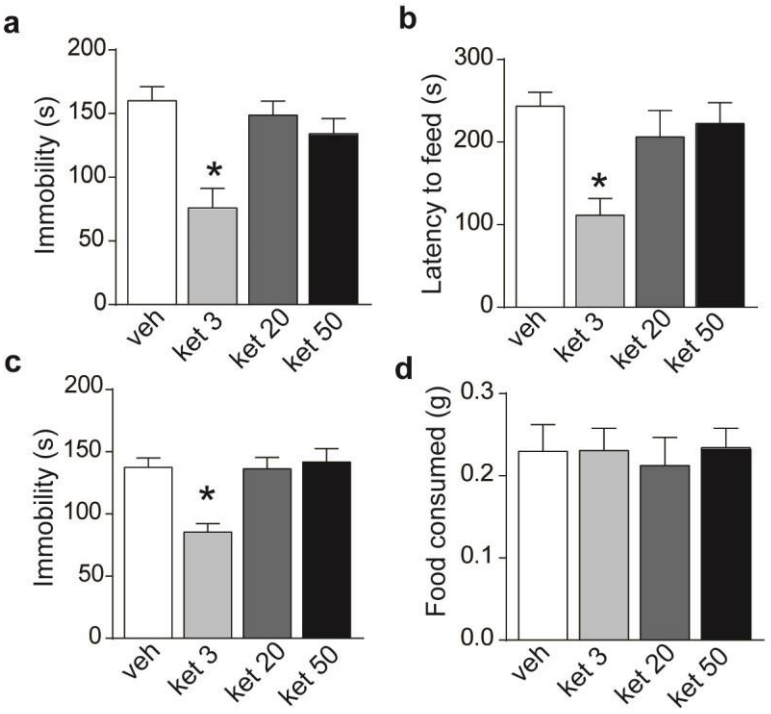
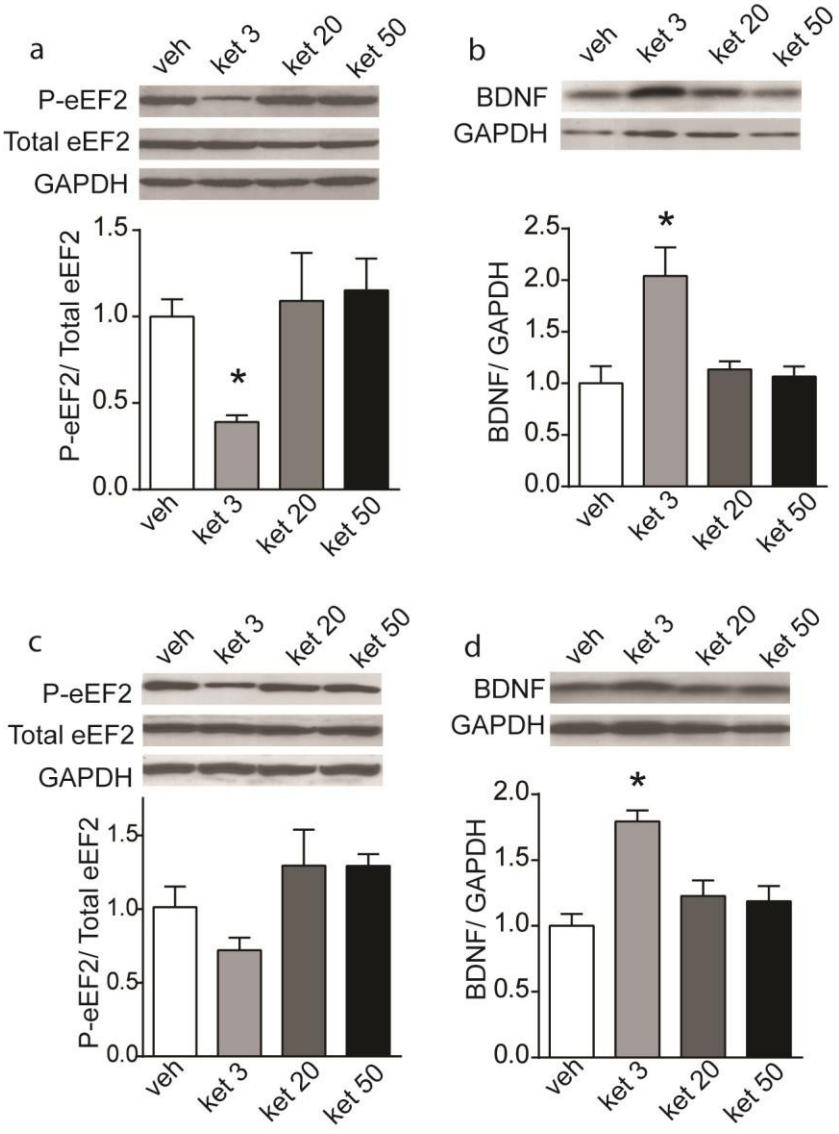


Figure 2-2 Ketamine's impact on acute and long lasting antidepressant responses is dose dependent. a, Immobility in FST was assessed 2 hours after ketamine injection. ANOVA, $F(3,29)=7.6$, $P=0.0007$ for treatment. Tukey's post hoc analysis demonstrates that the low 3.0 mg/kg dose of ketamine enhances mobility in comparison to vehicle, whereas the high dose ketamine at 20mg/kg and 50mg/kg do not alter immobility. *$P<0.05$. n=8-9 **b,** In NSF test, latency to feed was recorded 2 hours after ketamine administration. ANOVA, $F(3,29)= 6.487$, $P= 0.0017$ for treatment. Tukey's post hoc test reveals that low dose ketamine (3.0 mg/kg) produces a significant decrease in latency to feed in comparison to vehicle, whereas high dose ketamine (20mg/kg and 50 mg/kg) do not alter latency to feed *$P<0.05$. n=7-9. **c,** 24 hour analysis of FST behavior. ANOVA, $F(3,34)= 9.615$, $P<0.0001$ for treatment. Tukey's post hoc shows that 24hr after the low dose ketamine injection (3 mg/kg) a significant decrease in immobility in comparison to vehicle was observed. In contrast mice treated with high dose ketamine (20mg/kg and 50mg/kg) did not show altered immobility at 24 hours.*$P<0.05$. n=9-10 **d,** The amount of food consumed in the home cage was measured immediately after the 2 hour, NSF trial. ANOVA $F(3,29) =0.9427$, $P=0.9626$ for treatment. No significant changes were observed between groups. Significance was set at *$P<0.05$, n=7-9 per group.


a
veh
ket 3
ket 20
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P-eEF2
Total eEF2
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P-eEF2/ Total eEF2
1.5
1.0
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*
b
BDNF
GAPDH
BDNF/ GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
c
d

Figure 2-3. Ketamine induces acute changes in protein expression in a dose dependent manner 30 minutes after administration. Phosphorylated eEF2 and BDNF protein levels in the hippocampus (HC) and prefrontal cortex (PFC) were measured 30 min after intraperitoneal injection of either vehicle or ketamine at 3, 20, and 50 mg/kg. **a-b,** Densitometric analysis of hippocampal phospho-eEF2 levels (normalized to total eEF2) ***a,*** and BDNF normalized to GAPDH **b**. ANOVA $F(3,20) = 4.024$, $P=0.0216$ for treatment and ANOVA $F(3,20) = 8.020$, $P=0.0011$ for treatment, for phospho-eEF2 and BDNF, respectively. Tukey's post-hoc analysis shows that low ketamine (3.0 mg/ kg) significantly attenuates hippocampal phosphorylated eEF2 levels with a corresponding significant increase in hippocampal BDNF.n=6 peEF2; n=6 BDNF **c-d,** Densitometric analysis of prefrontal cortical phospho-eEF2 protein (normalized to total eF2)and BDNF protein normalized to GAPDH. ANOVA $F(3,19) = 4.987$, $P=0.102$ for treatment and ANOVA $F(3,20) = 11.18$, $P=0.0002$ for treatment, for phospho-eEF2 and BDNF, respectively. Tukey's post-hoc test demonstrates that low ketamine dose (3.0 mg/ kg) does not significantly decrease phospho-eEF2 in the prefrontal cortex compared to control, whereas significant increases in BDNF protein are observed in this same region. Significance was set at $*P<0.05$. n=5-6 peEF2; n=6 BDNF.

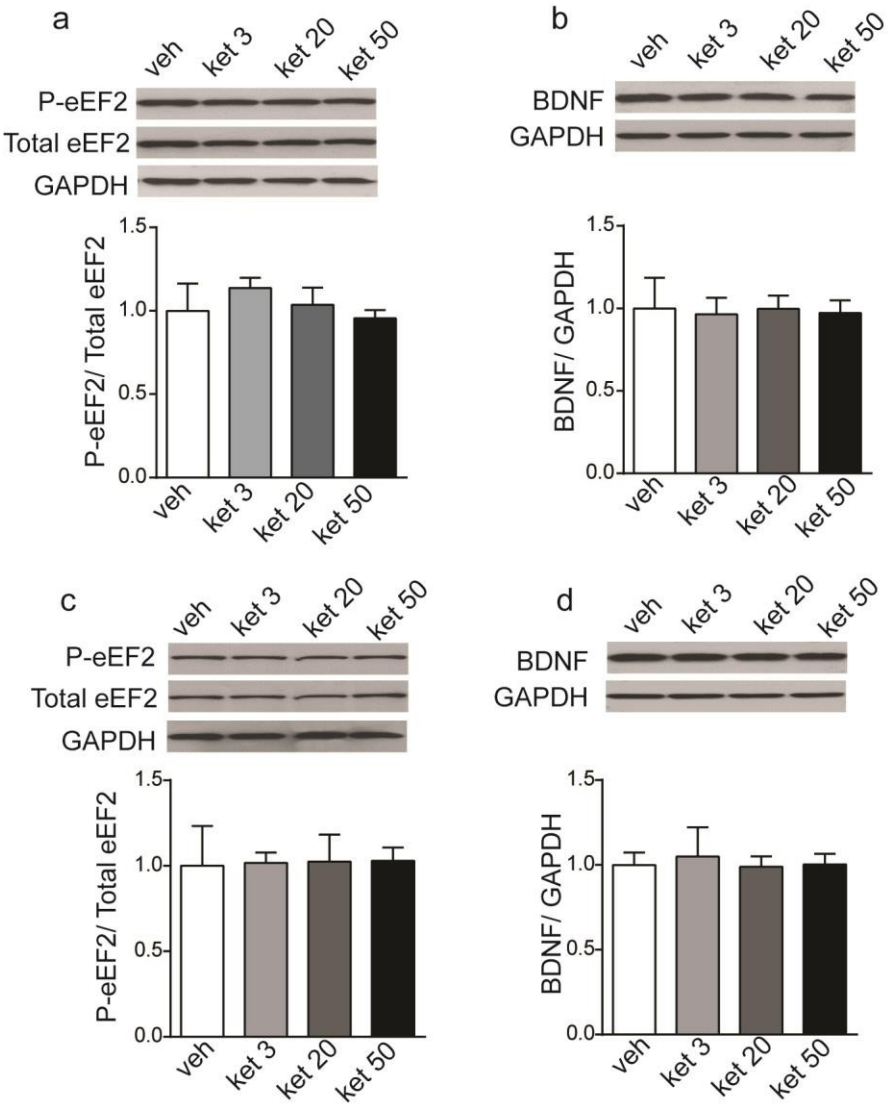

Figure 2-4. Ketamine dosage does not differentially impact protein expression at 2 hours. Phosphorylated eEF2, and BDNF protein levels in hippocampus (HC) and prefrontal cortex (PFC) were measured 2 hours after intraperitoneal injection of either vehicle or ketamine at 3, 20, and 50 mg/kg. **a-b,** Densitometric analysis of phosphorylated eEF2 normalized to total eEF2 in HC **a,** and hippocampal BDNF normalized to GAPDH **b.** ANOVA $F(3,16) = 0.9861$, $P=0.4242$ for treatment and ANOVA $F(3,20) = 0.02279$, $P=0.9952$ for treatment, for phospho-eEF2 and BDNF, respectively. No significant changes were observed. Significance was set at $^*P<0.05$. n=5 peEF2; n=6 BDNF. **c-d,** Densitometric analysis of phospho-eEF2 normalized to total eEF2 in the PFC **c,** and prefrontal cortical BDNF normalized to GAPDH **d**. ANOVA $F(3,16) =0.2013$, $P=0.8940$ for treatment and ANOVA $F(3,20)=0.06673$, $P=0.9769$ for treatment, for peEF2 and BDNF, respectively. No significant differences were observed between groups. Significance was set at $^*P<0.05$. n=5 peEF2; n=6 BDNF.

CHAPTER III

L-TYPE CALCIUM CHANNEL-MEDIATED ACTIVATION OF CALCINEURIN IS REQUIRED FOR KETAMINE'S RAPID ANTIDEPRESSANT EFFECTS

Introduction

Major depressive disorder (MDD) is a leading cause of disability worldwide with current estimates that approximately 7% of the population in a given year will develop depressive symptoms requiring treatment (Kessler et al., 2005). Traditional antidepressant medications are nearly all based on targeting the monoamine system, and while they are effective in some patients, they generally require several weeks of treatment before a clinical improvement is typically achieved (Brunoni et al., 2010). There is a crucial need for faster-acting and more effective antidepressants treatments, especially in patients at risk of suicide. Therefore, there is great interest in clinical data demonstrating that a single sub-psychomimetic dose of ketamine, an ionotropic glutamatergic *n*-methyl-*d*-aspartate (NMDA) receptor antagonist, produces rapid antidepressant responses in treatment resistant depressed (Berman et al., 2000; Zarate et al., 2006) or bipolar disorder (Price et al., 2009) patients with sustained effects lasting for up to 1-2 weeks in some individuals. These results support the premise that ketamine mediates rapid antidepressant effects by blocking NMDA receptors and impacting glutamatergic transmission. However, while current data has shown that ketamine is safe for the treatment of depression in a clinical setting, there is concern regarding its potential to induce psychosis as well as abuse liability. The identification of the mechanism by which ketamine potentiates the onset of

antidepressant responses may provide important target information for novel therapeutic approaches without the potential side effects associated with antagonism of NMDA receptors.

In previous work, we demonstrated that ketamine mediated blockade of NMDA receptors at rest deactivates eukaryotic elongation factor 2 (eEF2) kinase, resulting in a reduction of eEF2 phosphorylation and a desuppression of BDNF translation necessary to elicit synaptic and behavioral effects required for antidepressant efficacy (Autry et al., 2011). Importantly, we were able to show that eEF2 kinase inhibitors induce fast-acting behavioral antidepressant-like effects in wild type mice (Autry et al., 2011), and that ketamine does not elicit an antidepressant response in eEF2 kinase knockout mice (Nosyreva et al., 2013), demonstrating that inhibition of eEF2 kinase is both necessary and sufficient to elicit antidepressant-like responses of ketamine.

The inhibition of eEF2 kinase by pharmacological inhibitors or by ketamine administration leads to a decrease in the phosphorylation of eEF2, the only known target for eEF2 kinase (Ryazanov, 2002), which in turn triggers effects on dendritic protein translation that are required for behavioral and synaptic antidepressant effects (Autry et al., 2011; Nosyreva et al., 2013). This finding implies that the activity of a protein phosphatase balances the action of eEF2 kinase on eEF2 phosphorylation levels. However, to date the protein phosphatase involved in eEF2 dephosphorylation in neurons remains unknown. Given the proposed role of eEF2 phosphorylation as a key determinant of ketamine's rapid antidepressant action, we hypothesized that the protein phosphatase specific for phosphorylated eEF2 to balance eEF2 kinase action is a necessary component for ketamine mediated rapid antidepressant responses.

Materials and Methods

Mice

Male C57BL/6 mice aged 6-8 weeks old were habituated to the animal facility for one week prior to behavioral testing. The mice were habituated to the behavioral room for one hour prior to behavioral testing. Mice were kept on a 12 hour light/dark cycle and given access to food and water *ad libitum*. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Drugs

For *in vitro* studies, drugs were used at the following final concentrations: ketamine 10 µM, tetrodotoxin 1.0 µM (TTX), okadaic acid 0.1 µM and 1.0 µM, fostriecin 40 nM, cyclosporin A 1.0 µM, FK506 50 nM, nimodipine 10 µM, BAYK8644 1.0 µM, and BAPTA-AM and EGTA-AM 10 µM. For *in vivo* studies all drugs were given intraperitoneally (i.p.) at the following concentrations: ketamine 3.0 mg/kg in 0.9% saline, and nimodipine 5.0 mg/kg and FK506 5.0 mg/kg in 20% DMSO. Reagents were purchased from the following sources: ketamine, okadaic acid, fostriecin, cyclosporin A, and FK506 (Tocris Biosciences); and nimodipine, BAYK8644, BAPTA-AM, and EGTA- AM (Invitrogen).

Cell culture

Dissociated hippocampal cultures were prepared as previously described (Kavalali et al., 1999). Briefly, whole hippocampi were dissected from postnatal day 1-2 (P1-2) C57BL/6 mice. Tissue was trypsinized (10 mg/ml trypsin) for 10 min at 25°C, mechanically dissociated by pipetting,

and plated onto Matrigel coated 6 well plates for phosphatase inhibitor and Ca^{+2} buffering experiments or coverslips for electrophysiological experiments. Cytosine arabinoside (4 µM ARAC) was added at day 1 *in vitro* (DIV). The concentration of ARAC was then reduced to 2 µM at 4 DIV. All experiments were performed on 14-21 DIV cultures. All experiments were conducted by an observer blind to group assignments.

