Antidepressant Interactions of Ketamine and Glycogen Synthase Kinase-3 (GSK3) in Mice

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ANTIDEPRESSANT INTERACTIONS OF KETAMINE AND GLYCOGEN SYNTHASE KINASE-3 (GSK3) IN MICE

By
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A THESIS

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Master of Science

Coral Gables, Florida
May 2015
ANTIDEPRESSANT INTERACTIONS OF KETAMINE AND GLYCOGEN SYNTHASE KINASE-3 (GSK3) IN MICE

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Depression is a prevalent and debilitating mood disorder affecting nearly one in five people in the United States. Current medications used for treatment of depression are inadequate because they have a delayed onset of therapeutic benefit, low efficacy, several side effects, and require chronic administration. This presents the need for improved antidepressant therapies. Ketamine, a general anesthetic, was recently shown to have rapid-acting antidepressant effects at a sub-anesthetic dose. It is unknown how ketamine elicits an antidepressant effect, but several mechanisms have been proposed, including glycogen synthase kinase-3 (GSK3) inhibition and α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA) receptor activation. This thesis focuses on the role of GSK3 in the ketamine antidepressant effect by expanding previous findings to additional depressive-like behavior models in mice and examines the molecular role of GSK3 and AMPA receptors in response to ketamine in order to further understand the signaling that leads to ketamine’s antidepressant effect. I provide evidence that the antidepressant effect of ketamine requires the inhibition of GSK3 and the activation of AMPA receptors and support a mechanism involving interaction between GSK3 and AMPA receptor trafficking that leads to the
antidepressant effect of ketamine. This will provide possible strategies to develop much needed new antidepressant therapies.
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Chapter 1: Introduction

1.1. Depression

Depression is a highly prevalent, debilitating, recurring mental illness primarily affecting mood. In addition to changes in mood, depression causes abnormalities in cognition, neurovegetative functions (e.g. appetite, sleep, and concentration), and psychomotor activity, (e.g. impaired coordination and incoherent speech) (Fava & Kendler, 2000; Villanueva, 2013). Depression is the most common psychiatric disorder affecting approximately 20% of the United States population with the most severe forms, also referred to as major depressive disorder, affecting 2-5% of the population (Fava & Kendler, 2000; Kessler et al., 2003; Nestler et al., 2002b; Villanueva, 2013). Females are twice as likely as males to suffer from a form of depression. The onset of depression can begin at any age, but is most common in adults 25-45 years of age (Nestler et al., 2002a; Villanueva, 2013). A depressive episode lasts a period of at least two weeks and includes five out of nine symptoms defined by the American Psychiatric Association’s DSM 5, including low mood and irritability, loss of enjoyment in once pleasurable activities, change in appetite and weight, change in sleep patterns, decreased energy, feelings of worthlessness and guilt, difficulty concentrating, and thoughts of death and suicide. It is by these criteria that depression is clinically diagnosed (Nestler et al., 2002a). Depression lowers a person’s functionality making it one of the leading causes of years lost to disability, lost productivity, and a major health care expense (Fava & Kendles, 2000; Manji et al., 2001; Marcus & Olfsen, 2010). Due to the increased risk of
suicide, depression is a threatening illness and a leading cause of death worldwide (Nestler et al., 2002b).

The cause of depression is not fully understood, but it is believed to be a combination of factors. Depression is a highly heritable disorder and has a genetic risk factor of 40-50% (Berton & Nestler, 2006; Nestler et al., 2002a). However, there is a lack of fundamental understanding of the genes involved in increasing the risk of depression and therefore the specific gene(s) involved have not been identified (Nestler et al., 2002b). Non-genetic factors are also important in the susceptibility to depression. Environmental factors, particularly stress and emotional trauma, are often triggers for depressive episodes. In addition, the etiology of depression is influenced by biological factors, such as brain development and viral infections. Depression is also frequently comorbid with other diseases including cancer, diabetes, Cushing’s disease, Parkinson’s disease, and heart disease (Benton & Nestler, 2006; Fava & Kendler, 2000; Lason et al., 2013).

The pathophysiology of depression is complex because many brain regions are implicated in regulating emotions and mood. Due to the diversity of symptoms associated with depression, it is most likely that several brain regions play a role in the mood disorder (Kharade et al., 2010; Nestler et al., 2002a). Brain imaging and postmortem studies have implicated the prefrontal cortex, hippocampus, striatum, amygdala, and thalamus because they have shown abnormalities in depressed patients (Hastings et al., 2004; Manji et al., 2001; Nestler et al., 2002a). The hippocampus and prefrontal cortex contribute to the
impaired cognitive features of depression, such as memory impairments and feelings of worthlessness, while the striatum, amygdala, and thalamus are significant in emotional memory and contribute to anxiety and reduced enjoyment in activities (Benton & Nestler, 2006; Nestler et al., 2002a).

There are several additional factors believed to influence depression. One of the earliest suggested and most dominate theories is the monoamine hypothesis of depression which states that a deficiency of neurotransmitters (serotonin, norepinephrine, and dopamine) cause depression. It was based on the observation that early antidepressant agents increased the levels of these neurotransmitters (Kharade et al., 2010; Massart et al., 2012; Wainwright & Galea, 2013). The monoamine theory does not fully explain the cause of depression though because antidepressants developed based on this theory are only effective in approximately 50% of patients (Massart et al., 2012). The neurotrophic theory of depression proposes that stress causes a decrease in neurotrophin levels that play a role in mood regulation (Clark-Raymond & Halaris, 2013). Neurotrophins are involved in survival, differentiation, and maintenance of neurons (Jiang & Salton, 2014). Reduced levels of neurotrophins, particularly brain-derived neurotrophic factor (BDNF), have been associated with depressive-like behavior in animal models and have been observed in serum of depressed individuals (Jiang & Salton, 2014). Antidepressant treatments increase levels of BDNF (Autry, 2011; Schmidt et al., 2008). Another theory suggests abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis have a role in depression. The HPA axis is activated during a stressor and works as a feedback loop between
the hypothalamus, pituitary, and adrenal glands resulting in the release of cortisol, the glucocorticoid stress hormone. It is common among patients with mood disorders to have HPA axis hyperactivity leading to increased levels of cortisol which may result in structural changes of the brain, specifically the hippocampus (Massart et al., 2012; Varghese et al., 2001). Inflammation following stress is also suggested to have a role in depression. Depression is often comorbid with infectious, autoimmune, and neurodegenerative disorders and depressed patients frequently have high levels of pro-inflammatory cytokines (Leonard, 2010; Massart et al., 2012). The neuroplasticity theory of depression proposes that there are impairments in many facets of brain plasticity, including the birth, survival, migration, and integration of new neurons, causing depression (Wainwright & Galea, 2013). Stress, a main casual factor of depression, decreases neurogenesis and reports state that increasing neurogenesis relieves depressive symptoms (Jacobs et al., 2000). This theory encompasses several of the other proposed hypotheses as neural plasticity is a highly involved process influenced by several signaling pathways including neurotrophins, glucocorticoids, and neurotransmitters (Wainwright & Galea, 2013). The etiology and pathophysiology of depression is multifaceted making effective treatment of the mood disorder difficult.

1.2. Antidepressants and Other Treatments for Depression

1.2.1. Treatments for Depression

There are several beneficial treatments for depression, despite the complexity of the etiology of depression. The treatment for depression can
involve psychotherapy, such as cognitive and behavioral therapies, electroconvulsive therapy (ECT), and most commonly pharmacotherapy, such as antidepressant medications. Psychotherapies have shown efficacy in mild and moderate forms of depression and often are supplemented with antidepressants (Benton & Nestler, 2006). ECT involves pulsing electric currents through the brain to cause brief seizures that result in changes in brain chemistry. Although very effective, the use of ECT is limited to cases of depression that are resistant to other treatment options or for patients that show signs of psychosis (Fava & Kendler, 2000). Antidepressant medications are the most common and most effective treatment for depression.