In vitro inhibitor experiments

Hippocampal cultures were individually treated with and without the phosphatase inhibitors fostriecin, cyclosporin A, FK506, and low or high concentrations of okadaic acid for 1.5 h at 37°C. After 1 h, ketamine, ketamine/tetrodotoxin, or vehicle were added to the cultures for 30 min then washed with pre-equilibrated media for 1 h at 37°C. Neurons were subsequently quickly washed in phosphate buffered saline (PBS), re-suspended in 1 X sample buffer, containing, in mM, 62.5 Tris HCL, pH 6.8, at 25ºC, 2% w/v SDS, 10% glycerol, 50 DTT, 0.01% w/v bromophenol blue. Reactions were sonicated for 30 seconds and boiled for 5 minutes at 95º C, then 15uL per well were loaded onto 10% SDS- polyacrylamide gels and analyzed by immunoblotting. For the calcium buffering experiments and those utilizing nimodipine and BAYK8644 experiments were performed identically to the phosphatase inhibitor experiments.

Electrophysiology

Miniature excitatory postsynaptic currents (mEPSCs) were recorded from dissociated hippocampal cultures using the whole-cell voltage-clamp configuration. Data was acquired using a Multiclamp 700B amplifier and Clampex 10.0 software (Molecular Devices). Recordings were sampled at 100 µs, filtered at 2 kHz, and with a gain of 5. The pipette solution

contained the following (in mM): 110 K-gluconate, 20 KCl, 10 NaCl, 10 HEPES, 0.6 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 10 QX-314, pH 7.3 by KOH, 305 mOsm. A modified Tyrode's solution was used as the external solution (in mM): 150 NaCl, 4 KCl, 10 glucose, 2 $CaCl_2$, 1.25 $MgCl_2$, 10 HEPES, pH 7.4 with NaOH, 300 mOsm. To isolate mEPSCs, 1 µM tetrodotoxin and 50 µM picrotoxin were added to the modified Tyrode solution prior to recording. To assess the effect of Ca^{2+} chelators on mEPSC frequency and amplitude, 1 h prior to recording, coverslips were incubated in 10 µM BAPTA-AM, 10 µM EGTA-AM, or vehicle (0.1% DMSO) and then mEPSCS were recorded with the associated chelator or vehicle in the external solution. Data was analyzed using Clampfit 10.3 software and statistical analyses were done in GraphPad 5 (Prizm). All experiments were conducted by an observer blind to group assignments.

Protein quantification and Immunoblotting

Anterior hippocampal slices (2/mouse, ~1 mm thick) were dissected from C57BL/6 mice receiving saline, vehicle, ketamine (3.0 mg/kg), or FK506 (5.0 mg/kg) i.p. 30 minutes post-injection and rapidly frozen. Tissue was homogenized in a radioimmunopreciation assay (RIPA) buffer containing: Tris 50 mM, Igepal 1.0%, SDS 0.1%, sodium deoxycholate 0.5%, EDTA 4.0 mM, NaCL 150 mM, pH 7.4. Protease (Roche) and phosphatase inhibitors (sodium pyrophosphate 10 mM, sodium fluoride 50 mM, and sodium orthovanadate 2.0 mM) were added immediately prior to use. Total protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce). Samples were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with anti-phospho-T56 eEF2 antibody (peEF2; 1:2000; Cell Signaling), anti- total eEF2 (teEF2; 1:2000; Cell Signaling), anti- BDNF (1:200; Santa Cruz) and anti-GAPDH antibody (1:50,000; Cell Signaling) as previously described (Autry et al., 2011).

Immunoreactive bands were visualized by enhanced chemoluminescence and captured on autoradiography film (Light Labs). Digital images were produced by densitometric scans of the autoradiographs on a ScanJet 4300C (Hewlett Packard) and quantified using Image J software. ECL developed bands were exposed to film, and films were analyzed by ImageJ. Phosphorylated eEF2 and BDNF band intensities were taken as a ratio of total eEF2 and GAPDH values, respectively. Significant differences between treatment and control for protein levels were determined using a one way analysis of variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons.

Time course experiment

Separate cohorts of C57BL/6 adult male mice were i.p. injected with either vehicle, FK506 (5.0 mg/kg), or nimodipine (5.0 mg/kg) one hour prior to testing. Thirty minutes prior to testing, mice received either saline or ketamine injection (3.0 mg/kg) (n=10 per group). For 24 hour experiments, mice were given vehicle, FK506 (5.0 mg/kg), or nimodipine (5.0 mg/kg) 30 minutes prior to an injection of ketamine and tested in the FST one day later. All behavioral experiments were conducted and scored by an observer blind to group assignment.

Novelty suppressed feeding

The novelty suppressed feeding paradigm was performed as described previously (Autry et al., 2011). Briefly, group housed animals were food deprived for 24 hours prior to testing with free access to water. For the test, a single pellet of the mouse's regular food chow was placed in the center of 42 × 42 cm open field arena and individual mice were placed in the corner of the arena. The light level in the open field was maintained at 40 lux. Latencies to approach and begin

eating were recorded within a limit of 10 minutes. As soon as the mouse was observed to eat or the 10 minute period was reached, the mouse was removed from the arena and returned to the home cage. Animals were then individually housed in temporary home cages. As a control for appetite, the amount of food consumed within the temporary home cage was defined as weight of chow consumed in 5 minutes.

Forced swim test

The forced swim test was performed as previously described (Adachi et al., 2008; Autry et al., 2011). In brief, mice were placed in a 4-L Pyrex glass beaker containing 3-L of water at 24 ± 1º C for six minutes. Water was changed between subjects. All test sessions were recorded by a video camera positioned adjacent to the beakers. The videotapes were scored by an observer blind to group assignment during the last four minutes of the six minute trial.

Locomotor activity

Mice were placed individually in a standard mouse cage with fresh bedding. Locomotor activity was recorded for 120 minutes under red light by photocell beams coupled to computer acquisition software (San Diego Instruments).

Statistics

Data was analyzed by Analysis of Variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons unless otherwise specified. . Immunohistochemistry was quantified using Image J analysis combined with ANOVA. Data was presented as mean ± SEM and significance was set at $p < 0.05$

Results

Calcineurin dephosphorylates eEF2 in neurons

We systematically tested whether inhibition of key protein phosphatases reverses eEF2 dephosphorylation seen after resting NMDA receptor blockade. Dissociated hippocampal cultures were treated with protein phosphatase inhibitors for 1 hour in the presence of vehicle, ketamine, or ketamine/tetrodotoxin (TTX) to rule out effects due to evoked transmission. Previous data had suggested that protein phosphatase 2A (PP2A) dephosphorylates eEF2 in reticulocyte lysates (Browne and Proud, 2002; Redpath and Proud, 1989). We therefore used low concentrations of okadaic acid (OA) as well as the more selective PP2A inhibitor fostriecin to investigate the role of PP2A on phosphorylated levels of eEF2 in dissociated hippocampal cultures. Western blot analysis revealed that ketamine and ketamine/TTX did not alter total levels of eEF2, however both equally reduced levels of phosphorylated eEF2, suggesting that the decrease in eEF2 phosphorylation is not due to alterations in total levels of eEF2 (Figure 3-1a,b), in agreement with previous data (Autry et al., 2011). The indistinguishable levels of phosphorylated eEF2 between ketamine and ketamine/TTX treatment further suggests that evoked neurotransmission is not involved in the regulation of phosphorylated eEF2 by low dose ketamine, highlighting the distinct intracellular signaling cascades that are triggered by block of NMDA receptors at rest compared to evoked activity. Application of either low dose okadaic acid (0.1 μM) or fostriecin had no effect on reversing ketamine- or ketamine/TTX-mediated reductions in phosphorylated eEF2 suggesting that PP2A is not the main regulator of eEF2 dephosphorylation in hippocampal neurons (Figures 3-1a and 3-1b). We next used high dose OA to examine the contribution of protein phosphatase 1 (PP1) in ketamine-mediated dephosphorylation of eEF2. Again, we found that ketamine and ketamine/TTX both reduced

levels of phosphorylated eEF2 to a similar extent suggesting that evoked synaptic activity was not mediating ketamine's reductions in phosphorylated eEF2 (Figure 3-1c). We found that high doses of OA had no effect on ketamine-mediated dephosphorylation of eEF2 indicating PP1 does not regulate levels of phosphorylated eEF2 in this pathway (Figure 3-1c). To examine the contribution of calcineurin to phosphorylated eEF2 levels in hippocampal neurons, we tested the calcineurin inhibitors, cyclosporin A or FK506. In agreement with the previous data, ketamine and ketamine/TTX reduced the level of phosphorylated eEF2 to similar levels (Figure 3-1d and 3-1e). However, in contrast to the previous phosphatase inhibitor data, cyclosporin A and FK506 blocked ketamine mediated reductions in phosphorylated eEF2 levels and reversed levels of phosphorylated eEF2 slightly higher than baseline (Figure 3-1d and 3-1e). Taken together, these data identify calcineurin as the protein phosphatase involved in dephosphorylating eEF2 in hippocampal neurons.

Calcineurin is required for ketamine's rapid antidepressant response

We examined whether inhibition of calcineurin through its effects on eEF2 dephosphorylation impacts the ability of ketamine to trigger a fast acting antidepressant response. We pretreated C57BL/6 mice with vehicle or FK506, which has been demonstrated to cross the blood brain barrier (Murakami et al., 2004), 30 minutes before a low dose of ketamine, and assessed behavior 30 minutes or 24 hours after ketamine treatment. Previous work had shown that ketamine mediates a rapid antidepressant response in the forced swim test and the novelty suppressed feeding test (Autry et al., 2011; Li et al., 2010; Lindholm et al., 2012; Popik et al., 2008). In initial experiments we demonstrated that FK506 did not impact locomotor activity suggesting that the behavioral responses cannot be explained by differences in locomotor

activity (Supplementary Figure 3-1). In agreement with previous data, ketamine induced a rapid (30 minute) and a long lasting (24 hour) antidepressant response in separate cohorts of animals in the FST as assessed by a significant decrease in immobility (Figures 3-2a and 3-2b). We found the calcineurin inhibitor FK506 by itself had no effect on immobility in the FST at either time point (Figures 3-2a and b). However, FK506 coapplication prevented ketamine's antidepressant response in the FST at both 30 minutes (Figure 3-2a) and 24 hours after ketamine administration (Figure 3-2b). In the NSF test, ketamine significantly reduced the latency to acquire a food pellet in comparison to vehicle, suggestive of an antidepressant response (Figure 3-2c). We found that FK506 by itself did not alter the antidepressant response in these mice in this test (Figure 3-2c). However, we again found that the ketamine triggered antidepressant response was blocked by FK506 co-treatment (Figure 3-2c). The behavioral data in the NSF test was not compounded by changes in the amount of food consumed by the different drug treatments (Figure 3-2d). To examine whether inhibiting calcineurin impacts phosphorylated eEF2 levels *in vivo*, C57BL/6 mice were injected with vehicle or FK506 at 30 minutes prior to ketamine administration and then animals were sacrificed 30 minutes after ketamine treatment. In agreement with our previous findings (Autry et al., 2011), ketamine significantly reduced phosphorylated eEF2 levels while total levels of eEF2 levels were unchanged in the hippocampus (Figure 3-2e). We also found by Western blot that FK506 attenuated ketamine mediated decreases in phosphorylated eEF2 levels (Figure 3-2e), suggesting that calcineurin is involved in regulating levels of phosphorylated eEF2 *in vivo*. The reduction of phosphorylated eEF2 is indicative of desupression of protein translation. Previous work from our group demonstrated that inhibition of eEF2 kinase, which results in significantly decreased levels of phosphorylated eEF2, triggers an upregulation of BDNF protein levels necessary for the fast

acting antidepressant response of ketamine (Autry et al., 2011). We therefore examined BDNF protein levels in the hippocampus of these mice and found that the significant upregulation of BDNF protein by ketamine (Figure 3-2f) was blocked by FK506 pretreatment (Figure 3-2f).

L-type calcium channels are the primary source of calcium that drives calcineurin activity

Thus far, our data demonstrate that calcineurin dephosphorylates eEF2 and subsequently upregulates BDNF protein expression, necessary requirements for ketamine's fast acting antidepressant effects, and that inhibition of calcineurin blocks the rapid antidepressant response of ketamine. It is intriguing that eEF2 kinase and calcineurin, regulators of eEF2 phosphorylation, are both calcium-calmodulin dependent enzymes suggesting calcium may impact the balance of eEF2 phosphorylation in hippocampal neurons. To test this possibility we applied the fast acting calcium chelator, BAPTA-AM, to cultured hippocampal neurons in parallel with ketamine or ketamine/TTX. In agreement with our earlier data, ketamine and ketamine/TTX significantly reduced phosphorylated eEF2 to comparable levels (Figure 3-3a and 3-3b). We found BAPTA treatment alone significantly reduced levels of phosphorylated eEF2 similar to ketamine or ketamine/TTX (Figure 3-3a). We also found that BAPTA blocked ketamine or ketamine/TTX mediated reductions in phosphorylated eEF2 (Figure 3-3a). These data suggest that the ketamine mediated blockade of NMDA receptors that inhibits eEF2 kinase and decreases phosphorylation of eEF2 is countered by BAPTA likely inhibiting calcineurin activity. To further examine a role for calcium in controlling eEF2 phosphorylation levels we tested the effect of the slow acting calcium chelator, EGTA-AM, on cultured hippocampal neurons. We found that EGTA treatment alone significantly reduced levels of phosphorylated eEF2 to comparable levels seen with ketamine or ketamine/TTX treatment (Figure 3-3b).

However, EGTA did not reverse ketamine mediated reductions in phosphorylated eEF2 levels (Figure 3-3b) suggesting EGTA was not blocking the ability of calcineurin to dephosphorylate eEF2. To investigate whether the BAPTA and EGTA differences on hippocampal neurons are due to presynaptic glutamate release, we utilized an electrophysiology approach. We treated hippocampal cultures with BAPTA-AM or EGTA-AM under the same conditions as our biochemical analysis and recorded miniature excitatory post-synaptic currents (mEPSCs). We found that under the same conditions as above, BAPTA and EGTA treatment significantly reduced mEPSC frequency to comparable levels (Figures 3-3c and 3-3d) while having no effects on mEPSC amplitudes (Figure 3-3e) in comparison to vehicle treated cultures. These data show that, in agreement with earlier observations (Nosyreva and Kavalali, 2010; Xu et al., 2009), BAPTA and EGTA suppress spontaneous glutamate release in hippocampal neurons and therefore are likely to partially suppress eEF2 kinase activity. However, the equal effectiveness of BAPTA and EGTA on presynaptic release also suggests that the differential action of BAPTA and EGTA on ketamine-mediated dephosphorylation of eEF2 is likely due to postsynaptic effects. Taken together, these data demonstrate that the difference in eEF2 phosphorylation levels depends on whether calcium signals are buffered by the use of BAPTA or EGTA, suggesting specificity to the calcium source in impacting calcineurin activity.

This hypothesis is consistent with previous work that has demonstrated differences in postsynaptic signaling due to BAPTA suppressing rapid calcium rises near the sources of calcium entry whereas EGTA suppresses rises in bulk calcium microns away from the source (Deisseroth et al., 1996; Wheeler et al., 2012). Our findings show that in the presence of BAPTA, the ketamine mediated blockade of NMDA receptors that triggers inhibition of eEF2 kinase and decreased levels of phosphorylated eEF2 is reversed suggesting that BAPTA is

inhibiting the calcineurin effect. Since ketamine inhibits NMDA receptor mediated calcium influx and suppresses eEF2 kinase, the source of the calcium signal that is masked by BAPTA is distinct from NMDA receptors.

A potential candidate for the source of calcium that regulates calcineurin is the L-type voltage gated Ca^{2+} channel which has previously been shown to strongly impact postsynaptic signaling (Deisseroth et al., 2003). Moreover, prior work has documented that these channels are present on hippocampal neuronal dendrites (Kavalali et al., 1997) and show significant activity at near resting membrane potentials (Avery and Johnston, 1996; Kavalali and Plummer, 1996; Magee et al., 1996). In addition, calmodulin, the cofactor critical for activating calcineurin, is a critical binding partner for L-type calcium channels (Peterson et al., 1999; Zuhlke et al., 1999). To investigate whether the calcium mediated regulation of calcineurin on eEF2 phosphorylation levels is through L-type voltage gated channels, we pretreated hippocampal neurons with nimodipine, a selective L-type voltage gated calcium channel blocker. In the presence of nimodipine we found that it impacted the baseline phosphorylation of eEF2 (Figure 3-3f). However, we found that nimodipine reversed ketamine mediated reductions in phosphorylated eEF2 without alterations in total eEF2 levels (Figure 3-3f), thus partially mimicking the effect of calcineurin inhibition. To further investigate whether the calcium mediated regulation of calcineurin on eEF2 is through L-type voltage gated calcium channels, we pretreated hippocampal neurons with BAYK8644, a selective L-type voltage-gated calcium channel agonist. In the presence of BAYK8644, we found that it increased baseline levels of phosphorylated eEF2, suggesting that increased calcium entry may trigger eEF2 kinase activity. We additionally found that BAY8644 did not reverse ketamine mediated reductions in phosphorylated eEF2 (Figure 3-3g), implicating that the influx of calcium induced

by this selective L-type agonist may be triggering calcineurin activity, providing further support that L-type voltage-gated channels may regulate calcineurin activity.