1.2.2. First Generation Antidepressants

In the 1950s two classes of antidepressant drugs were discovered by chance; monoamine oxidase inhibitors (MAOIs) were developed from antitubercular drugs and tricyclic antidepressants (TCAs) were developed from antihistamine drug research when they were both discovered to have antidepressant properties unexpectedly (Nestler et al., 2002a). MAOIs are chemicals that act by inhibiting the activity of monoamine oxidase enzymes. This inhibition prevents the oxidative breakdown of monoamine neurotransmitters increasing their availability in the synapse to carry signals more efficiently (Baldessarini, 1985). MAOIs are often only used after other antidepressants have failed to improve a patient’s symptoms because they alter the metabolism of other drugs and tyramine present in food and have many side effects that can be as severe as hypertension (Baldessarini, 1985; Grady & Stahl, 2012).
TCAs are named for their chemical structure of three connecting rings of atoms. TCAs modulate mood by blocking the transporters for reuptake of neurotransmitters, principally serotonin and norepinephrine, increasing their synaptic concentration resulting in improved neurotransmission. TCAs are well absorbed after oral administration, but have several side effects including headache, dizziness, nausea, fatigue, seizures, and in some cases can be toxic at high doses (Baldessarini, 1985; Gillman, 2007).

1.2.3. Second Generation Antidepressants

Following the discoveries of MAOIs and TCAs, research on depression and antidepressant mechanisms increased. Pharmacological studies of TCAs provided evidence that the modulation of monoamine neurotransmitters was responsible for the antidepressant response in patients. This led to rationale drug development by pharmaceutical companies throughout the 1960s and 1970s focused on producing a drug that would specifically inhibit reuptake of serotonin to the presynaptic neuron, but have no effect on other neuroreceptors with the goal of creating a drug with antidepressant effects and fewer side effects. This resulted in the development of selective serotonin reuptake inhibitors (SSRIs), the first class of the second generation of antidepressants (Preskorn et al., 2004; Schechter et al., 2005). Following the development of SSRIs, drugs with similar reuptake inhibitory properties targeting the synaptic increase of additional monoamine neurotransmitters, norepinephrine and dopamine, began being produced. The goal of dual acting serotonin/norepinephrine reuptake inhibitors (SNRIs) and triple- reuptake inhibitors, which increase levels of serotonin,
norepinephrine, and dopamine, was to increase efficacy and further reduce side effects (Schechter et al., 2005).

1.2.4. Shortcomings of Antidepressants

Although selectivity, safety, tolerance, and efficacy have improved with the development of new antidepressant drugs, there are still several shortcomings of these medications (Benton & Nestler, 2006; Lieberman, 2003; Preskorn, 2012). Most clinically available antidepressants are based on the early research of TCAs and consequently development has focused on a similar mechanism of action targeting components of the monoamine neurotransmitter system to increase the synaptic availability of serotonin, norepinephrine, and dopamine (Lopez-Munoz & Alamo, 2009; Nestler et al., 2002a). Therefore, currently available antidepressants all suffer from the same limitations including the need for chronic administration, delayed onset of therapeutic benefits and, although improvements have been made, antidepressants are still lacking efficacy and continue to have many side effects (Duman & Li, 2012; Kavalali & Monteggia, 2012). Antidepressants are only effective in approximately 50% of patients and most patients require trials with multiple antidepressants before the optimal treatment to achieve remission of symptoms is found (Benton & Nestler et al., 2006; Gaynes et al., 2009; Murrough & Charney, 2012). Response rates decrease further in patients that do not show signs of remission after two antidepressant trials (Gaynes et al., 2009). In the United States, one-third of patients are defined as treatment-resistant because two or more antidepressant therapies failed to produce remission of symptoms (Covvey et al., 2012).
Antidepressants have a delay of onset to elicit an antidepressant response of several weeks (Duman & Li, 2012; Gelenberg & Chesen, 2000; Kavalali & Monteggia, 2012). Quitkin et al. (1996) showed that within the first two weeks of treatment there is no significant antidepressant effect. The suggested timeline for assessment of antidepressants effects is two-four weeks due to the delay in time of the response to antidepressants (Anderson et al., 2008; Bauer et al., 2007). These weaknesses of current antidepressants present the need for a better understanding of the mechanisms of depression to aid the development of antidepressant therapies that are fast acting and effective in treatment resistant patients.

1.3. Ketamine as an Antidepressant

1.3.1. Glutamatergic System

Depression and antidepressant research has led to a growth in knowledge about other mechanisms contributing to depression’s etiology in addition to monoamines (Benton & Neslter, 2006). There is accruing evidence that the glutamatergic system is involved in the neurobiology of mood disorders (Lapidus et al., 2013; Murrough & Charney 2012; Sanacora et al., 2008). This presents new prospects for antidepressant development. Glutamate mediates excitatory synaptic transmission in the brain that plays a major role in synaptic plasticity, learning, and memory, but in certain conditions can lead to neurotoxicity. Glutamate neurotransmission is required to sustain neuronal function. Evidence suggests that dysregulation of the glutamatergic system is a contributing factor to synaptic impairments in depression (Sanacora et al., 2008).
In the central nervous system there are two subtypes of glutamatergic receptors. The first group is the metabotropic glutamate receptors (mGluRs), G protein-coupled receptors. The second group is ionotropic glutamate receptors and there are three subtypes: kainite (KA) receptors, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, N-Methyl-D-aspartic acid (NMDA) receptors. AMPA receptors, and to a lesser extent KA receptors, are involved in mediating fast excitatory neurotransmission by opening a channel in response to glutamate to allow the passage of ions into or out of the postsynaptic neuron. NMDA receptors act similarly, but produce excitation over longer periods of time. AMPA receptors and NMDA receptors are co-expressed at mature synapses (Sanacora et al., 2008). NMDA receptors are crucial mediators of synaptic plasticity and antagonists of these receptors have been shown to have antidepressant effects (Paoletti et al., 2013; Trullas & Skolnick, 1990).

1.3.2. Ketamine

Ketamine is a noncompetitive NMDA receptor antagonist derived from phencyclidine hydrochloride (PCP) that was originally used as a general anesthetic (Krystal et al., 1994; Zunszain, 2012). Ketamine blocks the flow of calcium through the NMDA receptor by binding within the open channel at the PCP binding site (Krystal et al., 1994; Orser et al., 1997). At sub-anesthetic doses, ketamine has been shown to have rapid antidepressant effects, particularly in treatment resistant patients, in several clinical trials. A randomized controlled trial in the 1990s reported that ketamine given one time intravenously at a sub-anesthetic dose relieved depressive symptoms within hours (Berman et
al., 2000). A second trial with only treatment-resistant patients, again showed a low dose of ketamine to have antidepressant effects within two hours that lasted for up to a week (Zarate et al., 2006a). Additional evidence supporting the rapid-onset antidepressant effects of ketamine comes from clinical trials in which a majority of patients that were unresponsive to currently available antidepressants had mood improvement within twenty-four hours following a single low-dose intravenous ketamine treatment (DiazGranados et al., 2010; Larkin & Beautrais, 2011; Machado-Vieira et al., 2009a; Mathew et al., 2010; Price et al., 2009). Thus, there is much interest in ketamine because it has a fast-acting antidepressant effect and is effective in patients who did not previously respond to other antidepressant treatments. However, widespread routine clinical use of ketamine as an antidepressant is not practical because it is not easily self-administered, the long-term harmful effects are unknown, and it is classified as a schedule III substance due to its dissociative and psychosomatic effects and abuse potential (Murrough & Charney, 2012; aan het Rot et al., 2012; Zunszain et al., 2013; Covvey et al., 2012).

Studies of the antidepressant properties of ketamine are currently directed towards understanding the underlying mechanism to develop improved pharmacological therapies without the undesirable side effects. Using animal models, several actions of ketamine have been reported that may contribute to its antidepressant effect. It has been shown that the rapid antidepressant effect of ketamine is associated with increased AMPA receptor activity (Maeng et al., 2008), increased synaptogenesis through the activation of mammalian target of
rapamycin (mTOR), 4E binding protein 1 (4E-BP1), and 70-kDa ribosomal protein S6 kinase (p70S6K) pathway (Li et al., 2010), increased rapid synthesis of BDNF (Monteggia et al., 2013), and by inhibition of GSK3 (Beurel et al., 2011). Although all of these pathways are potential therapeutic targets, it remains to be determined if they act separately or are interrelated in response to ketamine.

1.4. New Possible Therapeutic Target: Glycogen Synthase Kinase-3 (GSK3)

1.4.1. GSK3

Originally discovered for its role in phosphorylating and inactivating the enzyme glycogen synthase, GSK3 was later found to be involved in a variety of cellular functions and to have more than fifty substrates (Embi et al., 1980; Jope & Johnson, 2004). GSK3 has previously been linked to mood disorders. Beurel et al. (2011) found that the inhibition of GSK3 by serine phosphorylation is required for the antidepressant effect of ketamine, prompting further investigation.

GSK3 is a serine/threonine protein kinase denoting two paralogous proteins, GSK3α and GSK3β, that are encoded by separate genes, but share 85% sequence homology and are 97% similar in the kinase domain (Frame & Cohen, 2001; Jope & Johnson, 2004; Li & Jope, 2010). Unlike most kinases, GSK3 is constitutively active and signals influencing GSK3 regulate it by inhibiting its activity (Jope & Johnson, 2004). The primary mechanism of regulation of GSK3 activity is phosphorylation of an N-terminal serine (Ser21 in GSK3α and Ser9 in GSK3β) that inhibits its activity. GSK3 activity is also regulated by its cellular localization and its presence in protein complexes (Jope & Johnson, 2004).
GSK3 activity can be regulated pharmacologically. Lithium, the drug most commonly used to treat bipolar disorder, was shown by Klein and Melton (1996) to inhibit GSK3. Lithium inhibits GSK3 in two ways: by competing with magnesium (Mg$^{++}$) which reduces catalytic activity and by increasing inhibitory serine phosphorylation (Figure 1.4.1.1) (De Sarno et al., 2002). The discovery that lithium inhibits GSK3 led to the idea that GSK3 may be dysregulated in mood disorders (Jope & Johnson, 2004). The therapeutic potential of inhibition of GSK3 led to the development of selective GSK3 inhibitors (Eldar-Finkelman & Martinez, 2011). Many of the GSK3 inhibitors are ATP-competitive, such as SB415286 and SB216763, but more promising are the GSK3 inhibitors that are not competitive with ATP, such as L803-mts, TDZD-8, and VPO.7, because they are less likely to inhibit other kinases and may be less toxic (Eldar-Finkelman & Martinez, 2011; King et al., 2013).

1.4.2. GSK3 Knockin Mouse Model

The homozygous GSK3α21A/21A/β9A/9A knockin mice, referred to as GSK3 knockin mice, are used experimentally to investigate the role inhibitory serine phosphorylation of GSK3 has in a process. GSK3 knockin mice express GSK3 that is maximally active, but within physiological levels because it is not over-expressed. In the GSK3 knockin mice the regulatory serines of both GSK3 isoforms (Ser21 in GSK3α and Ser9 in GSK3β) are mutated to alanines (Ala21 in GSK3α and Ala9 in GSK3β), preventing inhibition of GSK3 via phosphorylation of the inhibitory serine residues (Figure 1.4.1.1) (McManus et al., 2005). GSK3
knockin mice have normal reproduction and development and do not display any overt phenotype.

**Figure 1.4.1.1- GSK3 regulation and GSK3 knockin mice.**

### 1.4.3. GSK3 and Mood Disorders

Links between GSK3 and mood disorders have been shown in both animal models and humans. Administering selective GSK3 inhibitors to mice causes antidepressant-like behavioral effects (Kaidanovich-Beilin et al., 2004; Rosa et al., 2008). Behavioral measures in GSK3 transgenic mice also indicate a connection between dysregulated GSK3 and depression. GSK3 knockin mice, where GSK3 cannot be regulated by inhibitory serine phosphorylation, are more inclined to have depressive-like behaviors following stress compared to wild-type mice (Polter et al., 2010). Mice with GSK3β haploinsufficiency, lacking one copy of the gene encoding for GSK3β, have an antidepressant-like behavior phenotype (O’Brien et al., 2004). Administration of currently used antidepressants, fluoxetine or imipramine, monoamine reuptake inhibitor antidepressants, increased the inhibitory serine phosphorylation of GSK3 (Li et al., 2007). Humans with depression may have abnormal GSK3 activity as shown by a postmortem study of brain samples from depressed subjects in which an increase in GSK3 activity was found (Karege et al., 2007). Therefore,
understanding the role of GSK3 in response to ketamine may open a new avenue for the development of a new therapeutic strategy for depression.

1.5. Summary: Project Description and Goal of Thesis

Depression is a devastating mood disorder affecting approximately one out of five people, making it one of the most severe problems facing the health care system (Greden, 2001; Kessler et al., 2003). The underlying causes of depression are not well understood due to the heterogeneous nature of the mood disorder and as a result, the available treatments have substantial shortcomings, most significantly, poor efficacy in terms of failure to produce a therapeutic response or to maintain an antidepressant response for an adequate time and delayed onset of therapeutic benefit (Duman & Li, 2012; Kavalali & Monteggia, 2012; Krishnan and Nestler, 2008). Antidepressant treatments are only effective in approximately half of patients, with most patients requiring multiple trials of medications (each trial lasts 2-4 weeks) before determining the optimal treatment (Murrough & Charney, 2012). Patients who do not show signs of remission after two antidepressant trials are considered treatment-resistant patients (Gaynes et al., 2009). Therefore, development of antidepressant therapies that are both fast-acting and have a higher efficacy will provide great benefit to current therapies.

The majority of the current antidepressants act on the monoamine neurotransmitter system, principally serotonin. There are effective antidepressant treatments, although, they take time to show efficacy (Krishnan and Nestler, 2008). Therefore, other neurotransmitter systems have been targeted to develop new faster-acting antidepressants. There is increasing evidence that the
glutamatergic system, which is involved in mood disorders, presents potential new targets for antidepressants (Sanacora et al., 2008). Antagonists of NMDA receptors have been shown to have rapid antidepressant effects (Trullas & Skolnick, 1990), one such being ketamine. An intravenously administered sub-anesthetic dose of ketamine relieved depressive symptoms within hours in many treatment-resistant patients (Berman et al., 2000; Zarate et al., 2006a). Although ketamine is a promising treatment, widespread routine clinical use of ketamine as an antidepressant therapy is not practical because it is difficult to self-administer and the effects of long-term use are unknown (Murrough & Charney, 2012). However, understanding the antidepressant properties of ketamine could provide a new antidepressant therapy strategy.