L-type calcium channels impact ketamine's rapid antidepressant response

The parallels between the actions of nimodipine and FK506 suggests that L-type Ca^{2+} channels may be the source of Ca^{2+} at rest leading to activation of calcineurin and ultimately dephosphorylation of eEF2 by ketamine that is necessary for rapid antidepressant efficacy. To test this hypothesis, we examined the effects of inhibiting L-type voltage gated calcium channels on ketamine mediated antidepressant-like behavior. In initial experiments, we demonstrated that nimodipine did not impact locomotor activity (Supplementary Figure 3-1). We tested the ability of nimodipine to influence ketamine mediated antidepressant responses using the FST and NSF paradigms. Mice were pretreated with vehicle or nimodipine 30 minutes prior to ketamine administration and behavior was assessed 30 minutes or 24 hour later in separate cohorts of animals. In agreement with our earlier data, ketamine produced a rapid (30 minutes) and long lasting (24 hours) antidepressant effect following ketamine administration in the FST (Figures 3-4a and 3-4b). We found that nimodipine alone did not affect baseline behavior in the FST at either time point (Figures 3-4a and 3-4b). In contrast, nimodipine blocked ketamine mediated antidepressant responses at 30 minutes or 24 hours in this behavioral paradigm (Figures 3-4a and 3-4b). To further explore the involvement of L-type calcium channels on ketamine mediated antidepressant responses, we also tested nimodipine in the NSF paradigm. Again, we found that nimodipine by itself did not impact this behavioral measure (Figure 3-4c). In agreement with our previous data, we found that nimodipine blocked the ability of ketamine to mediate an antidepressant effect in the NSF paradigm (Figure 3-4c) without any effect on the amount of

food consumed (Figure 3-4d). We next examined whether nimodipine impacted ketamine mediated changes in eEF2 phosphorylation in the hippocampus. We found that nimodipine blocked ketamine-mediated decreases in phosphorylated eEF2 levels in the hippocampus within 30 minutes following drug treatment with no effect on total eEF2 levels (Figure 3-4e). Moreover, we found that nimodipine blocked ketamine-mediated increases in BDNF protein levels in the hippocampus at this same time point (Figure 3-4f).

Discussion

In this study, we demonstrate that the activity of the calcium calmodulin-dependent phosphatase, calcineurin, controls ketamine-mediated decreases in eEF2 phosphorylation levels *in vitro* and *in vivo* and that it is required for ketamine to trigger fast-acting antidepressant responses. Additionally, we show that the fast calcium buffer BAPTA but not the relatively slow buffer EGTA reverses ketamine-mediated reductions in phosphorylated eEF2, while both BAPTA and EGTA equally reduce mEPSC frequency, suggesting that the source of postsynaptic calcium entry which regulates calcineurin is independent of NMDA receptors. We demonstrate that L-type Ca^{2+} channel function is required to activate calcineurin and elicit ketamine's behavioral effects. These results support the premise that L-type calcium-mediated calcium influx at rest activates calcineurin, which in turn dephosphorylates eEF2 thus augmenting BDNF signaling to elicit ketamine's rapid antidepressant effect.

The most salient and potentially clinically relevant finding in our study was the robust effect of nimodipine on ketamine mediated antidepressant responses. In the FST and NSF tests, inhibition of L-type Ca^{2+} channels blocked ketamine-mediated antidepressant like effects. Moreover, nimodipine reversed ketamine-mediated reductions in phosphorylated eEF2 levels both *in vitro* and *in vivo* leading to suppression of ketamine-mediated increases in BDNF protein levels. These findings are consistent with earlier work that documented significant activity of L-type Ca^{2+} channels at near resting membrane potentials in hippocampal neurons (Kavalali and Plummer, 1996; Magee et al., 1996) and their potential to influence neuronal signaling at near resting membrane potentials (Wang et al., 2011). Moreover, calmodulin, the cofactor critical for activating calcineurin, is a critical binding partner for L-type calcium channels (Peterson et al., 1999; Zuhlke et al., 1999). The current findings uncover a parallel L-type calcium channel

mediated calcium signaling pathway that dephosphorylates eEF2 and competes with the previously identified NMDA receptor dependent signaling that activates eEF2K function (Supplementary Figure 3-2) (Scheetz et al., 2000; Sutton et al., 2007). The balance between these two calcium signaling pathways determines the degree of eEF2 phosphorylation and the extent of eEF2-dependent protein translation, which in turn gauges the efficacy of ketamine-mediated rapid antidepressant responses in preclinical mouse models (Autry et al., 2011). This premise is also consistent with a recent study from our group which showed that ketamine mediated rapid antidepressant responses were blocked in eEF2 kinase knockout mice and ketamine failed to increase BDNF protein levels in eEF2 kinase deficient hippocampi (Nosyreva et al., 2013). Taken together, these data provide additional support that ketamine's fast-acting behavioral responses requires eEF2 kinase and suggest that NMDAR blockade and deactivation of eEF2K is instructive to ketamine's behavioral effect. However, it is important to note that inhibition of calcineurin alone does not produce a fast acting antidepressant effect but that inhibition of calcineurin blocks ketamine's behavioral effects, suggesting that the effect of calcium entry through L-type calcium channels on calcineurin is permissive to ketamine's behavioral effects, rather than instructive.

Altered intracellular calcium levels have been suggested to play a role in the pathophysiology of affective disorders such as depression (Dubovsky, 1993; Eckert et al., 1993). Previous studies have shown that nimodipine can trigger an antidepressant response in some animal studies (Brunoni et al., 2011; Pazzaglia et al., 1998; Post et al., 1997) and, in agreement with our current study, not in others (Czyrak et al., 1989). This apparent discrepancy among studies could be due to the dosing of the drug, the animal species, the background of the animal, and the behavioral test, among other possibilities. However, our findings that nimodipine blocks

ketamine-mediated behavioral responses has clinical support. A double-blind placebo-controlled study demonstrated that pretreatment with nimodipine modulated ketamine responses (Krupitsky et al., 2001). This study found that nimodipine reduced the capacity of ketamine to induce psychosis, negative symptoms, altered perception, dysphoria, verbal fluency impairment, and learning deficits, suggesting that antagonism of L-type calcium channels attenuates the behavioral effects of ketamine in humans. Our present findings provide mechanistic insight as to how nimodipine may block ketamine's antidepressant responses.

In conclusion, our results identify calcineurin as a key regulatory component of ketamine-mediated rapid antidepressant responses. The crucial role for calcineurin in the regulation of eEF2 phosphorylation bolsters the premise that ketamine acts via inactivation of eEF2 kinase leading to a decrease in phosphorylation of eEF2 and a subsequent rapid increase in BDNF protein which is necessary for rapid antidepressant responses. The bidirectional regulation of eEF2 phosphorylation and subsequent increase in BDNF expression further expand this model (Supplementary Figure 3-2). Finally, as neuronal voltage-gated calcium channels are already implicated in the pathophysiology of a number of neuropsychiatric disorders and can be pharmacologically or molecularly targeted by a wide range of pathways (Lipscombe et al., 2004; Ma et al., 2013), their role in ketamine's rapid antidepressant action opens up novel avenues for treatment advance against major depressive disorder.

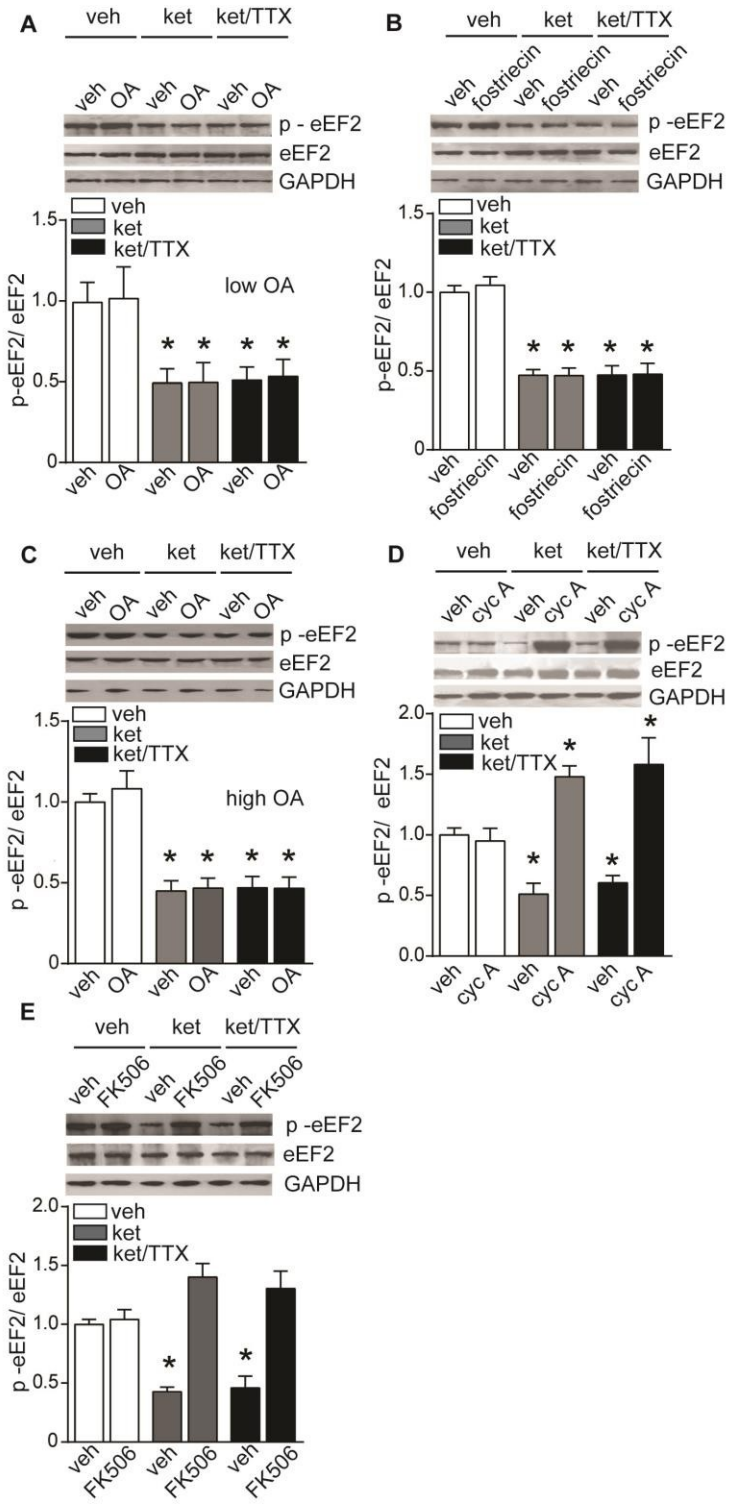

Figure 3-1. Inhibition of calcineurin blocks ketamine-mediated reductions in p-eEF2 levels. **A-E,** Primary hippocampal cultures were treated with either OA (0.1 μM), fostriecin (40 ηM), OA (1 μM), cyc A (1 μM), or FK506 (50 ηM) for 1 hour prior to being treated in parallel with

vehicle, ketamine (10 μM), or ketamine and TTX (10 and 1 μM, respectively). **A,** Densitometric analysis of p-eEF2 (normalized to total eEF2) in primary hippocampal cell culture following OA treatment (0.1 μM) in the presence and absence of vehicle, ketamine, or ketamine/TTX (ANOVA $F(5,42)=14.72$, $P<0.0001$ for treatment). Tukey's post-hoc analysis shows that ketamine and ketamine/ TTX siginificantly reduce p-eEF2 levels and that this effect is not reversed by low OA *$P<0.05$. N=8/group. **B,** Densitometric analysis of p-eEF2 (normalized to total eEF2) after fostriecin treatment (40 ηM) in the presence and absence of vehicle, ketamine, or ketamine /TTX (ANOVA $F(5,42)=28.53$, $P<0.0001$for treatment). Tukey's post-hoc analysis reveals that ketamine and ketamine/TTX decrease p-eEF2 levels and that fostriecin does not attenuate ketamine–mediated decreases in p-eEF2. *$P<0.05$ (N=8/group). **C,** Densitometric analysis of p-eEF2 (normalized to total eEF2) after OA treatment (1 μM) in the presence and absence of vehicle, ketamine, or ketamine/TTX (ANOVA $F(5,36)=16.57$, $P<0.0001$ for treatment). Tukey's post-hoc test demonstrates that ketamine and ketamine/TTX reduce p-eEF2 levels and that high OA does not block ketamine-mediated reductions in p-eEF2 levels. * $P<0.05$; N=7/group p ***D,*** Densitometric analysis of p-eEF2 (normalized to total eEF2) in primary hippocampal cell culture following cyc A treatment in the presence and absence of vehicle, ketamine or ketamine/TTX (ANOVA $F(5,30) =14.07$, $P<0.0001$ for treatment). Tukey's post-hoc analysis shows that ketamine and ketamine/ TTX significantly reduce p-eEF2 levels and that the ketamine effect is reversed by cyc A. *$P<0.05$. N=6. ***E,*** Densitometric analysis of p-eEF2 (normalized to total eEF2) in primary hippocampal cell culture following FK506 treatment in the presence and absence of vehicle, ketamine, or ketamine/TTX (ANOVA $F(5,30)=16.57$, $P<0.0001$ for treatment).Tukey's post-hoc test demonstrates that ketamine-mediated reductions in p-eEF2 are blocked by FK506. Significance was set at *$P<0.05$. N=6 /group. The values represent mean ± SEM in all figures.

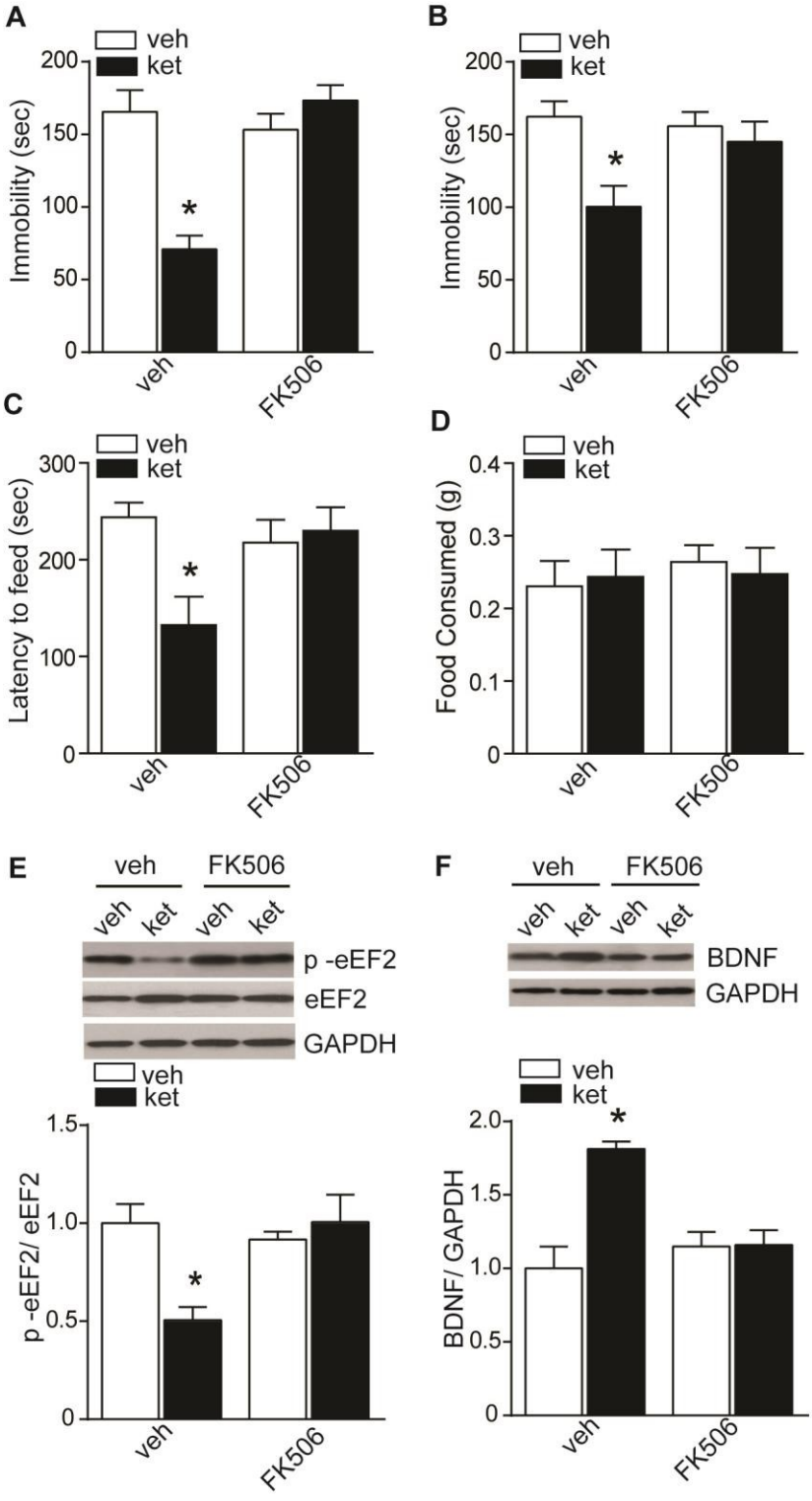
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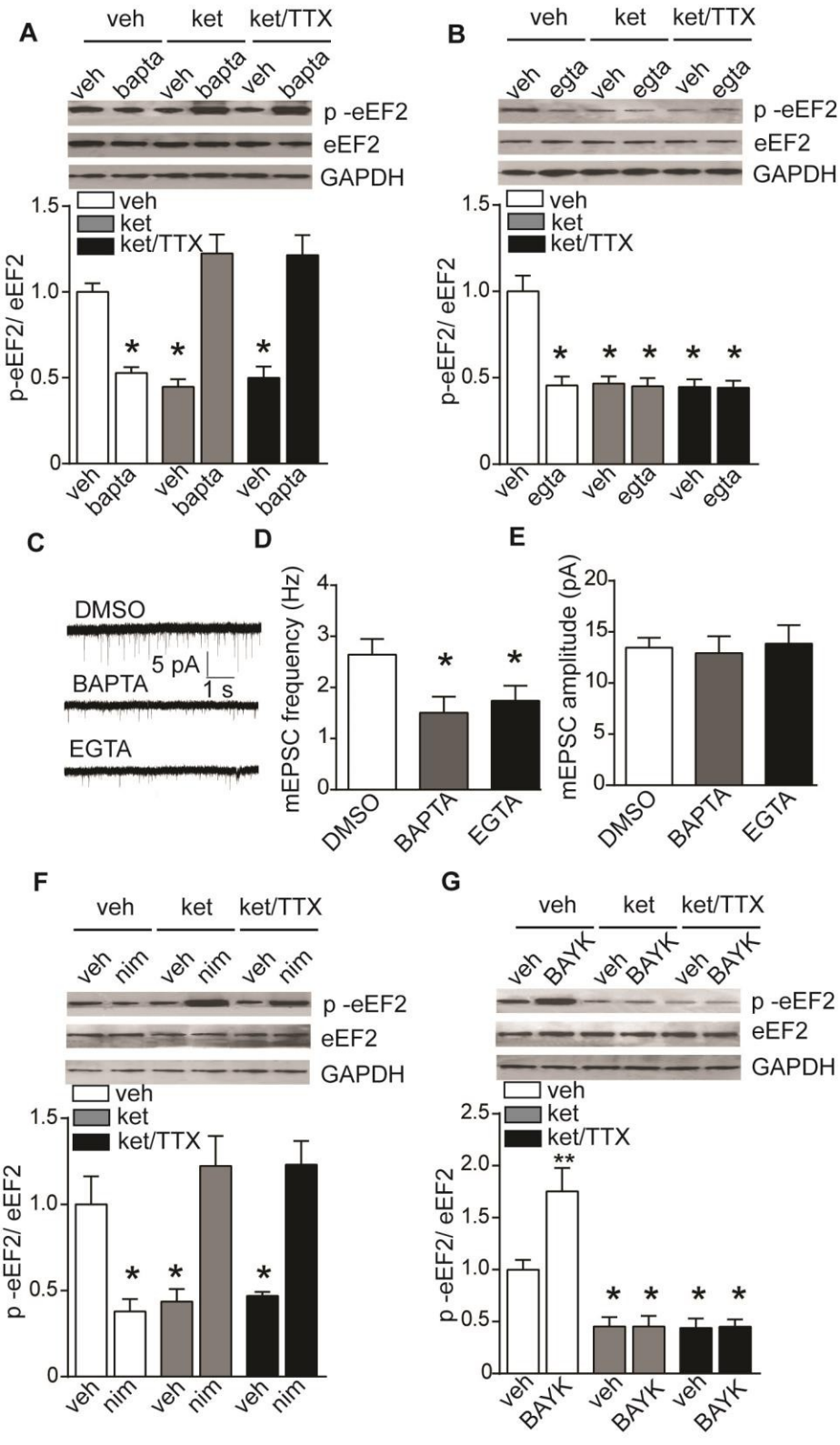
Figure 3-2. Inhibition of calcineurin blocks ketamine-meditated behavior and ketamine-mediated changes in p-eEF2 and BDNF protein levels *in vivo*. **A-F,** FK506 (5 mg/kg) was administered i.p 30 min prior to ketamine (3 mg/kg). **A,** Immobility in FST was assessed 30 min after ketamine injection. ANOVA, $F(3,33)=16.35$, $P<0.001$ for treatment, Tukey's post hoc test reveals that ketamine potentiates mobility in comparison to vehicle and that FK506 blocks ketamine-mediated behavior at 30 min (*$P<0.05$; N=9-10/group). **B,** Twenty-four hour analysis of FST (ANOVA, $F(3,39)=5.130$, $P=0.047$ for treatment). Tukey's post hoc demonstrates that ketamine decreases immobility in comparison to vehicle and that FK506 blocks ketamine mediated behavior at 24 h (* $P<0.05$; N=10/group). ***C,*** In the NSF test, latency to feed was recorded 30 min after ketamine administration (ANOVA, $F(3,39)=5.130$, $P=0.0097$ for treatment).Tukey's post hoc analysis shows that ketamine decreases latency to acquire a food pellet in comparison to vehicle and that FK506 blocks ketamine mediated NSF behavior (*$p<0.05$; N=10/group). **D,** The amount of food consumed in the home cage was measured immediately after the 30 min NSF trial (ANOVA $F(3,39)=0.1755$, $P=0.9123$ for treaatment). No significant changes were observed between groups. (N=10/group)**. E-F,** Hippocampal p-eEF2 and BDNF protein levels were measured 30 min after ketamine administration. **E,** Densitometric analysis of p-eEF2 levels normalized to total eEF2 (ANOVA $F(3,20)=4.899$, $P=0.0103$ for treatment).Tukey's post hoc test demonstrates that ketamine reduces p-eEF2 levels and that FK506 blocks ketamine-mediated reductions in hippocampal p- eEF2 levels (*$P<0.01$; N=5-6/group). **F,** Densitometric analysis of BDNF levels normalized to GAPDH (ANOVA $F(3,20)=7.370$, $P=0.0016$ for treatment).Tukey's post-test shows that ketamine increases BDNF levels and that FK506 blocks ketamine-mediated increases in BDNF levels (*$P<0.05$; N=5-6/group).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mEPSC amplitude (pA)
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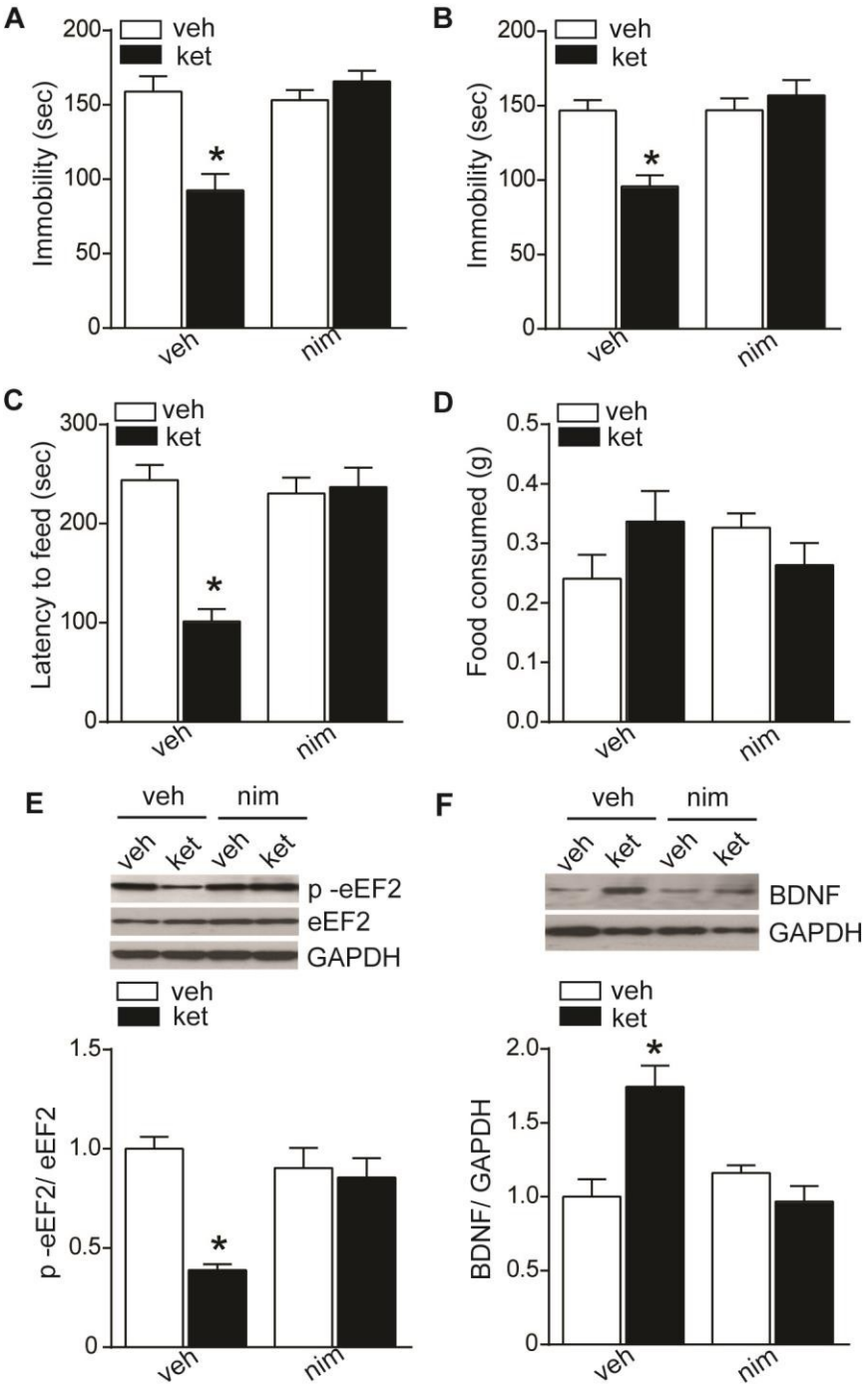
Figure 3-3. BAPTA but not EGTA blocks ketamine-mediated reductions in p-eEF2 levels. **A-B,** Primary hippocampal cultures were pretreated (1 hour) with either BAPTA (10 μM) or EGTA (10 μM) prior to being treated with either vehicle, ketamine (10 μM), or ketamine/ TTX (10 and 1 μM, respectively). **A,** Densitometric analysis of p-eEF2 (normalized to total eEF2) following BAPTA treatment in the presence and absence of vehicle, ketamine, or ketamine/ TTX (ANOVA $F(5,30)=22.14$, $P<0.0001$ for treatment).Tukey's post-hoc analysis reveals that BAPTA both reduces basal p-eEF2 levels and blocks ketamine-mediated reductions in p-eEF2 (*$P<0.05$; N=6/group). **B,** Densitometric analysis of p-eEF2 (normalized to total eEF2) following EGTA treatment in conjunction with vehicle, ketamine, or ketamine/ TTX (ANOVA $F(5,42)=16.23$, $P<0.0001$ for treatment). Tukey's post-hoc analysis shows that EGTA reduces basal p-eEF2 levels but does not attenuate ketamine-mediated reductions in p-eEF2 (*$P<0.05$; N=8/group). **C-E**, mEPSCs were recorded in the presence of vehicle (0.1% DMSO), BAPTA (10 μM), or EGTA (10 μM). **C,** Representative mEPSC traces. **D,** BAPTA and EGTA equally reduce mEPSC frequency compared to vehicle treated controls (Student's t-test $P=0.0219$ and $P=0.0496$ for BAPTA and EGTA treatment, respectively). Significance was set at*$p<0.05$. **E,** BAPTA and EGTA do not affect mEPSC amplitude compared to vehicle treated controls (Student's t-test $p=0.765$ and $p=0.722$ for BAPTA and EGTA treatment, respectively). Significance was set at*$P<0.05$. **F,** Primary hippocampal cultures were treated (1 hour) with nimodipine (10 μM) prior to treatment with vehicle, ketamine, or ketamine/ TTX, as previously described. Densitometric analysis of p-eEF2 levels (normalized to total eEF2) following nimodipine treatment in the presence and absence of vehicle, ketamine, and ketamine /TTX (ANOVA $F(5,30)=11.4$, $P<0.0001$ for treatment). Tukey's post hoc test demonstrates that nimodipine reduces basal p-eEF2 levels and blocks ketamine-mediated reductions in p-eEF2. Significance was set at *$p<0.05$. N=6 /group. **G.** Primary hippocampal cultures were treated (1 hour) with BAYK8644(1.0 μM) prior to treatment with vehicle, ketamine, or ketamine/ TTX, as previously described. Densitometric analysis of p-eEF2 levels (normalized to total eEF2) following BAYK8644 treatment in the presence and absence of vehicle, ketamine, and ketamine /TTX (ANOVA $F(5,30)=12.6$, $P<0.0001$ for treatment). Tukey's post hoc reveals that BAYK8644 increases basal p-eEF2 levels and does not blocks ketamine-mediated reductions in p-eEF2. Significance was set at *$P<0.05$. N=6 /group. The values represent mean ± SEM in all figures.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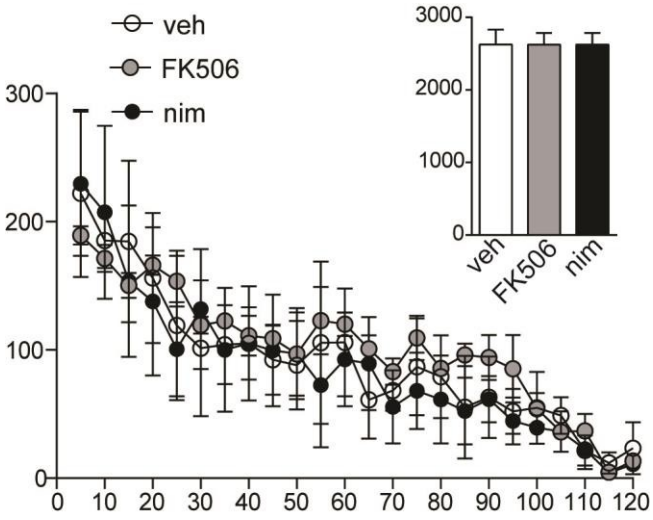
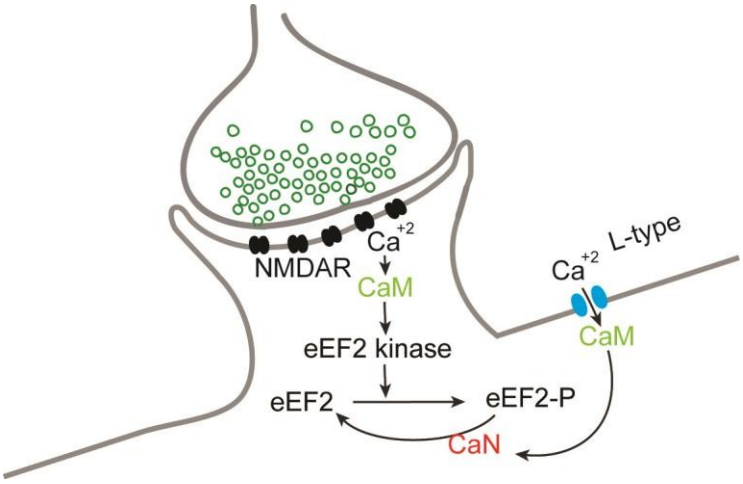
p -eEF2/ eEF2
BDNF/ GAPDH

Figure 3-4. Inhibition of L-type Ca^{+2} channels with nimodipine blocks ketamine mediated antidepressant like behavior and ketamine mediated changes in p-eEF2 and BDNF levels *in vivo*. **A-F,** Nimodipine (5 mg/kg) was administered i.p 30 min prior to ketamine (3 mg/kg). **A,** Immobility in FST was assessed 30 min after ketamine injection (ANOVA, F(3,39)=23.91, P<0.0001 for treatment). Tukey's post hoc analysis reveals that ketamine potentiates mobility in comparison to vehicle and that nimodipine blocks ketamine-mediated behavior at 30 min (*P<0.05; N=10/group). **B,** Twenty-four hour analysis of FST (ANOVA, $F_{3,39} = 11.04$, P<0.0001 for treatment). Tukey's post hoc analysis shows that ketamine decreases immobility in comparison to vehicle and that nimodipine blocks ketamine-mediated behavior at 24 h (*P<0.05; N=10/group). **C,** In the NSF test, latency to feed was assessed 30 min after ketamine administration (ANOVA, F(3,39)=18.64, p<0.0001 for treatment). Tukey's post hoc analysis demonstrates that ketamine decreases latency to acquire a food pellet in comparison to vehicle and that nimodipine blocks ketamine mediated NSF behavior (*p<0.05; N=10/group). **D,** The amount of food consumed in the home cage was measured immediately after the 30 min NSF trial (ANOVA F(3,39)=0.1545, P=0.9261 for treatment). No significant changes were observed between groups. (N=10/group). **E-F,** Hippocampal p-eEF2 and BDNF protein levels were measured 30 min after ketamine administration. **E,** Densitometric analysis of p-eEF2 levels normalized to total eEF2 (ANOVA F(3,19) = 17.88, P<0.0001 for treatment). Tukey'spost hoc test reveals that ketamine reduces p-eEF2 levels and that nimodipine blocks ketamine-mediated reductions in hippocampal p- eEF2 levels (**p< 0.01; N=5/group). ***F,*** Densitometric analysis of BDNF levels normalized to GAPDH (ANOVA F(3,19)= 36.11, P<0.0001 for treatment). Tukey's post-test shows that ketamine increases BDNF levels and that nimodipine blocks ketamine-mediated increases in BDNF levels (**p< 0.01. N=5/group).