Several mechanisms have been proposed for how ketamine elicits a rapid antidepressant effect. For this thesis, I was interested in understanding the role of GSK3 in the ketamine antidepressant effect. I address the role of GSK3 in the ketamine antidepressant effect in mice behaviors using GSK3 genetically modified mice, GSK3 knockin mice. So far the implication of GSK3 in the ketamine response was achieved in the learned helplessness paradigm of depression-like behavior, and determining if this effect is also observed in other depression-like behaviors may provide a better understanding of the action of GSK3. Because GSK3 has been shown to regulate AMPA receptor trafficking (Du et al., 2010; Gould et al., 2008), I examined the interactions between GSK3 and AMPA receptor trafficking to determine if there is cross-talk between these pathways in response to ketamine. Altogether, I determine if GSK3 inhibition is
sufficient to induce ketamine’s antidepressant effect and the effect of GSK3 inhibition on AMPA receptor activation in response to ketamine. This would provide a new opportunity to examine GSK3 inhibitors as antidepressants.
Chapter 2: Materials and Methods

2.1. Mice

C57BL/6 wild-type and homozygous GSK3α^{21A/21A}/β^{9A/9A} knockin (GSK3 knockin) mice, along with strain- and age-matched wild-type mice were housed three to five per cage in a 24 h light-dark cycle, temperature-controlled animal facility with free access to food and water. Mice were housed and treated in accordance with National Institutes of Health and the University of Miami Institutional Animal Care and Use Committee guidelines. GSK3 knockin mice contain serine-to-alanine mutations in the regulatory serines of both GSK3 isoforms (S21A-GSK3α and S9A-GSK3β) that disable the inhibitory serine phosphorylation of GSK3 (Figure 1.4.1.1) (McManus et al., 2005). Both isoforms are expressed at normal levels so GSK3 retains maximal activities within the normal physiological range. GSK3 knockin mice reproduce and develop normally and no overt phenotype has been reported. Where indicated, mice (age 8-12 weeks) were injected intraperitoneally (i.p.) (injected in 10 µL/g body weight) with 10 mg/kg ketamine or 10 mg/kg 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Tocris Bioscience).

2.2 Learned Helplessness Mouse Model of Depressive-Like Behavior (LH)

The learned helplessness paradigm of depression-like behavior involves a training session with inescapable mild foot shocks (180 foot shocks, 0.3 mA shock amplitude, 6 s duration, 5-45 s interval) administered in shuttle boxes (15x17x27 cm) with a stainless steel bar grid floor connected to an internal shock source (Gemini II Avoidance System). During the training, mice are confined to one side of the shuttle box by a gate separating the shuttle box into two
chambers. The learned helplessness behavior is apparent from 24 h up to two weeks after the training foot shock paradigm when the mice are placed in the shuttle box chambers and foot shocked again, but are able to escape. The testing day consists of 30 trials of 24 s foot shocks, interspaced with 30 s of rest. Mice begin confined to one chamber of the shuttle box and upon initiation of the foot shock the gate separating the two chambers opens giving the mice access to the second chamber where no shock is being administered. Depressive-like learned helplessness behavior entails failure to escape the foot shock more than 15 times out of the 30 trials.

2.3. Novelty Suppressed Feeding Test (NSF)

In the novelty suppressed feeding test food is removed from the cage 24 h before testing. Mice are weighed at the start of food deprivation and again before testing to assess body weight loss. For testing, mice are placed for 10 min in a brightly lit novel open field (50×50×50 cm box containing bedding) with a food pellet at the center on a slightly (1 cm) elevated platform. Mice are placed in the open field directly from their home cage. The latency for each mouse to begin feeding within the 10 min is recorded. Upon returning to their home cage, the latency to eat and the total amount of food consumed during a 5 min period is analyzed to test whether feeding differences in the novel environment were due to differences in hunger/motivation.

2.4. Tail Suspension Test (TST)

In the tail suspension test, the mice are attached by the tail for a period of 6 min and monitored for the amount of time they remain immobile. The tail
suspension test is initiated by securing mice by the tail to a hanging vertical metal bar that uses a strain gauge to detect movement using adhesive tape positioned about 4 cm from the tail tip in a 32x 33x 33 cm box with an open front to allow visual observation during the test. Mice are suspended individually for 6 min and the duration of immobility below a preset threshold is determined for the final 4 min of the test using an automated tail suspension test apparatus (Med Associates).

2.5. Immunoblotting

Mice are decapitated and the prefrontal cortex and hippocampus brain regions are rapidly dissected and homogenized in ice-cold Triton lysis buffer (containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 μg/mL of leupeptin, aprotinin, and pepstatin A, 1 mM orthovanadate, 50 mM NaF, 0.1 μM okadaic acid, 1 mM PMSF) and centrifuged at 14,000 rpm, 10 min at 4°C to prepare tissue lysates. Protein concentrations are determined by the Bradford assay method (BioRad) or bicinchoninic acid (BCA) assay method (Thermo Scientific). Samples (5-20 ug) are mixed with Laemmli sample buffer (2% SDS), boiled for 5 min, resolved in SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking, the membranes are incubated with antibodies to PSD-95, GluR1, GluR2, GluR3, GluR4, NMDAR1, NMDAR2A, and phospho-Ser9-GSK3β (cell signaling), GSK3β and EGFR (BD Transduction), pT19 PSD-95 (Abcam), or β-actin (Sigma). Immunoblots are incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG. HRP is detected on film by enhanced
chemiluminescence. The intensity of the signal is quantitated by densitometry using computer software.

2.6. Membrane Extraction

Following the instructions of the ProteoExract® Transmembrane Protein Extraction Kit, tissue was fractionated into cytosolic and membrane extracts. Brain hippocampus tissue is collected on ice and rinsed with 2 mL ice-cold PBS by centrifugation at 4°C for 2 min at 100xg two times. PBS is removed and tissue is homogenized with 5 µL protease inhibitor cocktail and 2 mL ice-cold extraction buffer until there are no intact pieces of tissue visible. The homogenate is incubated with gentle agitation for 10 min at 4°C then centrifuged at 4°C for 5 min at 1,000xg. The supernatant is collected as the soluble cytosolic protein fraction. The pellet is suspended in 5 mL ice-cold PBS and centrifuged at 4°C for 5 min 1,000xg. The supernatant is removed and the pellet is suspended in 0.2 mL ice-cold extraction buffer 2 and 5 µL protease inhibitor cocktail. Samples are incubated with gentle agitation for 15 min at 4°C than centrifuged at 4°C for 15 min at 16,000xg. The supernatant is collected. The lysate enriched in integral membrane proteins, such as AMPA receptors, is analyzed by a bicinchoninic acid (BCA) protein assay and immunoblotting.

2.7. Statistical Analysis

Data are expressed as means ± SEM. Statistical significance between groups was analyzed with a one-way multiple range analysis of variance (ANOVA) for multiple comparisons with the appropriate post-hoc test or with
unpaired Student’s t-test using Prism software. Differences between groups were considered significant at a p value < 0.05.
Chapter 3: GSK3 Phosphorylation is Required for the Antidepressant Effect of Ketamine

3.1. Overview

Current antidepressants have delayed onset of therapeutic effects, low efficacy, and require chronic administration. Therefore, the discovery that a sub-anesthetic dose of the NMDA receptor antagonist ketamine has a rapid antidepressant effect, acting within 24 hours, generated much enthusiasm in the field. Ketamine provides a new target for the development of antidepressant medications because unlike most available antidepressants it targets the glutamatergic system instead of the monoamine system (Benton & Neslter, 2006; Sanacora et al., 2008). Understanding the mechanism whereby ketamine exerts its rapid antidepressant action would have major implications in the development of new faster acting antidepressant drugs.