Supplemental Figure 3-1. FK506 and nimodipine do not alter locomotor behavior. C57BL/6 mice were intraperitoneally injected with either vehicle, nimodipine (5 mg/kg) or FK506 (5 mg/kg) and immediately assessed for locomotor activity by recording the number of photo beam breaks over 120 min in either 5 min increments or the entire 120 min period (inset). No significant difference between groups was found when total locomotor activity was analyzed (ANOVA $F(2,29)=0.5193$, $P=0.6010$ for treatment) or when locomotor activity was analyzed in 5 minute bins. Significance was set at $^{*}P<0.05$. N=10/group



Supplemental Figure 3-2. Model demonstrating the parallel L-type calcium channel mediated calcium signaling pathway that dephosphorylates eEF2 and competes with the previously identified NMDA receptor dependent signaling that activates eEF2K function (Scheetz et al., 2000; Sutton et al., 2007). The balance between these two calcium signaling pathways determines the degree of eEF2 phosphorylation and the extent of eEF2-dependent protein translation, which in turn gauges the efficacy of ketamine-mediated rapid antidepressant responses in preclinical mouse models

CHAPTER IV

THE MOLECULAR MECHANISMS UNDERLYING THE FAST-ACTING ANTIDEPRESSANT EFFECT OF SCOPOLAMINE

Introduction

Major depressive disorder (MDD) is one of the most prevalent forms of mental illness with a morbid risk of over 6.7% of the general population (Kessler et al., 2005). MDD is a heterogeneous disorder that is characterized by dysregulated mood and/or anhedonia with intense feelings of despair and sadness, agitation, self-deprecation, and suicidal ideation (Association, 2013) . Antidepressants, such as selective serotonin reuptake inhibitors, are the most common form of treatment for MDD, however the precise mechanism by which these drugs work is largely unknown. Moreover, the time they take to reach clinical effect can take weeks to months, and some patients never truly respond, leaving a critical need for more rapidly acting antidepressants with sustained efficacy.

Recent clinical data have demonstrated that scopolamine, a muscarinic acetylcholine receptor antagonist, produces a fast and long lasting antidepressant response that is safe and well tolerated in depressed, (Drevets and Furey, 2010; Ellis et al., 2014; Furey and Drevets, 2006; Furey et al., 2010; Gillin et al., 1991) as well as bipolar patients (Furey et al., 2012). Depressed patients administered three intravenous infusions of scopolamine (4ug/kg) over a 3 to 5 day period reported marked improvement in major depressive symptoms the third day after initial treatment, with effects lasting over two weeks (Drevets and Furey, 2010; Ellis et al., 2014; Furey and Drevets, 2006). This work builds on earlier clinical data showing that a single dose of scopolamine administered intramuscularly to depressed patients produces an antidepressant

response the day after administration (Gillin et al., 1991). While this earlier data demonstrated the rapid antidepressant effects of scopolamine over 20 years ago, a dearth of studies specifically investigating scopolamine's effects on mood followed, possibly due to concern over side effects associated-with blocking muscarinic acetylcholine receptors, causing the field to shift towards serotonergic compounds as antidepressants. Interestingly, it wasn't until the serendipitous discovery by Furey and Drevets in 2006 that suggested that scopolamine produces rapid antidepressant effects in depressed patient populations, which have renewed the interest in scopolamine as a rapid antidepressant.

The goal of our study therefore was to elucidate the molecular and cellular mechanisms that underlie scopolamine's rapid antidepressant effects. To investigate the mechanism by which scopolamine produces a rapid antidepressant response, we mice in the learned helplessness paradigm; a test of antidepressant efficacy. We found that scopolamine produces a fast acting antidepressant effect in mice at twenty-four hours that is dependent on *bdnf* transcription. We also demonstrated that scopolamine induced increases in phosphorylated Ser421 MecP2 in the hippocampus 24 hours after administration. Utilizing an acute hippocampal slice pharmacology approach, we demonstrate that scopolamine-induced increases in phosphorylated S421 MecP2 are mediated by antagonism of the M1 muscarinic acetylcholine receptor, thus providing mechanistic insight into scopolamine-mediated MecP2 dependent transcriptional mechanisms involved in fast acting antidepressant responses. Moreover, we show that inhibition of calcineurin triggers scopolamine-mediated increases in phosphorylated MecP2 at position S421 in an M1 dependent manner, suggesting that calcineurin is involved in regulating the phosphorylation of MecP2 at Ser421 and providing further support for this pathway as a key requirement for scopolamine's antidepressant response.

Materials and Methods

Mice

C57BL/6 male mice aged 6-8 weeks old were habituated to the animal facility for one week prior to behavioral testing. Mice were kept on a 12 hour light/dark cycle and given access to food and water *ad libitum*. Inducible BDNF knockout mice were generated from a trigenic cross of NSA-tTA, TetOP-Cre, and floxed BDNF mice as previously described (Monteggia et al., 2004). For all behavioral testing, adult male mice were age and weight-matched, and groups were balanced by genotype. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Drugs

All drugs used for behavioral experiments were administered intraperitoneally (i.p.). Concentrations were the following: scopolamine, 0.15mg/kg in 0.9% saline, and Actinomycin D, 0.5 mg/kg in 5% EtOH. Drugs for *in vitro* studies were used at the following final concentrations: scopolamine 220 nM, pirenzepine 50 nM, 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (AF-DX116) 150 nM, 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide (4 DAMP) 1 nM, tropicamide 20 nM, physostigmine 2.0 μM, and FK506 50 nM. Reagents were purchased from the following sources: scopolamine and Actinomycin D (Sigma Aldrich); pirenzepine, physostigmine, AFDX116, 4-DAMP, tropicamide, and FK506 (Tocris Biosciences).

Learned helplessness.

The learned helplessness test was performed as previously described (Autry et al., 2011). Briefly, 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 (0.5 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 was recorded for fifteen trials.

Locomotor activity.

Mice were placed individually in a standard mouse cage with fresh bedding. Locomotor activity was recorded for 120 minutes under red light by photocell beams coupled to computer acquisition software (San Diego Instruments).

Slice pharmacology.

Adult male C57BL/6 mice aged 6 weeks were sacrificed by decapitation. The brains were rapidly removed and placed in ice cold oxygenated hippocampal dissection buffer, containing, in mM: 3 KCL, 1 NaH_2PO_4, 26 $NaHCO_3$, 212 sucrose, 5 $MgCL_2$, and 0.5 $CaCL_2$. Horizontal slices (350 microns) were prepared using a vibrating blade microtome (Leica Microsystems). Hippocampi were dissected from the slices in ice- cold oxygenated dissection buffer. Hippocampal slices were placed in ASCF, containing in mM: 124 NaCL, 5 KCL, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2 $CaCL_2$, and 1 $MgCL_2$, pH 7.4, and preincubated at 30 degrees under constant oxygenation with 95% O_2/5% CO_2 for 1 h. After recovering in ASCF for 1 h, slices

were pretreated with vehicle or physostigmine (2uM) for 1 h, in order to induce cholinergic tone. Slices were then treated with either vehicle or the muscarinic acetylcholine receptor antagonist, scopolamine (220nM) for 1 hour. After one hour, slices were washed for 3 h with ASCF under constant oxygenation with 95% O_2/5% CO_2. Slices were then transferred to eppendorf tubes, frozen on dry ice, and stored at -80ºC until assayed. In the antagonist experiments, slices were treated with pirenzepine (50nM), AFDX-116 (150nM), 4DAMP (1nM), or tropicamide (20 nM) after pretreatment with vehicle or physostigmine. In the FK506 experiments, slices were pretreated with physostigmine prior to being treated with vehicle or FK506 (50 nM) for one hour. Slices were then treated with either scopolamine or pirenzepine as described above.

Frozen tissue slices were homogenized in a modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCL pH 7.4, 1.0 % Igepal, 0.1 % SDS, 0.5% Na-deoxycholate, 150 mM NaCL, 4 mM EDTA, 50 mM NaH_2PO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche). Small aliquots of the homogenate were retained for protein determination by the BCA protein assay method (Pierce). Equal amounts of protein (20 μg) were resolved by SDS/polyacrylamide gel electrophoresis (10% polyacrylamide gels), transferred to nitrocellulose membranes, and probed with anti–phospho S421 MecP2 antibody (1:1000; Phosphosolutions), anti-MecP2 antibody(1:3000; Thermo Scientific), and anti-GAPDH antibody (1:50,000; Cell Signaling Technology). Immunoreactive bands were visualized by enhanced chemoluminescence and captured on autoradiography film (Light Labs). Densitometric scans of the autoradiographs were produced on a ScanJet 4300C (Hewlett Packard, Palo Alto, CA) and quantified using Image J software. Significant differences between treated and control slices were determined by a one way analysis of variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons.

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. The conditions for cDNA construction, amplification of BDNF and GAPDH, and sequences for the primers were described previously (Adachi et al., 2008). Fold change in BDNF expression of the coding exon IV is normalized to GAPDH. For data analysis, the fold change BDNF expression relative to GAPDH was calculated as mean ± SEM.

Protein quantification and Immunoblotting.

Anterior hippocampal slices (2/mouse, ~1 mm thick) were dissected and rapidly frozen. Tissue was homogenized in a radioimmunopreciation assay (RIPA) buffer containing: Tris 50 mM, Igepal 1.0%, SDS 0.1%, sodium deoxycholate 0.5%, EDTA 4.0 mM, NaCL 150 mM, pH 7.4. Protease (Roche) and phosphatase inhibitors (sodium pyrophosphate 10 mM, sodium fluoride 50 mM, and sodium orthovanadate 2.0 mM) were added immediately prior to use. Total protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce)**.** Samples were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with anti-phospho-S421 MecP2 antibody (1:1000; Phosphosolutions), anti- total MecP2 C-terminal antibody (1:3000; Thermo Scientific), anti- BDNF (1:1000; Abcam) and anti-GAPDH antibody (1:50,000; Cell Signaling) as previously described (Autry et al., 2011). Immunoreactive bands were visualized by enhanced chemoluminescence and captured on autoradiography film (Light Labs). ECL developed bands were exposed to film, and films were analyzed by ImageJ. Phosphorylated MeCP2 and BDNF band intensities were taken as a ratio of total MecP2 and GAPDH values,

respectively. Significant differences between treatment and control for protein levels were determined using a one way analysis of variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons.

Hippocampal slice electrophysiology.

Hippocampal slices were prepared as previously described (Na et al., 2012). Input-output relationship was determined by providing an ascending series of stimulus input intensities (40-240 μA) until the maximum field excitatory post-synaptic potential (fEPSP) response was determined. An input stimulus intensity that induced 40-50% of the maximum response was used for measuring paired pulse ratio (PPR) and LTP. An input intensity that induced the maximum response was used for high frequency stimulation (HFS)-induced LTP. PPR was induced by giving 2 pulses at decreasing interpulse intervals (500, 400, 200, 100, 50, 30, 20 ms) and analyzed by dividing the fEPSP slope of pulse 2 by pulse 1. Following 20 minutes of stable baseline fEPSP slope, LTP was induced with two 100 Hz trains of 100 pulses with an intertrain interval of 20 seconds.

Immunostaining

C57B/6 mice were injected with vehicle or scopolamine (0.15 mg/kg). Twenty four hours after administration animals were euthanized and then trans-cardial perfused with PBS followed by 4% paraformaldehyde. Brains were obtained and post-fixed in 4% paraformaldehyde for 24 hours. Brains were then sunk into 30% sucrose solution and then sectioned on a freezing microtome at 40 microns and subsequently preserved in 1XPBS/0.01% sodium azide. Floating sections were washed in 1X PBS/ 0.1% Triton for 1 hour followed by antigen unmasking in

boiling sodium citrate/0.05% Tween20, pH 6.0 for 15 minutes. Sections were rinsed (3x5 min 1X PBS/0.03% Triton) and endogenous peroxidase activity was quenched in 1% H_20_2 for 30 minutes at room temperature. Sections were washed in 1X PBS/0.03% Triton. Tissue was then blocked for 30 min in 3% normal goat serum/1XPBS/0.03% Triton, followed by primary antibody rabbit anti-pMeCP2 (diluted 1:3000 in blocking solution; Phosphosolutions) and incubated for 24 hours at 4 degrees C. After rinsing in1X PBS/0.03% Triton (3x5 min), Alexa Flour 488 was applied at 1:200, for 2-4 hours at room temperature, followed by washing (3x5min in 1XPBS/0.03% Triton). Slices were then counterstained with the nuclear stain, DRAQ5 (5μM, 15 min) and mounted on superfrost plus slides, dried for 2 hours, and coverslipped in Aqua polymount mountant.

Statistics.

Data was analyzed by Analysis of Variance (ANOVA) combined with Tukey's post hoc analysis for multiple comparisons unless otherwise specified. Data is presented as mean ± SEM and significance is set at $*P < 0.05$.

Results

Scopolamine produces a rapid antidepressant effect in mice

To examine whether scopolamine produced an antidepressant effect in wild-type C57BL/6 mice, we tested them in the learned helplessness (LH), a behavioral paradigm predictive of antidepressant efficacy (Bodnoff et al., 1989; Seligman and Maier, 1967). We initially did a dose response and found that scopolamine at 0.15 mg/kg was the lowest dose that produced a significant antidepressant response (data not shown). Therefore in all subsequent experiments scopolamine was used at a dose of 0.15mg/kg, unless otherwise specified. Scopolamine elicited a significant decrease in the latency to escape compared to vehicle treated mice in the LH paradigm twenty four hours after treatment, indicative of an antidepressant response (Figure 4-1a). We focused on the 24 hour point as clinically a single dose of scopolamine does not produce a rapid antidepressant response in depressed patients within 2 hours, but rather antidepressant effects are observed the following day (Gillin et al., 1991). We found that acute scopolamine significantly increased locomotor activity for thirty minutes after administration after which activity levels returned to baseline, suggesting that the antidepressant-like responses at 24 hours are not due to differences in locomotor activity (Figure 4-1b).

Scopolamine significantly increases phosphorylated S421 MeCP2

BDNF is an endogenous downstream target gene of several transcription factors, including cyclic response element binding protein (CREB) and methyl-CpG-binding protein 2 (MecP2). Changes in phosphorylated serine 133 CREB (pCREB) (Fukuchi et al., 2014; Samoilov et al., 2014) and phosphorylated serine 421 MecP2 (pMecP2) levels (Cohen et al., 2011; Deng et al., 2010; Li et al., 2011; Zhou et al., 2006) have been shown to impact *Bdnf* gene expression. To

examine whether an increase in BDNF transcription is mediated through CREB or MecP2, C57Bl/6 mice were treated with scopolamine and sacrificed 24 hours later. Scopolamine did not alter pCREB levels in the hippocampus relative to total CREB levels (Figure 4-2a), however scopolamine significantly increased pMecP2 in the hippocampus relative to total MecP2 levels as detected by Western blot analysis (Figure 4-2b) and immunostaining (Figure 4-2c-d). We did not observe changes in pMecP2 levels in the prefrontal cortex or the nucleus accumbens, 24 hours after scopolamine administration (Supplementary Figure 4-1a, b).

Previous work has shown that phosphorylation of MecP2 at serine 421 in the hippocampus is mediated by activation of calcium/calmodulin-dependent protein kinase II (CaMKII)(Buchthal et al., 2012; Zhou et al., 2006). To determine whether acute scopolamine activates CaMKII to trigger MecP2 phosphorylation at serine421 *in vivo*, we pretreated C57BL/6 mice with vehicle or the CaMKII selective inhibitor KN62 for 30 minutes prior to scopolamine administration and subsequently assessed molecular changes 24 hours later. We found that KN62 attenuated the scopolamine mediated increases in pMecP2 in the hippocampus comparable to vehicle treatment (Figure 4-3a). We further found that scopolamine treatment had no appreciable effects on CaMKII phosphorylation at threonine 286 compared to vehicle (Figure 4-3b), suggesting that the increases in pMecP2 levels by scopolamine are not due to the induction of CaMKII kinase activity. KN62 pretreatment significantly reduced CaMKII phosphorylation at threonine286 in both vehicle and scopolamine treated conditions, demonstrating that KN62 crosses the blood brain barrier following peripheral administration.

M1 selective AchR antagonists increase phosphorylation of MeCP2 at position Ser421

Our data to this point shows that scopolamine triggers phosphorylation of MecP2 that is required for the transcriptional dependent effects necessary for the antidepressant response. Scopolamine is a nonselective muscarinic acetylcholine receptor antagonist that acts on all five muscarinic receptor subtypes, M1-M5, making it unclear whether a particular receptor subtype mediates the increase in pMecP2 levels and ultimately the antidepressant effects. Selective muscarinic acetylcholine receptor antagonists have been identified to the M1-M4 subtypes but have poor penetration to the brain, limiting the feasibility of mimicking the effect of scopolamine by peripheral administration (Wess, 2004). Mice with global deletion of a muscarinic acetylcholine receptor subtype (M1-M5) display a variety of behavioral deficits (Gomeza et al., 1999a; Gomeza et al., 1999b; Hamilton et al., 1997; Matsui et al., 2000; Yamada et al., 2001a) that may confound their use in delineating the antidepressant efficacy of scopolamine (Gerber et al., 2001; Gomeza et al., 1999b; Stengel et al., 2000; Yamada et al., 2001a; Yamada et al., 2001b). Therefore, we used hippocampal slice pharmacology to examine whether blocking a specific muscarinic acetylcholine receptor triggers MecP2 phosphorylation at serine421. A critical consideration was that hippocampal slices have limited cholinergic tone thus treatment with specific muscarinic acetylcholine receptor antagonists may not result in significant changes in intracellular signaling. Indeed, although scopolamine treatment by itself produced a trend towards an increase in pMeCP2 levels it was not significant (Figure 4-4a). To offset this complication, acute hippocampal slices were pretreated with physostigmine (2 μM for 1 hour) to inhibit acetylcholinesterase activity and exacerbate cholinergic tone which by itself does not alter pMecP2 levels (Figure 4-4a). Acute pretreatment of hippocampal slices with physostigmine followed by scopolamine administration resulted in a significant increase in pMecP2 relative to vehicle treatment (Figure 4-4a), providing proof of principle for detecting scopolamine mediated

pMecP2 in a slice preparation. To determine the contribution of the muscarinic acetylcholine receptor subtypes on triggering phosphorylation of MecP2 at serine421, we utilized selective antagonists for the M1-M4 subtypes. The M1 selective antagonist, pirenzepine, in the presence of physostigmine triggered a significant increase in pMecP2 similar to scopolamine (Figure 4-4b). In contrast, the selective antagonist of M2 (11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (AF-DX116)), M3 (1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP)), or M4 (tropicamide) in the presence of physostigmine did not alter pMecP2 levels (Supplemental Figure 4-2 a-c). Taken together, these data strongly implicate the M1 receptor subtype though its ability to stimulate pMecP2 in the antidepressant effects of scopolamine.

Inhibition of calcineurin triggers scopolamine mediated increases in phosphorylated MeCP2 levels

The M1 muscarinic acetylcholine receptor is a Gq-coupled receptor that activates phospholipase C and in turn the inositol trisphosphate signal transduction pathway leading to an increase in intracellular calcium. It is easy to envision how an M1 receptor agonist could activate CaMKII causing a subsequent increase in pMecP2 levels. However, our data shows that scopolamine by blocking the M1 receptor triggers pMecP2 without increasing CaMKII activity (Figure 4-3d), which is necessary for the antidepressant effects. The amount of MecP2 phosphorylated at serine421 would be determined by the action of CaMKII balanced with an as yet unidentified protein phosphatase. Since CaMKII is a calcium/calmodulin-dependent protein kinase we examined whether the calcium/calmodulin dependent protein phosphatase, calcineurin, is involved in determining the levels of pMecP2. Hippocampal slices were

pretreated with physostigmine followed by the calcineurin inhibitor, FK506, either in the presence or the absence of scopolamine (Figure 4-4c) or pirenzepine (Figure 4-4d). We found that FK506 treatment triggered a significant increase in pMecP2 levels demonstrating that inhibition of calcineurin augments phosphorylation of MecP2 at serine421. Moreover, FK506 treatment in the presence of scopolamine (Figure 4-4c) or pirenzepine (Figure 4-4d) occluded the effect on pMecP2 levels, demonstrating that scopolamine through its action of blocking the M1 receptor subtype inhibits calcineurin to increase the level of MecP2 phosphorylation at serine 421. To determine whether inhibition of calcineurin impacts pMecP2 levels *in vivo*, we treated C57BL/6 mice with FK506 and sacrificed them 24 hours later. Western blot analysis revealed that FK506 treatment results in a significant increase in pMecP2 levels in the hippocampus relative to vehicle treated mice (Figure 4-4e).

In summary, these data demonstrate that calcineurin dephosphorylates pMecP2 and that scopolamine, through blockade of M1 muscarinic acetylcholine receptors, increases neurotransmitter release presynaptically, which leads to a downstream postsynaptic inhibition of calcineurin, culminating in increases in pMecP2 levels, increased *bdnf* transcription, and antidepressant efficacy.

Discussion

A significant impact of our study is that it provides key insight into the cellular mechanism by which scopolamine mediates its fast acting antidepressant responses. Importantly, we show that scopolamine produces a fast acting antidepressant effect in animal models. We additionally demonstrate that scopolamine increases phosphorylated MecP2 levels at position S421 *in vivo*. We demonstrate that scopolamine and the selective M1 receptor antagonist, pirenzepine, increase levels of phosphorylated S421 MecP2 in acute hippocampal slices. Furthermore, we show that inhibition of calcineurin activity increases phosphorylated S421 MecP2 levels *in vitro* and *in vivo*. Taken together, we demonstrate that scopolamine, through blockade of M1 muscarinic acetylcholine receptors, increases neurotransmitter release presynaptically, which leads to downstream post-synaptic effects, causing post-synaptic M1-dependent inhibition of calcineurin activity, that enhance S421 MecP2 phosphorylation thereby triggering increases in *bdnf* transcription, which produce the antidepressant effect.

The scopolamine-induced rapid antidepressant responses that we observed in our behavioral paradigms are consistent with clinical data showing that scopolamine produces a fast acting antidepressant response in depressed patients within an approximate 24 hour time period (Drevets and Furey, 2010; Ellis et al., 2014; Furey and Drevets, 2006; Furey et al., 2010; Gillin et al., 1991). In this study, we found that scopolamine mediates an antidepressant effect in animal models within twenty four hours. The timing of scopolamine's antidepressant actions is consistent with our findings that scopolamine-mediated antidepressant responses are induced by a transcriptional dependent mechanism. Additionally we observed increased levels of phosphorylated S421 MecP2 at 24 hours which is necessary to trigger increases in *bdnf* transcription at that same time point. This is in contrast to our previous work with ketamine in

which we found that acute ketamine-mediated antidepressant effects occur 30 minutes after administration (Autry et al., 2011). While this study demonstrates that scopolamine's antidepressant effects are regulated through *bdnf* transcription, ketamine's rapid antidepressant responses were found to be triggered by a rapid and transient increase in BDNF protein translation rather than transcription (Autry et al., 2011). Ketamine-mediated NMDAR blockade at rest was shown to deactivate eukaryotic elongation factor 2 (eEF2) kinase, resulting in reduced eEF2 phosphorylation leading to a desuppression of BDNF translation (Autry et al., 2011), whereas scopolamine-mediated antidepressant behavior does not appear to be mediated by this same molecular pathway.

It is of interest to note that scopolamine and ketamine block differential receptor subtypes. Scopolamine antagonizes metabotropic muscarinic acetylcholine receptors, whereas ketamine blocks ionotropic NMDA receptors. Metabotropic receptors mediate slower and wider range signaling events by coupling to second messenger systems upon ligand binding; in contrast, ionotropic receptors traduce faster cellular events through the opening or closing of an ion channel pore upon ligand binding. Hence, the timing of both scopolamine-mediated and ketamine-mediated behavioral responses is consistent with the receptor-type on which they act. Given the divergent signaling pathways associated with NMDA and metabotropic acetylcholine receptors, it is anticipated that the immediate effectors of scopolamine and ketamine differ. Despite this fact, one point of convergence that appears to be critical for both scopolamine- and ketamine-mediated fast-acting antidepressant responses is BDNF.

Interestingly, both scopolamine and ketamine produce antidepressant effects (up to 1 week, preclinically) that extend well beyond their respective half-lives, suggesting the induction of changes in synaptic plasticity. The fast acting antidepressant effects of ketamine have been

shown to be dependent on glutamate, as ketamine- mediated behavioral effects are blocked by pretreatment with NBQX, an antagonist of AMPA receptors (Autry et al., 2011; Li et al., 2010). Similarly, in this study, we found that scopolamine-mediated antidepressant responses were blocked by co-application of NBQX, which is in agreement with previous work by Voleti and colleagues (Voleti et al., 2013). These data suggest that both ketamine- and scopolamine-mediated antidepressant effects require AMPAR receptors. However, the timing in which ketamine- and scopolamine- mediated behavioral effects are blocked by NBQX differs. NBQX blocks ketamine's behavioral responses 30 minutes after ketamine administration, suggesting that AMPA receptor insertion is required for the ketamine effect (Autry et al., 2011), whereas we show that NBQX blocks scopolamine's behavioral responses 24 hours after scopolamine administration (data not shown) and not after 30 minutes (data not shown), indicating that AMPA-receptor maintenance is necessary for scopolamine-mediated behavior. Moreover, acute ketamine application at rest has been shown to potentiate subsequent evoked synaptic responses in hippocampal slices (Autry et al., 2011), further indicating that increased AMPA-mediated neurotransmission underlies ketamine's antidepressant-like effects. In contrast, scopolamine does not potentiate evoked synaptic responses in acute hippocampal slices (data not shown); rather, scopolamine increases the probably of neurotransmitter release (data not shown), a presynaptic mechanism.

While more work needs to be done looking at scopolamine mediated changes in synaptic efficacy, recent work has shown that scopolamine administration produces an enhancement of serotonin- and orexin-induced excitatory postsynaptic currents in layer V pyramidal neurons (Voleti et al., 2013). Interestingly, both scopolamine (Voleti et al., 2013) and ketamine (Li et al., 2010) have been demonstrated to induce small but significant increases in dendritic spine

number and dendritic head diameter, which are considered potential structural correlates of synaptic plasticity. Additionally, micro-dialysis experiments have shown that both scopolamine (Voleti et al., 2013) and ketamine (Moghaddam et al., 1997) increase extracellular glutamate in the prefrontal cortex. Taken together, these results suggest that scopolamine, similar to ketamine, increases glutamatergic synaptic plasticity.

Our finding that blockade of M1 muscarinic acetylcholine receptors increases phosphorylated S421 MecP2 levels analogously to scopolamine suggests that scopolamine functions in an M1-dependent manner. Our data is in agreement with a previous study which demonstrated that the M1 selective antagonist, telenzepine, produced a significant antidepressant response in the FST (Voleti et al., 2013). However, this group's data must be interpreted with caution as to date there is no data on the bioavailability of telenzepine in the brain when administered peripherally, hence this group may be blocking more than the M1 receptor in their study. Previous work has shown that it is the activation of M1 muscarinic acetylcholine receptors produces increases in phosphorylated S421 MecP2 levels through the actions of CaMKII (Buchthal et al., 2012; Power and Sah, 2002; Zhou et al., 2006). However, our study indicates that M1 acetylcholine receptor blockade induces increases in phosphorylated S421 MecP2 levels, in contrast to the previous data (Buchthal et al., 2012; Power and Sah, 2002; Zhou et al., 2006).

Our data leads to the question as to how tonic inhibition of a signaling pathway can invoke increases in phosphorylated S421 MecP2. Our work is the first to suggest that the inhibition of the protein phosphatase, calcineurin, increases phosphorylated S421 MecP2 under muscarinic receptor blockade. Our data indicates that upon application of the calcineurin inhibitor, FK506, an increase phosphorylated S421 MecP2 can be detected; moreover, in the presence of scopolamine or pirenzepine, FK506 occludes the effect of both scopolamine and pirenzepine on

phosphorylated MeCP2 levels. This mechanism provides a plausible explanation to the conundrum as to how receptor blockade can lead to increases in phosphorylation of a site that has previously been shown to be activity dependent. Thus, M1 mediated inhibition of calcineurin activity may trigger a reduction in dephosphorylated S421 MecP2, increasing phosphorylated S421 MecP2, which is required for BDNF transcription, and the subsequent antidepressant effects of scopolamine.

In summary, a more thorough understanding of the mechanism by which scopolamine produces its antidepressant response is crucial for the development of fast -acting antidepressants. To reduce the need for repeated interventions, it will also be critical to identify the molecular determinants which can be targeted to prolong the antidepressant responses of scopolamine, as well as ketamine. Furthermore, identification of convergent pathways between scopolamine and ketamine may facilitate the discovery of novel fast-acting therapeutic strategies for the treatment of major depressive disorder.

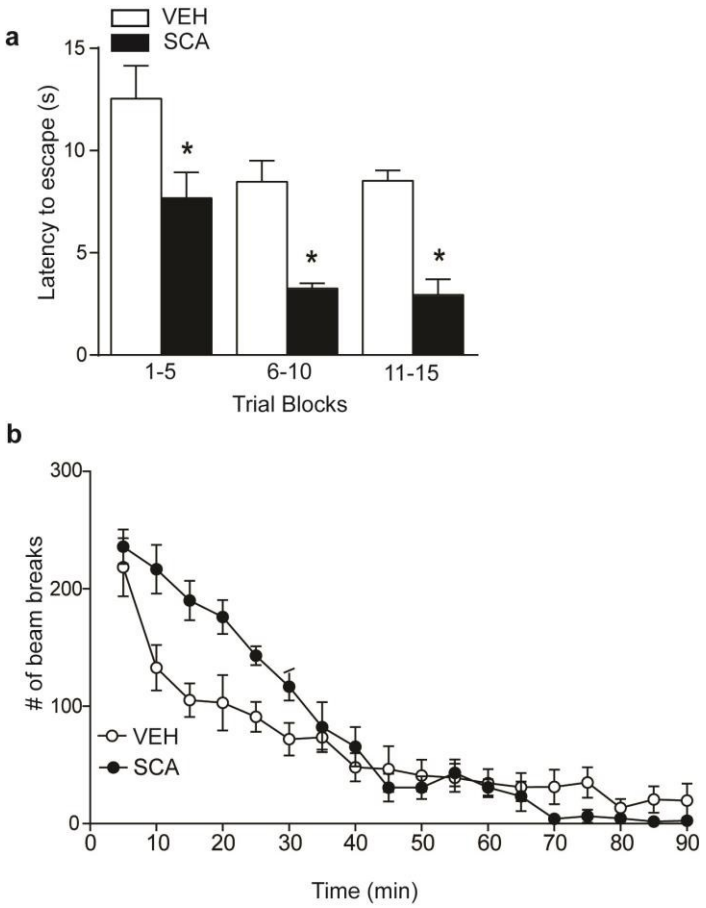


Figure 4-1. Scopolamine treatment produces an antidepressant effect in the learned helplessness paradigm and alters acute, but not long term, locomotor activity. a-b, Adult male C57BL/6 mice were intraperitoneally injected with either saline or scopolamine (0.15mg/kg). **a,** Learned helplessness activity was assessed 24 hours after scopolamine injection and analyzed in three trial blocks. Two way ANOVA ($F(14,255) = 3.90$, $P < 0.0001$ for treatment revealed a significant difference between vehicle and scopolamine treated mice in all trial blocks. Significance was set at * $P < 0.05$. n=10/group. **b,** Locomotor activity was analyzed in 5 minute bins immediately after an acute injection of scopolamine for 90 minutes. Two way ANOVA $F(17, 102) = 62.73$, $P=0.001$ for treatment. Tukey's post hoc test showed that locomotor activity was significantly increased in scopolamine treated animals from the time spanning 10-30 minutes post scopolamine injection compared to vehicle treated animals. *$P<0.05$. N=10/group.