GSK3 has previously been linked to mood disorders. Lithium, a mood stabilizer prescribed for bipolar disorder, inhibits GSK3 and this action is believed to contribute to the therapeutic actions of the drug (Jope, 2003). This discovery led to the idea that GSK3 may be dysregulated in mood disorders (Jope & Johnson, 2004). Animal studies provide further evidence that GSK3 plays a role in mood disorders including the antidepressant-like behavioral effects of mice treated with selective GSK3 inhibitors and increased susceptibility to depressive-like behavior following stress in GSK3 knockin mice where GSK3 is expressed maximally (Kaidanovich-Beilin et al., 2004; Polter et al., 2010). Additional evidence includes administration of monoamine reuptake inhibitor
antidepressants (fluoxetine or imipramine) increasing the inhibitory serine phosphorylation of GSK3 and abnormal GSK3 activity in humans with depression (Karege et al., 2007; Li et al., 2007). This evidence provides the basis for investigating the role GSK3 plays in the antidepressant actions of ketamine.

The involvement of GSK3 in ketamine’s rapid antidepressant effect was first implicated by our laboratory using the learned helplessness model of depression in mice. The ketamine antidepressant action in the learned helplessness model in mice is abolished in mice expressing constitutively active GSK3 (GSK3 knockin mice) that is unable to be inhibited after ketamine treatment. A single sub-anesthetic dose of ketamine was also shown to induce the inhibitory serine phosphorylation of GSK3 in wild-type mice (Beurel et al., 2011). However, it remains to be determined if modulating GSK3 activity is sufficient to mediate ketamine’s antidepressant action. Therefore, I induced depression using the stress of the learned helplessness mouse model of depressive-like behavior and investigated if ketamine alleviates multiple depressive-like behaviors in mice that model different aspects of depression, including the novelty suppressed feeding test and the tail suspension test or if the antidepressant action of ketamine is limited to certain aspects of depressive behavior.

3.2. Results and Discussion

Using the sub-anesthetic dose of ketamine (10 mg/kg, i.p.) previously reported to have rapid antidepressant effects in rodents (Li et al., 2010; Maeng et al., 2008), I confirmed previous reports (Beurel et al., 2011) that ketamine
increases serine phosphorylation of both isoforms of GSK3, GSK3α and GSK3β, indicators of inhibition of GSK3. Adult male wild-type mice were treated by intraperitoneal injection with saline (vehicle) or ketamine (10 mg/kg) and 30 min later the brain prefrontal cortex and hippocampus were collected for tissue lysate preparation and protein analysis by immunoblotting. After 30 min, ketamine increased serine phosphorylation of GSKβ in the prefrontal cortex (Figure 3.2.1a) and GSK3α and GSK3β in the hippocampus (Figures 3.2.1b and 3.2.1c) of mice. This finding suggests the involvement of inhibition of GSK3 in the rapid antidepressant effect of ketamine.

Figure 3.2.1 - Ketamine increases inhibitory serine phosphorylation of GSK3. 8-12 week old wild-type mice treated with 10 mg/kg ketamine (Ket) or saline (Veh) for 30 min and brain prefrontal cortex (a) and hippocampus (b and c) were collected and tissue extracts were immunoblotted for phospho-Ser21 GSK3α or phospho-Ser9 GSK3β and total GSK3α or GSK3β. Blots were quantified and the ratio of phospho-Ser9 GSK3β/GSK3β (a and b) or phospho-Ser21 GSK3α/GSK3α (c) was calculated. Bars represent means ± SEM (n = 4-5/group, *p ≤ 0.05, Student’s t-test, comparing to control (wild-type veh)).

In addition to being effective in the learned helplessness test, I found that ketamine exerted antidepressant effects in additional mouse models of depressive-like behaviors including the novelty suppressed feeding test and tail
suspension test, and that the ketamine antidepressant effect was abolished in GSK3 knockin mice (where GSK3 cannot be inhibited by serine phosphorylation), similar to the results obtained with the learned helplessness test (Beurel et al., 2011). I had four groups of adult male mice including wild-type mice treated with saline (vehicle), wild-type mice treated with ketamine (10 mg/kg, i.p.), GSK3 knockin mice treated with saline, and GSK3 knockin mice treated with ketamine (10 mg/kg, i.p.). All mice were subjected to the inescapable foot shock training day of the learned helplessness protocol to induce a depressive-like state and 24 h later treated with vehicle or ketamine (10 mg/kg, i.p.). At 24 h, 48 h and 72 h after treatment, I assessed depressive-like behavior with the learned helplessness, the novelty suppressed feeding and the tail suspension tests, respectively (Figure 3.2.2). Treatment with ketamine between inescapable and escapable shock treatments matches previous reports that ketamine is effective in alleviating learned helplessness depressive-like behavior in this paradigm (Beurel et al., 2011; Koike et al., 2011; Li et al., 2010; Maeng et al., 2008).

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**Figure 3.2.2- Experimental setup for investigating the requirement of GSK3 inhibition in ketamine’s antidepressant response.** Wild-type and GSK3 knockin mice received foot shocks in the training day of the learned helplessness paradigm, 24 h later were treated with saline or ketamine, 24 h later were tested for depressive-like behavior in the learned helplessness model, 24 h later were tested for depressive-like behavior in the novelty suppressed feeding test, and 24 h later were tested for depressive-like behavior in the tail suspension test.
Ketamine reduced failures to escape of wild-type mice in the learned helplessness model of depressive-like behavior, indicative of an antidepressant effect. The antidepressant-like effect of ketamine was not as strong in the GSK3 knockin mice treated with ketamine as the number of failures to escape was not significantly reduced confirming previous reports (Beurel et al., 2011) (Figure 3.2.3a). Ketamine reduced the immobility time in the tail suspension test in wild-type mice, also suggesting an antidepressant effect of ketamine, as immobility is associated with depressive-like behavior in this test. The response to ketamine was abolished in the GSK3 knockin mice (Figure 3.2.3b). Similarly, ketamine reduced the latency to eat in the novelty suppressed feeding test in wild-type mice, indicative of an antidepressant effect of ketamine, as longer latency to eat is associated with depressive-like behavior and this response was abolished in the GSK3 knockin mice (Figure 3.2.3c). In the novelty suppressed feeding test, the mice in all groups tested lost similar percentages of weight during the 24 h starvation period (Figure 3.2.3d), consumed similar amounts of food once returned to the home cage (Figure 3.2.3e), and showed no significant differences in suppressed appetite, the total amount of food eaten compared to the weight of the mouse (Figure 3.2.3f) confirming the results of the novelty suppressed feeding test were due to treatment and not an artifact of the tests parameters, such as hunger as a motivation to eat. Overall, this data shows that inhibitory serine phosphorylation of GSK3 induced by ketamine is required for the antidepressant actions of ketamine.
Figure 3.2.3 - GSK3 inhibition is required for the antidepressant effect of ketamine. 8-12 week old male wild-type and GSK3 knockin mice were subjected to inescapable foot shocks and treated i.p. 24 h later with 10 mg/kg ketamine (Ket) or saline (Veh). Mice were tested in learned helplessness paradigm (a), tail suspension test (b), and novelty suppressed feeding test (c). For the novelty suppressed feeding test, % weight lost (d), food consumption (e), and suppressed appetite (f) were tested as controls to validate test. Bars represent means ± SEM (n = 4-5/group for learned helplessness; n = 7-9/group for novelty suppressed feeding and tail suspension tests, *p ≤ 0.05, one-way ANOVA with Dunnett’s post hoc test).

These results demonstrate that ketamine inhibits GSK3 in the mouse brain and that inhibitory serine phosphorylation of GSK3 is necessary for antidepressant effects in learned helplessness, novelty suppressed feeding, and tail suspension test models of depressive-like behavior. This finding supplements the evidence already linking active GSK3 to increased susceptibility of mood disorders such as depression and the possibility that inhibition of GSK3 is a viable target for novel therapies. This finding provides understanding of how
ketamine exerts its antidepressant effect in mouse models of depressive-like behavior and suggests a possible mechanism of treating and controlling depression by the use of specific inhibitors of GSK3.

3.3. Future Directions

These experiments offer insight into the involvement of GSK3 in the rapid antidepressant effect of ketamine. The discovery that ketamine has antidepressant properties is exciting because as an NMDA antagonist it targets the glutamatergic system instead of the monoamine system which most current antidepressants modulate. Understanding the mechanism by which ketamine exerts its antidepressant effects will advance the field and provide new targets for antidepressant drug development. Based on the above experiments, one such target is the inhibition of GSK3. With the knowledge that inhibitory serine phosphorylation of GSK3 increases in response to ketamine and that inhibition of GSK3 is required for antidepressant-like effects in three mouse models of depression depicting different aspects of depressive behavior following ketamine treatment, selective GSK3 inhibitors, of which there are several available, could be tested for their antidepressant effects. The experiment involving the learned helplessness, novelty suppressed feeding, and tail suspension tests could be repeated replacing the ketamine treatment with specific GSK3 inhibitors (Figure 3.2.2). This will confirm that GSK3 inhibition is sufficient to mediate an antidepressant response in mouse models of depressive-like behavior and strengthen the case for research of GSK3 inhibition as a target for rapid-acting antidepressant drug development.
In addition to investigating the antidepressant effect of ketamine to identify novel targets for therapeutic strategies, ketamine research can be used to identify mechanisms to prolong antidepressant effects. One of the drawbacks of current antidepressants is that they require chronic administration and that can lead to noncompliance to take medications. Ketamine research could lead to a target for drug development that requires one initial treatment with effects lasting for weeks to overcome daily administration. In clinical trials ketamine has rapid antidepressant effects acting within 24 h and has been reported to have antidepressant effects lasting up to two weeks in some patients (DiazGranados et al., 2010; Larkin & Beautrais, 2011; Machado-Vieira et al., 2009a; Mathew et al., 2010; Price et al., 2009; Zarete et al., 2006a). Maeng et al. (2008) reported antidepressant effects of ketamine in rodents lasted for two weeks in the forced swim test model of depressive-like behavior. The first step is to confirm the length of ketamine’s antidepressant effect in various mouse models of depression to determine if the antidepressant effect lasts in multiple aspects of depressive-like behavior. These studies can be started with animal models using depressive-like models of behavior such as learned helplessness, novelty suppressed feeding, and the tail suspension test by beginning as I have with inducing depression with foot shock stress and retesting weekly until an antidepressant response is not observed. A control will be required to ensure the antidepressant effect has waned and not that the mice have recovered from the induced depressive-like state. Determining the length of ketamine’s antidepressant response could be useful for patients that do not want to wait the
two to four weeks current antidepressants take to start relieving depressive symptoms. An initial dose of ketamine could be used until current antidepressants take effect. Ketamine could be useful to relieve patient’s symptoms quickly in the short term and, in the future, as more is learned about the mechanism of action, be helpful in developing improved antidepressants that act quickly and have prolonged therapeutic benefits without requiring chronic administration. Determining the duration of the antidepressant effect of ketamine and the mechanism that makes it possible is another important step to developing improved antidepressant medications.
Chapter 4: Ketamine’s Antidepressant Effect Involves Signaling Between GSK3 and AMPA Receptors

4.1. Overview

AMPA receptors are transmembrane proteins composed of subunits, GluR1, GluR2, GluR3, and GluR4. A receptor is formed at the cell surface by the joining of four subunits, most commonly a dimer of one subunit type with a dimer of another type (Malinow & Melenka, 2002). AMPA receptors are involved in the response to glutamate in the synapse and regulate excitatory synaptic strength and plasticity. Direct activation of AMPA receptors can result in overstimulation which can induce seizures and lead to neurotoxicity; therefore, indirect AMPA receptor potentiators have been used to stimulate AMPA receptors instead (Zarate & Manji, 2008). Li et al. (2001) showed the antidepressant effects of AMPA receptor potentiators providing evidence of AMPA receptor activation in an antidepressant response.

AMPA receptor activation has been implicated in the antidepressant effect of ketamine. In the forced swim test, a model used to test for depressive-like behavior in rodents, ketamine reduced the immobility time, suggesting an antidepressant-like effect. Pretreatment with the AMPA receptor antagonist 2,3 dihydroxy-6-nitro-7-sulfamoyl benzo[f]quinoxaline-2,3-dione (NBQX) abolished the decreased immobility associated with antidepressant-like behavior in response to ketamine (Maeng et al., 2008). Koike et al. (2011) reported that NBQX reversed the antidepressant-like behavior of ketamine in the learned
helplessness paradigm and tail suspension test confirming the requirement of AMPA receptor activation in ketamine’s antidepressant effect.

AMPA receptor trafficking and localization are important for the glutamate response (Mahado-Vieira et al., 2009a). One enzyme implicated to play a role in AMPA receptor trafficking is GSK3. AMPA receptor internalization has been shown to be inhibited by GSK3 inhibitors in vitro (Du et al., 2010). In vivo lithium treatment increased surface expression of AMPA receptors. This provides a connection between GSK3 and AMPA receptors (Gould et al., 2008). Therefore, GSK3 inhibition in response to ketamine may stabilize the AMPA receptor at the cell surface in synapses.

Trafficking of AMPA receptors is a dynamic process and is regulated partially by AMPA receptor-interacting proteins, one of which is postsynaptic density-95 protein (PSD-95) (Anggono & Huganir, 2012). PSD-95 is an abundant scaffold protein in the postsynaptic density of excitatory synapses that helps organize glutamate receptors, NMDA and AMPA receptors, and positively influence the strength of synapses promoting synapse maturation (Kim & Sheng, 2004). Alterations of PSD-95 at the synapse results in changes in synaptic strength and the number of AMPA receptors present at the cell surface (El-Husseini et al., 2002). PSD-95 is regulated by phosphorylation at different sites causing it to accumulate at the cell surface or detach from the membrane. For example, accumulation of PSD-95 at the synapse is regulated by Ser295 phosphorylation mediated by a protein kinase member of the MAPK family (Kim et al., 2007; Nelson et al., 2013). The phosphorylation of several additional N-
terminal domain residues has been implicated as important in PSD-95 localization regulation including Thr19 (Morabito et al., 2004). Nelson et al. (2013) reported the kinase responsible for phosphorylating Thr19 of PSD95 is GSK3 and that phosphorylation at this site destabilizes PSD-95 at the synapse leading to internalization of AMPA receptors during long term depression, the weakening of synapses, in neurons. This provides a connection between the two proposed mechanisms of ketamine’s antidepressant action, inhibition of GSK3 and AMPA receptor activation, which are explored in this chapter.