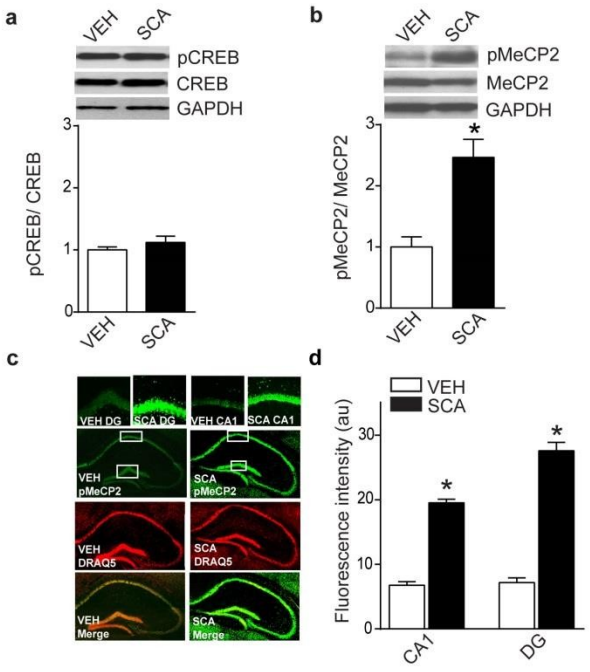


Figure 4-2. Scopolamine induces an increase in phosphorylated S421 MeCP2 levels. a-c. C57BL/6 mice were administered either vehicle (0.9 % saline) or scopolamine (0.15 mg/kg) i.p. **a,** Levels of phosphorylated CREB at position Ser133 in the hippocampus (HC) was measured 24 hours after injection. Densitometric analysis of phosphorylated CREB normalized to total CREB. Student's *t*-test P = 0.3098 indicates that no significant differences were found between groups. Significance was set at *P< 0.05. n=7/group. **b,** Levels of hippocampal phosphorylated MeCP2 at position Ser421 were measured 24 hours after injection. Densitometric analysis of pMeCP2 normalized to total MeCP2. Student's *t*-test P=0.0035, demonstrates that scopolamine increases pMeCP2 levels. Significance was set at *P< 0.05. n=7/group. **c,** Images of the hippocampus 24 h after vehicle or scopolamine treatment; scale bar = 100 microns. (green: phosphorylated S421 MeCP2, red: DRAQ5). Boxed in regions indicate the dentate gyrus and the CA1, respectively. **d,** Image J analysis of average fluorescence intensity. CA1, Student's *t*-test P<0.01, dentate gyrus P<0.01. Significance was set at *P<0.05. n=3 brains VEH; n=3 brains SCA.

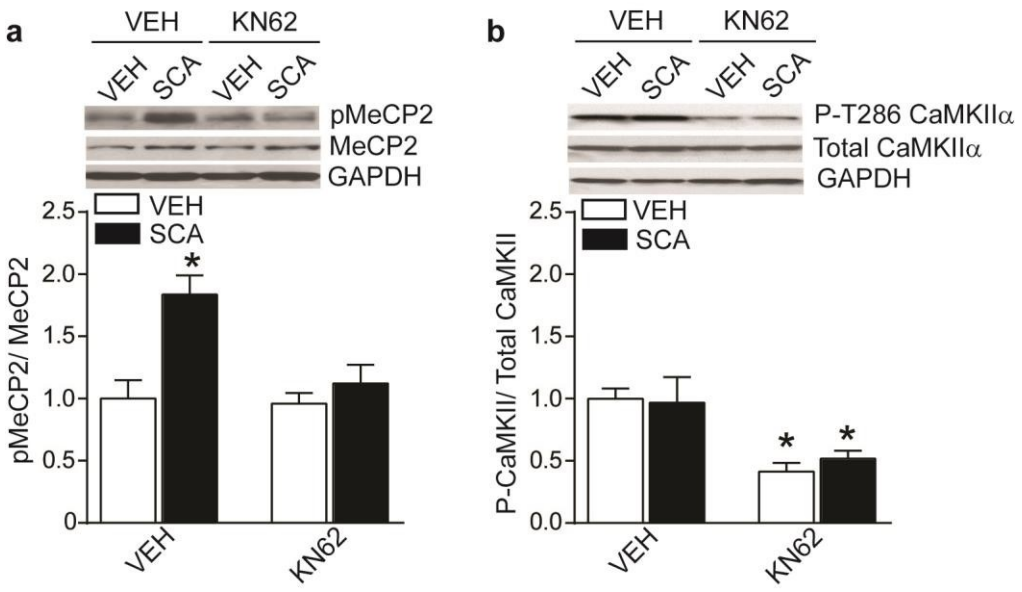
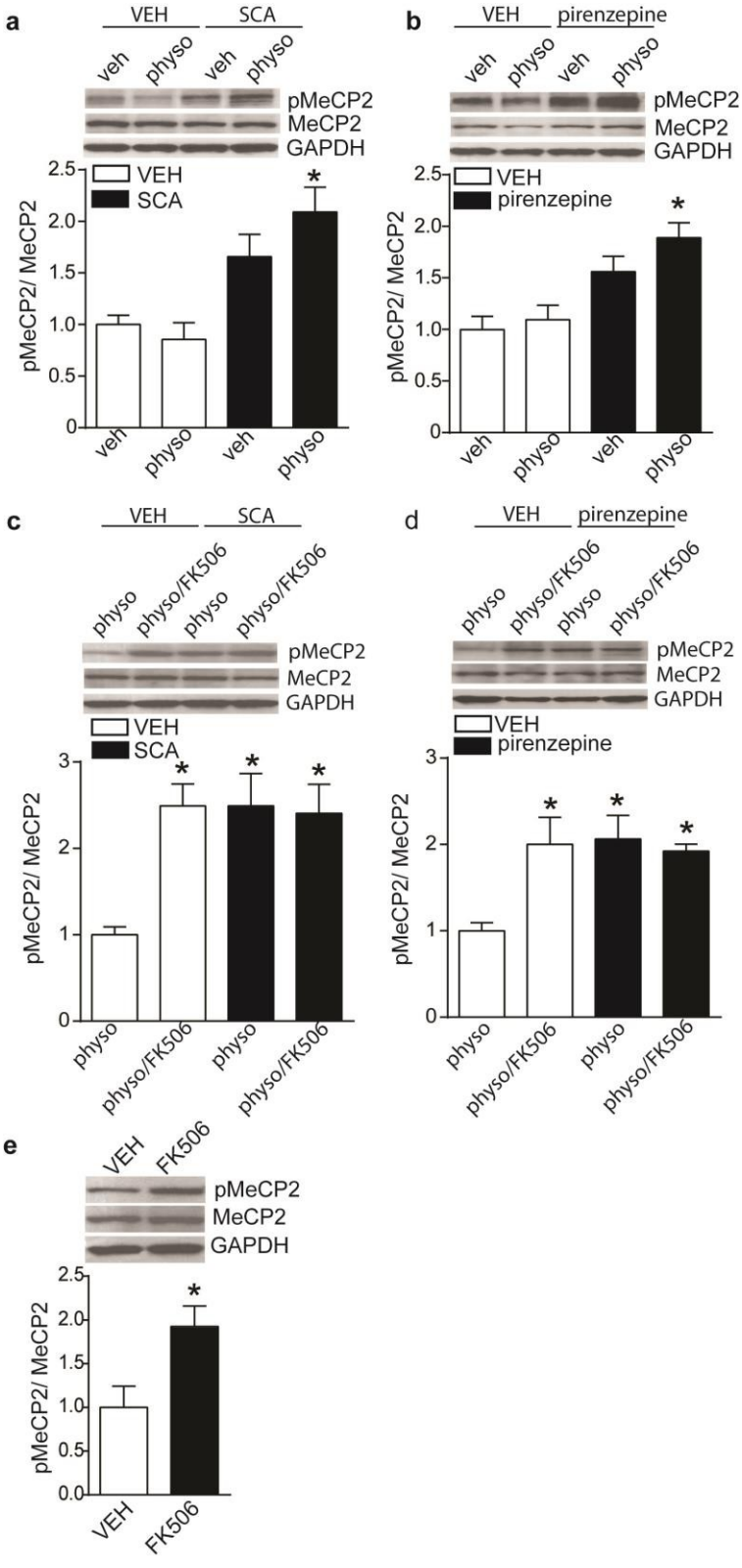


Figure 4-3. The effect of CaMKII inhibition on scopolamine mediated increases in p-S421 MeCP. a-b, KN62 (5.0 mg/kg) was administered to C57BL/6 mice i.p 30 min prior to scopolamine (0.15 mg/kg). **a,** Hippocampal phosphorylated S421 MeCP2 protein levels were measured 24 hours after scopolamine administration. Densitometric analysis of pMeCP2 levels normalized to total MeCP2. ANOVA $F(3,36) = 8.087$, $P=0.0003$ for treatment, Tukey's post hoc test demonstrates that scopolamine increases pMeCP2 levels relative to vehicle and that KN62 blocks scopolamine-mediated increases in pMeCP2. *$P< 0.05$. n=10/group. **b,** Levels of phosphorylated T286 CaMKII α were measured 24 h after scopolamine application. Densitometric analysis of pCaMKII α normalized to total CaMKII α. ANOVA $F(3,28)=6.218$, $P=0.0023$ for treatment. Tukey's post hoc test reveals that KN62 attenuates the phosphorylation of pCaMK2α in both vehicle and scopolamine treated groups. *$P<0.05$ n=8/group.


a
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SCA
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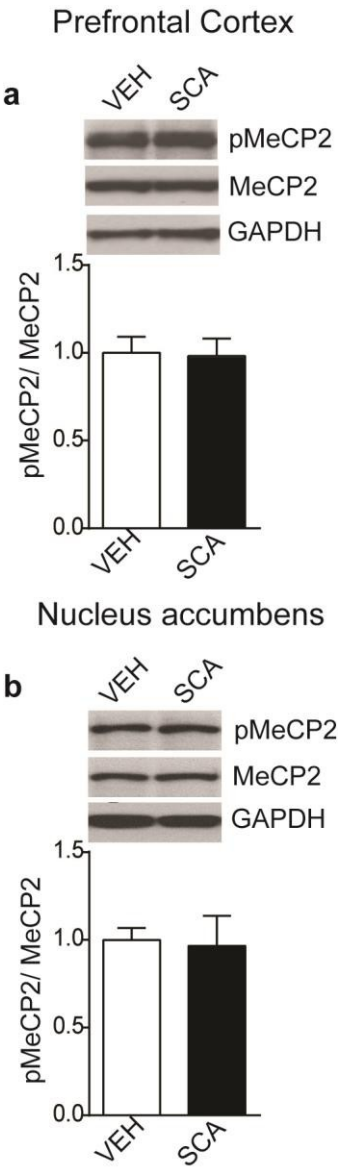
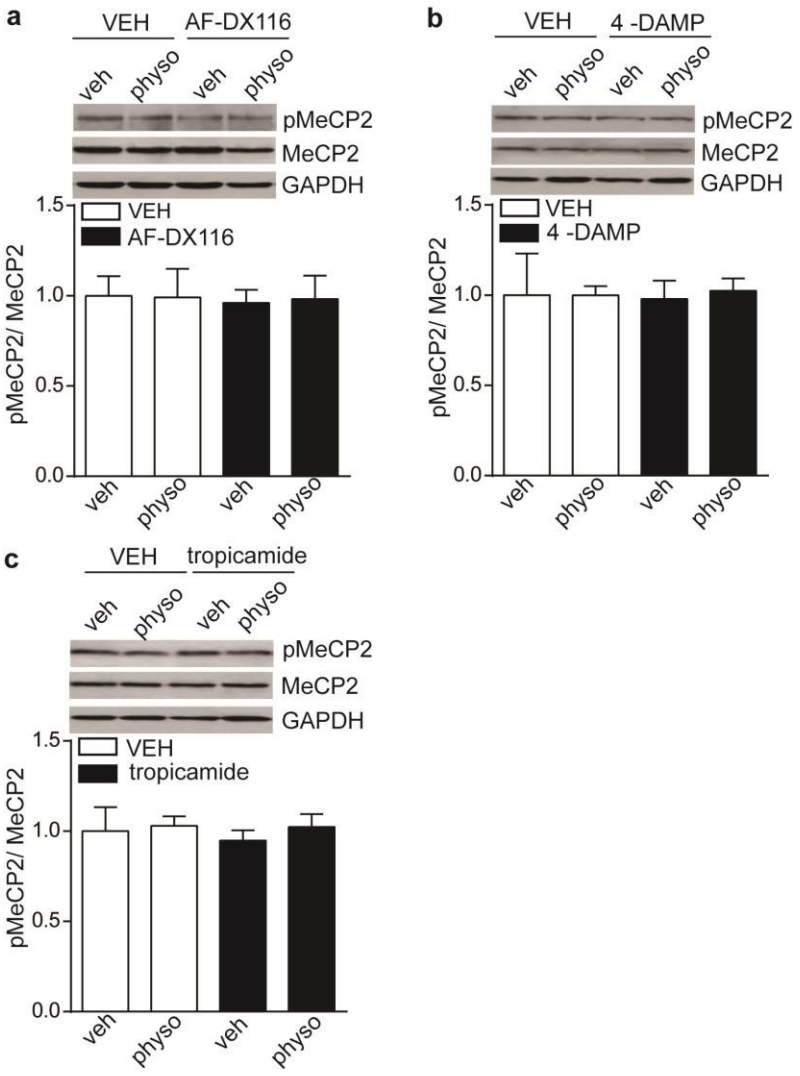
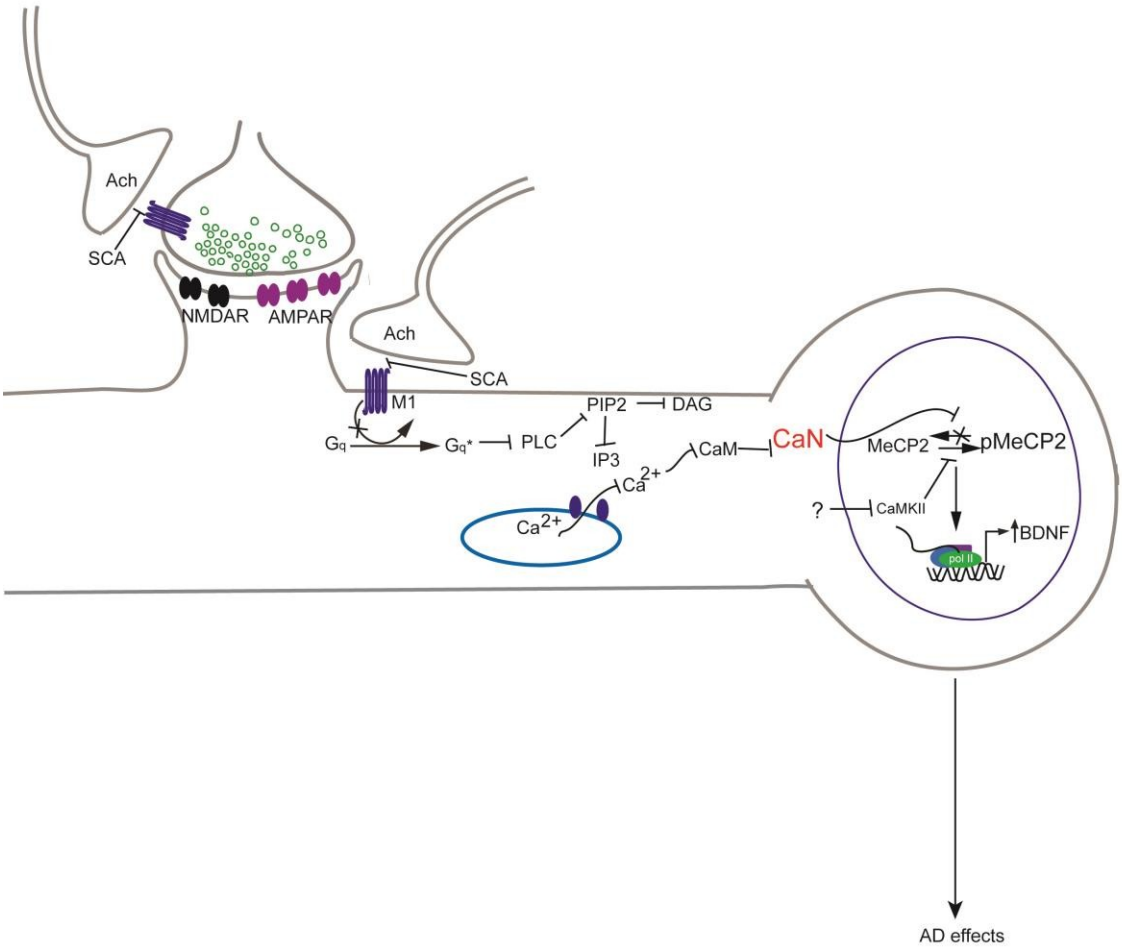
Figure 4-4. M1 muscarinic acetylcholine receptor blockade increases pMeCP2 levels and inhibition of calcineurin activity triggers scopolamine mediated increases in pMeCP2. **a-b,** Acute hippocampal slices (350 microns) were treated with vehicle or physostigmine (2 µM) for 1 hour prior to being treated for 3 hours with either vehicle or scopolamine (220 nM) or vehicle or physostigmine (2 µM). **a,** Densitometric analysis of pMeCP2 levels (normalized to total MeCP2) in acute hippocampal slices following scopolamine treatment in the presence and absence of physostigmine, ANOVA $F(3,12)= 8.078$, $P=0.0033$ for treatment, Tukey's post-hoc analysis, $^*P<0.05$. n=4/group. **b,** Densitometric analysis of pMeCP2 levels (normalized to total MeCP2) in acute hippocampal slices following pirenzepine treatment in the presence and absence of physostigmine, ANOVA $F(3,12)= 7.638$, $P=0.0041$ for treatment, Tukey's post-hoc test ,$^*P<0.05$. (N=4/group). **c-d,** Acute hippocampal slices were pre-treated with physostigmine (2 µM) for 1 hour prior to being treated for 1 hour with FK506 (50 nM). Slices were then incubated with either vehicle or scopolamine (220 nM) or vehicle or physostigmine (2 µM) for 3 hours. **c,** Densitometric analysis of pMeCP2 levels (normalized to total MeCP2) in acute hippocampal slices following physostigmine and FK506 treatment in the presence and absence of scopolamine, ANOVA $F(3,12)= 5.520$, $P=0.001292$ for treatment, Tukey's post-hoc analysis, $^*P<0.05$. N=4/group. **d,** Densitometric analysis of pMeCP2 levels (normalized to total MeCP2) in acute hippocampal slices following physostigmine and FK506 treatment in the presence and absence of pirenzepine. ANOVA $F(3,12)= 7.251$, $P=0.0049$ for treatment, Tukey's post-hoc test, $^*P< 0.05$. n=4/group. **e,** Levels of hippocampal pMeCP2 were measured 24 hours after FK506 (5 mg/kg) administration. Densitometric analysis of pMeCP2 normalized to total MeCP2. Student's *t*-test $P=0.0018$ reveals that FK506 increases pMeCP2 in comparison to vehicle. Significance was set at $^*P<0.05$. n=6/group.