Understanding the interplay between GSK3 and AMPA receptors and identifying proteins involved in regulating AMPA receptor trafficking could have a major impact in understanding depression mechanisms. Therefore, in this chapter I investigate a possible mechanism involving GSK3 and AMPA receptors in ketamine’s antidepressant effect. First, I confirm that AMPA receptor activation is required for the antidepressant effect of ketamine in the tail suspension test and expand it to another mouse model of depression, the novelty suppressed feeding test (Koiki et al., 2011; Maeng et al., 2008). The involvement of GSK3 in regulating AMPA receptor trafficking has also previously been implicated; therefore, I investigated if regulation of the AMPA receptor by its antagonist NBQX had any effect on the inhibitory serine phosphorylation of GSK3 in response to ketamine. To further understand the interaction between GSK3 and AMPA receptors in response to ketamine, I investigated individual AMPA receptor subunits changes at the cell surface. GSK3 knockin mice were tested to determine if changes in the AMPA receptor subunits are due to GSK3 inhibition
in response to ketamine. In the GSK3 knockin mouse model GSK3 cannot be inhibited by serine phosphorylation, therefore, if ketamine modulates an AMPA receptor subunit in the same manner in both wild-type and GSK3 knockin mice, the response is not dependent on the inhibition of GSK3, but if an AMPA receptor subunit is modulated in wild-type mice and not in GSK3 knockin mice then the change requires inhibition of GSK3 to occur. Finally, this chapter will investigate the role of PSD-95 in the interaction of GSK3 and AMPA receptors in response to ketamine based on the report by Nelson et al. (2013) that states GSK3 regulation of PSD-95 contributes to mobilization of AMPA receptors. I hypothesize that the inhibition of GSK3 decreases the phosphorylation of PSD-95 at the Thr19 site leading to stabilization of AMPA receptors in the membrane at the synapse. Inhibition of GSK3 leads to stabilization of the AMPA receptor in the membrane promoting activation.

4.2. Results and Discussion

The role of AMPA receptors in the antidepressant effect of ketamine has previously been implicated in the learned helplessness mouse model of depressive-like behavior and tail suspension test (Koiki et al., 2011; Maeng et al., 2008.) I confirm that AMPA receptors are required for the antidepressant effect of ketamine. I have four groups of adult wild-type mice with treatments of saline (vehicle), ketamine (10 mg/kg, i.p.), NBQX (10 mg/kg, i.p.), or NBQX and ketamine. Mice were subjected to the inescapable foot shock stress to induce a depressive-like state, 24 h later treated with the AMPA receptor antagonist NBQX (10 mg/kg, i.p.) and 1 h later with ketamine (10 mg/kg, i.p.). At 48 h and
72 h after treatment depressive-like behavior was assessed with the novelty suppressed feeding test and the tail suspension test, respectively (Figure 4.2.1).

Figure 4.2.1- Experimental setup for investigating requirement of AMPA receptor in the ketamine antidepressant response. Adult male wild-type mice received foot shocks on the training day of the learned helplessness paradigm, 24 h later were treated with saline, NBQX, ketamine, or NBQX and ketamine. 48 h later mice were tested for depressive-like behavior in the novelty suppressed feeding test and 24 h later were tested for depressive-like behavior in the tail suspension test.

As expected, ketamine reduces the latency to eat in the novelty suppressed feeding test and reduces the immobility time in the tail suspension test indicative of an antidepressant response (Figure 4.2.2). Pretreatment with NBQX blocks the antidepressant-like effect of ketamine as mice have similar latencies to eat and immobility times compared to the control mice only given the saline vehicle (Figure 4.2.2a and 4.2.2b). The mice in all groups tested lost similar percentages of weight during the 24 h starvation period (Figure 4.2.2c), consumed similar amounts of food once returned to the home cage (Figure 4.2.2d), and showed no significant differences in suppressed appetite (Figure 4.2.2e) confirming the results of the novelty suppressed feeding test were due to treatment and not an artifact of the test’s parameters, such as hunger. The treatment of NBQX alone has no effect on the depressive-like behavior of the
mice as the behavioral outcomes of the treated group are similar compared to
the vehicle controls confirming that NBQX alone does not affect depressive-like
behavior. These results show that when the AMPA receptor is antagonized the
antidepressant effect of ketamine is abolished; therefore, AMPA receptor
activation is required for the antidepressant effect of ketamine.

Figure 4.2.2- AMPA receptors are required for the antidepressant effect of
ketamine. 8-12 week old male wild-type mice were subjected to inescapable
footshocks and treated i.p. 24 h later with 10 mg/kg NBQX or saline (Veh) and 1h
later with ketamine (Ket) or saline (Veh). Mice were then tested with the novelty
suppressed feeding test 48h after treatment (b) and tail suspension test 72h after
treatment (a). For the novelty suppressed feeding test, % weight lost (c), food
consumption (d), and suppressed appetite (e) were tested as controls to validate
test. Bars represent means ± SEM (n = 13-15 for Veh, Ket, and NBQX+Ket
groups and n = 3 for NBQX group, *p ≤ 0.05, one-way ANOVA with Dunnett’s
post hoc test).
I have shown that the antidepressant effect of ketamine in mouse models of depression requires both GSK3 inhibition by serine phosphorylation and activation of AMPA receptors, but it is unknown if these pathways are interrelated or function separately in response to ketamine. In order to determine if AMPA receptor activation is required for inhibitory serine phosphorylation of GSK3 in response to ketamine, I analyzed the consequence of blocking AMPA receptors with NBQX and examined the levels of phospho-Ser9 GSK3β. I hypothesized that if GSK3 is downstream of the AMPA receptor, NBQX would block the ketamine induced serine phosphorylation of GSK3. To investigate this, adult wild-type mice were pretreated with NBQX (10 mg/kg, i.p.) for 60 min, and then treated with ketamine (10 mg/kg, i.p.) for 30 min, and brain hippocampus was collected and tissue extracts prepared for immunoblotting of phospho-Ser9 GSK3β and total GSK3β.

Figure 4.2.3 shows that as expected inhibitory serine phosphorylation of GSK3β increased in response to ketamine. Pretreatment with NBQX does not affect the increased serine phosphorylation of GSK3β. This demonstrates that activation of AMPA receptors is not required for GSK3 inhibition in response to ketamine and suggests that the increased phosphorylation of GSK3 in response to ketamine is upstream or independent of AMPA receptors.
Figure 4.2.3- GSK3 phosphorylation in response to ketamine is independent of the AMPA receptor. 8-12 week old male wild-type mice pretreated i.p. with 10 mg/kg NBQX or saline (veh) for 1h and 10 mg/kg ketamine (Ket) or saline and brain hippocampus was collected and tissue extract were immunobotted for phospho-Ser9 GSK3β and total GSK3β. Blots are quantified and the ratio phospho-Ser9 GSK3β/GSK3β was calculated. Bars represent means ± SEM (n = 3-4/group).

Activation of AMPA receptors is not required for GSK3 inhibition in response to ketamine, but it is possible GSK3 plays a role in AMPA receptor activation. GSK3 inhibitors have been implicated to have a role in AMPA receptor localization (Du et al., 2010; Gould et al., 2008). To investigate the role of GSK3 in AMPA receptor trafficking in response to ketamine I examined the levels of the individual AMPA receptor subunits present in the membrane. I have four groups of adult male mice including wild-type treated with saline, wild-type mice treated with ketamine (10 mg/kg, i.p.), GSK3 knockin mice treated with saline, and GSK3 knockin mice treated with ketamine. GSK3 knockin mice are used to determine if changes in an AMPA receptor subunit are dependent on inhibition of GSK3 since inhibitory-serine phosphorylation is not possible in these mice. Treatment with ketamine was for 30 min and the hippocampus was collected for tissue lysate
analysis by immunoblotting. As integral membrane proteins, AMPA receptors need to be extracted from the membrane prior to analysis by immunoblotting.