Supplemental Figure 4-1. Scopolamine does not alter levels of pMeCP2 in the prefrontal cortex or the nucleus accumbens. a-b. C57BL/6 mice were administered either vehicle (0.9% saline) or scopolamine (0.15mg/kg) i.p. Levels of pMeCP2 were measured 24 hours after injection. **a,** Densitometric analysis of pMeCP2 normalized to total MeCP2 in the prefrontal cortex. Student's *t*- test, P=0.2884, indicates that no significant differences were found between vehicle and scopolamine treated groups. Significance was set at *P<0.05. n=5/group. **b,** Densitometric analysis of pMeCP2 normalized to total MeCP2 in the nucleus accumbens. Student's *t* –test, P=0.4556, reveals that no significant differences were detected between vehicle and scopolamine treated groups. Significance was set at *P<0.05. n=10/group. The values represent the mean ± SEM.



Supplemental Figure 4-2. Inhibition of M2, M3, or M4 acetylcholine receptor subtypes do not alter pMecP2 levels. a-c, Acute hippocampal slices (350 microns) were treated with vehicle (ASCF) or physostigmine (2 µM) for 1 hour prior to being treated for 3 hours with either vehicle (0.008% DMSO) or AFDX-116 (150nM), vehicle (0.001% dmso) OR 4-DAMP (1nM), or vehicle ().001% DMSO) and tropicamide (20 nM). **a,** Densitometric analysis of pMeCP2 normalized to total MeCP2 after AFDX-116 treatment in the presence and absence of vehicle of physostigmine, ANOVA = $F(3,14)= 1.755$, $P=0.2018$ for treatment. *$P<0.05$. n= 4 veh; n=4 physostigmine; n=5 veh AFDX-116; n=5 physostigmine/AFDX-116. **b,** Densitometric analysis of pMeCP2 normalized to total MeCP2 after 4-DAMP treatment in conjunction with vehicle or physostigmine, ANOVA $F(3,14) =0.05467$, $P=0.9820$ for treatment. *$P<0.05$ n=4 veh; n=4 physostigmine; n=5 veh/ 4-DAMP, n=5 physostigmine/4-DAMP. c, Densitometric analysis of vehicle or physostigmine, ANOVA $F(3,14) = 0.2055$, $P=0.8897$ for treatment. *$P<0.05$. n=4 veh; n=4 physostigmine; n=5 veh/tropicamide; n=5 physostigmine/tropicamide.



Supplemental Figure 4-3. Working Model. Our data suggests a model in which blockade of M1 muscarinic acetylcholine receptors, increases neurotransmitter release presynaptically, which leads to a downstream postsynaptic inhibition of calcineurin, culminating in increases in pMeCP2 levels, *bdnf* transcription, and antidepressant efficacy.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The preceding chapters describe our recent observations that more clearly define the role of BDNF signaling in mechanisms underlying the fast-acting antidepressant action of ketamine and scopolamine. These data extend our understanding of how BDNF is involved in the pathology of MDD and in the therapeutic effect of fast-acting antidepressant agents. There is, however, a vast amount of knowledge anticipated to be discovered on a mechanistic level, both synaptically and biochemically, regarding the nature of MDD and the mechanisms of rapid and effective antidepressant treatments.

We began our investigation by asking whether ketamine might impact animal behavior in a dose dependent manner. In order to pursue this line of inquiry, we administered a range of ketamine dosages (3, 20 and 50 mg/kg) to C57BL/6 mice and found differential effects on locomotor activity, prepulse inhibition (PPI), forced swim, and novelty suppressed feeding behavior. We observed that high ketamine (20 or 50 mg/kg) increased total locomotor activity, whereas low ketamine (3 mg/kg) did not. We also showed that high ketamine (20mg/kg and 50 mg/kg) produced deficits in PPI behavior, whereas low ketamine (3 mg/kg) did not alter PPI behavior. Moreover, we found that only low ketamine (3mg/kg) produced significant antidepressant –like effects in both the FST and the NSF tests, whereas high ketamine doses did not. Furthermore, we demonstrated that low dose ketamine (3 mg/kg) reduced eEF2 phosphorylation and increased BDNF protein 30 minutes after ketamine injection, whereas high dose ketamine (20mg/kg and 50mg/kg) evoked no change in eEF2 phosphorylation or BDNF.

We believe that these data provide compelling evidence that ketamine specifically activates eEF2 at low but not at high doses, thereby suggesting that ketamine may activate divergent signaling pathways in a dose dependent manner, which may be responsible for ketamine's differential impact on behavior, including antidepressant efficacy.

In the future, it may be important to determine the cellular mechanisms by which high dose ketamine functions. A form of regulation that has previously been proposed for the action of high dose ketamine suggests that NMDA receptors present on inhibitory interneurons are tonically active and drive inhibition onto excitatory networks (Moghaddam and Javitt, 2012). Blockade of these NMDA receptors therefore leads to a decrease in inhibition that in turn "disinhibits" excitatory networks. This hypothesis for high dose ketamine, however, has yet to be tested. It will be exciting to test this hypothesis for high dose ketamine, as it may lead to a more thorough understanding of ketamine action as well as the discovery of novel targets for the treatment of depression and/or psychotic disorders.

While it is relatively straightforward to conceive how activation of NMDA receptors leads to synaptic and behavioral plasticity, it is more difficult to envision how an NMDA receptor blocker, such as ketamine, can lead to synaptic and behavior plasticity. The action of a blocker implies that there is ongoing tonic activity of NMDA receptors that lead to signaling events, which in turn is suppressed by the blocker that either inhibits these signaling events or leads to a desupression of an alternative pathway. To explain the rather unusual effect of ketamine at the neuronal level, our studies have focused on a more synapse specific effect of ketamine as the underlying basis for its antidepressant effect. Previously, we demonstrated that ketamine-mediated blockade of NMDA receptors at rest deactivated eukaryotic elongation factor 2 (eEF2) kinase, resulting in a reduction of eEF2 phosphorylation and a desuppression of BDNF

translation necessary to elicit synaptic and behavioral effects, which were required for antidepressant efficacy (Autry et al., 2011). However, the protein phosphatase involved in eEF2 dephosphorylation in neurons remained elusive. Given the proposed role of eEF2 phosphorylation as a key determinant of ketamine's rapid antidepressant action, we hypothesized that the protein phosphatase specific for phosphorylated eEF2 to balance eEF2 kinase action was a necessary component for ketamine mediated rapid antidepressant responses.

Next we demonstrated that the activity of the calcium calmodulin-dependent phosphatase, calcineurin, controlled ketamine mediated decreases in eEF2 phosphorylation levels *in vitro* and *in vivo* and that it was required for ketamine to trigger fast acting antidepressant responses. We additionally showed that the fast calcium buffer BAPTA but not the relatively slow buffer EGTA reversed ketamine mediated reductions in phosphorylated eEF2, while both BAPTA and EGTA equally reduced mEPSC frequency, suggesting that the source of postsynaptic calcium entry which regulates calcineurin is independent of NMDA receptors. We demonstrated that L-type Ca^{2+} channel function is required to activate calcineurin and elicit ketamine's behavioral effects. These results support the premise that L-type calcium mediated calcium influx at rest activates calcineurin, which in turn dephosphorylates eEF2 thus augmenting BDNF signaling to elicit ketamine's rapid antidepressant effect.

The crucial role for calcineurin in the regulation of eEF2 phosphorylation bolsters the premise that ketamine acts via inactivation of eEF2 kinase leading to a decrease in phosphorylation of eEF2 and a subsequent rapid increase in BDNF protein which is necessary for rapid antidepressant responses. The bidirectional regulation of eEF2 phosphorylation and subsequent increase in BDNF expression further expand this model. Finally, as neuronal voltage-gated calcium channels are already implicated in the pathophysiology of a number of

neuropsychiatric disorders and can be pharmacologically or molecularly targeted by a wide range of pathways (Lipscombe et al., 2004; Ma et al., 2013), their role in ketamine's rapid antidepressant action opens up novel avenues for treatment advance against major depressive disorder.

A key question raised by this study is whether calcineurin can be manipulated *in vivo* to enhance ketamine-mediated antidepressant efficacy. Future studies in the lab will thus take a viral-mediated approach to investigate whether knockdown of the regulatory subunit of calcineurin (calcineurin B) will enhance the activity of the catalytic domain of calcineurin (calcineurin A) and, in turn, antidepressant efficacy. This will be done by selectively expressing an AAV virus in the dentate gyrus of the hippocampus, which expresses both the catalytic domain of calcineurin (calcineurin A) and the knockdown of calcineurin B and assessing animal behavior in tests of antidepressant efficacy. Based on our previous data, which demonstrated that inhibition of calcineurin blocks ketamine-mediated behavior, we predict that expression of this virus will increase the catalytic activity of calcineurin and potentiate the behavioral response to ketamine. We also predict to see baseline antidepressant effects in the absence of ketamine in animals expressing this virus. We further may expect that Western blot analysis or immunohistochemical analysis of tissue expressing this virus will reveal reduced phosphorylated eEF2 expression and enhanced BDNF expression even in the absence of ketamine. Alternative future approaches to this same question may involve the acquisition and generation of transgenic mice in which the Tet operon drives the expression of calcineurin lacking the autoinhibitory domain (calcineurin B) and/or the acquisition of transgenic mice in which the Tet operon drives expression of only the calcineurin autoinhibitory domain**.** Based on our previous studies, we predict that transgenic mice expressing the autoinhibitory domain for calcineurin will not

respond to ketamine in tests of antidepressant efficacy; whereas we predict that transgenic mice expressing only the catalytic domain of calcineurin will demonstrate potentiated ketamine-mediated behavioral responses. The caveat to this approach is that it would not address the circuitry involved in ketamine – mediated behavior. In contrast, the previously described AAV viruses, which manipulate the activity of the catalytic domain of calcineurin and/or the autoinhibitory domain, may provide substantial insight into the circuitry involved in ketamine's behavioral effects as these constructs can be expressed in localized brain regions in a temporally restricted manner.

Another intriguing future direction is the identification of the mechanisms which contribute to ketamine's long lasting behavioral effects. While ketamine induces a rapid antidepressant effect, the antidepressant effect of ketamine is reported to last for up to two weeks, which is long after the 2-3 hour half-life of the drug. Although, we have not thoroughly tested the possibility that off-target effects may contribute to the long lasting antidepressant-like effects of ketamine, our lab has previously shown that targeting eEF2 kinase with its specific inhibitor, rottlerin, produces a long lasting antidepressant effect, similar to ketamine, suggesting specificity to the ketamine response (Autry et al., 2011). We have also shown that eEF2 kinase is required for the ketamine effect (Nosyreva et al., 2013). Based on this data, we posit 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; this permits further downstream BDNF-mediated plasticity. This potential mechanism is intriguing and would be essential to test in order to further clarify the notion that homeostatic processes contribute to ketamine's long lasting effects. To test this mechanism, wild-type, knockdown, and mutant eEF2 kinase lentiviruses and adeno-

associated viruses are being generated in order to characterize their electrophysiological parameters in primary hippocampal cell culture and behavior *in vivo*.

Our lab has previously shown that the fast acting effect of ketamine requires a transient upregulation in protein translation rather than transcription. It is reasonable to predict, however, that transcriptional mechanisms play a role in mediating longer lasting effects of ketamine. Our lab has recently begun to investigate whether transcription is required for long-term ketamine action. In an initial study, we administered ketamine (3mg/kg) to MecP2 knockout mice and their littermate controls, and asked if the transcription factor, MecP2, was required for ketamine mediated behavior 7 days later. In littermate controls we observed that ketamine significantly reduced immobility time compared to vehicle, suggesting a long-term antidepressant effect. In contrast, MecP2 knockout mice did not respond to ketamine, indicating that MecP2 is necessary for the long lasting effects of ketamine. The transcription factor Mef2C was also explored as a putative regulator of long-term ketamine action. In contrast to MecP2 knockout mice, we observed decreased immobility in Mef2C knockout mice and in littermate controls 7 days after acute ketamine administration, indicative of an antidepressant effect. Thus, Mef2C does not appear to be required for long-term ketamine antidepressant effects, lending specificity to the regulation of ketamine action by MecP2. However, future studies investigating the specificity of MecP2 to ketamine action and the time point when transcription is required for the long lasting effect of ketamine are critical, as acquisition of this information may provide information regarding molecular determinants that are essential to ketamine-mediated processes.

It is important to note that evidence suggests that ketamine may disrupt Regulator of G protein signaling 4 (RGS4) activity; a signal transduction protein that controls the function of monoamine, opiate, muscarinic, and other G-protein coupled receptors (Stratinaki et al., 2013).

It has also been documented that ketamine increases dopaminergic activity and potentially decreases acetylcholine activity (Lydic and Baghdoyan, 2002). Future investigations into the time-frame at which ketamine elicit these effects would be intriguing; because the notion that neuromodulators might play a role in ketamine's long lasting effects is not illogical. Moreover, future investigations into convergent and/or divergent pathways may allow the identification of additional molecular targets that could be used to facilitate therapeutic effects.

Although considerable progress has been made within a relatively short period of time toward the goals of understanding the mechanisms mediating ketamine's antidepressant effects, much more knowledge is needed. Indeed, a number of avenues are being pursued, including mechanistic preclinical studies, clinical trials with different formulations of ketamine, as well as more selective and better tolerated non-ketamine NMDA antagonists and other non-NMDA glutamatergic modulators. In this regard, it will be critical to fully delineate the specific synaptic mechanisms underlying ketamine action as this information will likely uncover additional synaptic proteins that can be targeted to elicit a rapid antidepressant response (Kavalali and Monteggia, 2012). While ketamine appears to benefit patients with treatment-resistant depression, further studies into ketamine's safety and feasibility are needed to determine its ultimate clinical utility (Rush, 2013).

Finally, our studies have explored the underlying cellular and molecular mechanism involved in scopolamine-mediated fast acting antidepressant action. In this study, we showed that scopolamine produced a fast acting antidepressant effect in animal models which is dependent on *bdnf* transcription. We additionally showed that scopolamine increases neurotransmitter release. Moreover, we demonstrated that scopolamine increases

phosphorylated MecP2 at position S421 *in vivo*. We demonstrated that scopolamine and the selective M1 receptor antagonist, pirenzepine, increase phosphorylated S421 MecP2 in acute hippocampal slices. Furthermore, we showed that inhibition of calcineurin activity increase phosphorylated S421 MecP2 levels *in vitro* and *in vivo*. Based on this study, we propose that scopolamine, through blockade of M1 muscarinic acetylcholine receptors, increases neurotransmitter release presynaptically, which leads to downstream post synaptic effects, causing post-synaptic M1 dependent inhibition of calcineurin activity that enhances S421 MecP2 phosphorylation, thereby triggering increases in *bdnf* transcription, which produce the antidepressant effect.

In this study we showed that scopolamine consistently increased phosphorylation of MecP2 at S421. Initial future work on this project will involve confirming whether MecP2 and pS421 MecP2 are required for scopolamine's antidepressant effect. We also plan to assess the impact of the inhibition of CaMKII on scopolamine-mediated behavioral responses. We further plan to assess whether inhibition of calcineurin can potentiate the effect of low dose scopolamine in tests of antidepressant efficacy.

We also identified *bdnf* as a gene that is required for scopolamine's antidepressant effects. In the future we intend to determine if *bdnf* is sufficient for the antidepressant response of scopolamine, as, alternatively, other genes may also impact scopolamine-mediated behavior. Other future directions include investigating the molecular mechanisms which regulate the longer term antidepressant effects at 1 and 2 weeks after scopolamine administration. Future plans include looking into neuromodulators and the possibility of other transcription factors in controlling scopolamine mediated antidepressant efficacy at those time points. Finally, microRNAs are emerging are novel antidepressant targets (O'Connor et al., 2013). Thus, we

intend to assess cotranscriptional processing of microRNAs in relation to MecP2, as MecP2 has been shown to regulate microRNA processing and alter the expression of transcription factors through this mechanism.

In summary, a more thorough understanding of the mechanisms by which scopolamine produces its antidepressant response is crucial for the development of fast -acting antidepressants. It will also be critical to identify the molecular determinants which can be targeted to prolong the antidepressant responses of both ketamine and scopolamine. Furthermore, the identification of convergent pathways between scopolamine and ketamine may facilitate the discovery of novel fast-acting therapeutic strategies with long-term efficacy for the treatment of major depressive disorder.

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