Of the four AMPA receptor subunits, only GluR2 is increased in the membrane of wild-type mice in response to ketamine (Figure 4.2.4b). In GSK3 knockin mice there is no significant change of this subunit between the vehicle and ketamine treated groups, therefore increasing the GluR2 subunit requires the inhibitory serine phosphorylation of GSK3 which is not possible in the GSK3 knockin mice. The increase of the GluR2 subunit is dependent on inhibition of GSK3 because GluR2 is increased in wild-type mice, but not in GSK3 knockin mice in response to ketamine. The GluR1 subunit has a tendency to decrease in wild-type mice in response to ketamine, but it is not dependent on inhibition of GSK3 because the same tendency is seen in the GSK3 knockin mice (Figure 4.2.4a). GluR3 and GluR4 do have significant changes in either wild-type or GSK3 knockin mice (Figure 4.2.4c and 4.2.4d). In response to ketamine the AMPA receptor, specifically the GluR2 subunit, is stabilized at the membrane in a manner dependent on GSK3 inhibition contributing to AMPA receptor activation required for the antidepressant effect of ketamine.
Figure 4.2.4 - AMPA receptor subunit GluR2 increases in the membrane in response to ketamine. 8-12 week old male wild-type and GSK3 knockin mice treated with saline (Veh) or 10 mg/kg ketamine (Ket) for 30 min and hippocampus was collected and tissue extracts were immunoblotted for (a) GluR1, (b) GluR2, (c) GluR3, and (d) GluR4. Bars represent means ± SEM (n = 6-9/group, *p ≤ 0.05, Student’s t-test, comparing to control (wild-type veh)).

The NMDA receptor is the other main ionotropic glutamate receptor and ketamine is an antagonist of this receptor, so in addition to investigating changes of the AMPA receptor in the membrane in response to ketamine, I also investigated subunits of the NMDA receptor. The NMDA receptor subunits, NR1 and NR2A, in wild-type or GSK3 knockin mice do not change in response to ketamine in the membrane (Figure 4.2.5). Ketamine antagonizes the NMDA
receptor, but does not affect the distribution of the subunits in the membrane, therefore the amount of NMDA receptors present at the cell surface is not a factor in the antidepressant effect.

Figure 4.2.5 - NMDA receptor subunits do not change in the membrane in response to ketamine. 8-12 week old male wild-type and GSK3 knockin mice were treated with saline (Veh) or 10 mg/kg ketamine (Ket) for 30 min and hippocampus was collected and tissue extracts were immunoblotted for (a) NR1 or (b) NR2A. Bars represent means ± SEM (n = 6-9/group).

The AMPA receptor GluR2 is increased in the membrane dependent on inhibition of GSK3 in response to ketamine (Figure 4.2.4b). In long term depression, the deterioration of synapses in which AMPA receptors are less active, GSK3 phosphorylates PSD-95 at the Thr19 site destabilizing it at the synapse leading to mobilization of AMPA receptors (Nelson et al., 2013). To investigate if GSK inhibition reduces phosphorylation of Thr19 PSD-95 in response to ketamine I examined levels of phospho-Thr19 PSD-95 in the membrane. I hypothesized that since active GSK3 phosphorylated PSD-95 at the Thr19 site leading to destabilization of AMPA receptors in the membrane in long
term depression, then the inhibition of GSK3 in response to ketamine will prevent
the phosphorylation of Thr19 PSD-95 stabilizing membrane AMPA receptors, specifically GluR2.

In response to ketamine phospho-Thr19 PSD-95 in wild-type mice is decreased (Figure 4.2.6). This effect does not occur in GSK3 knockin mice, so it is dependent on GSK3 inhibition; inhibitory serine phosphorylation is required for the decreased of phospho-Thr19 PSD-95 in response to ketamine. The level of phospho-Thr19 PSD-95 in the GSK3 knockin mice is increased in both the vehicle and ketamine treated groups compared to the wild-type vehicle control group. Since GSK3 is maximally active in GSK3 knockin mice it is expected that phosphorylation of PSD-95 is increased because active GSK3 phosphorylates Thr19 of PSD-95 (Nelson et al., 2013). In response to ketamine GSK3 is inhibited by serine phosphorylation and this affects the phosphorylation of PSD-95 leading to its stabilization in the membrane.

Interpretation of this data leads to a proposed mechanism of ketamine’s antidepressant action involving GSK3, PSD-95, and AMPA receptors. I report that ketamine inhibits GSK3 by increasing serine phosphorylation, AMPA receptor activation is required for the antidepressant effect of ketamine, and the AMPA receptor subunit GluR2 is stabilized and phosphorylation of Thr19 PSD-95 is decreased in the membrane dependent on inhibition of GSK3 in response to ketamine. Based on this I propose a possible mechanism for ketamine’s antidepressant effect (Figure 4.2.7). Ketamine inhibits GSK3 preventing
phosphorylation of Thr19 PSD-95, stabilizing it at the membrane and preventing mobilization of the AMPA receptors from the membrane of post synapses.

Figure 4.2.6 - PThr19 PSD-95 decreases in the membrane in response to ketamine. 8-12 week old male wild-type and GSK3 knockin mice were treated with 10 mg/kg ketamine (Ket) or saline (Veh) for 30 min and hippocampus was collected and tissue extracts were immunoblotted for phospho-Thr19 PSD-95 or total PSD-95. Blots were quantified and the ratio of phospho-Thr19 PSD-95/PSD-95 was calculated. Bars represent means ± SEM (n = 6-9/group, *p ≤ 0.05, Student’s t-test, comparing to control (wild-type veh)).

Figure 4.2.7- Proposed mechanism of action in response to ketamine. Ketamine inhibits GSK3 through serine phosphorylation. Inhibited GSK3 is unable phosphorylate Thr19 PSD-95, stabilizing it in the membrane and leading to stabilization of the AMPA receptor in the postsynaptic membrane.
4.3. Future Directions

This set of experiments offers insight into a possible mechanism for ketamine's antidepressant effect involving GSK3 and AMPA receptors. Using the AMPA receptor antagonist, NBQX, in the novelty suppressed feeding and tail suspension tests I showed that AMPA receptor activation is necessary for the ketamine antidepressant effect. I previously identified GSK3 as a possible target for antidepressant development, possibly through the use of specific GSK3 inhibitors. In this chapter I connected the inhibition of GSK3 with activation of AMPA receptors in response to ketamine, specifically the subunit GluR2 is increased in the membrane. Changes in the GluR2 subunit have previously been found in models of depression. Expression of GluR2 mRNA is decreased in the mild chronic unpredictable stress model of depression and is reversed by the SSRI fluoxetine (Yu et al., 2014). This evidence suggests increasing GluR2 as a possible target for improved antidepressants. GluR2 is one of four AMPA receptor subunits. Increasing other subunits in the membrane would also increase AMPA receptor activation, a requirement for the antidepressant effect of ketamine. Although ketamine was not shown to increase other subunits of the AMPA receptor, other drugs with antidepressant effects may. Other ways to increase membrane expression of AMPA receptors have the potential to have antidepressant effects.

AMPA receptors usually are composed of a dimer of one subunit joined with the dimer of a second subunit. I showed that only the GluR2 subunit is increased in the membrane in response to ketamine, but the subunit it joins with
and type of receptor that is made is important for the function. While it is possible that the receptors form GluR2/GluR2, this is rare and it is more likely GluR2 is joining with other subunits to form a functional AMPA receptor. This can be determined by immunoprecipitation experiments to investigate associations between GluR2 and the other AMPA receptor subunits. Immunoprecipitation can also be used to provide more evidence for the proposed mechanism by confirming associations between GSK3 and PSD-95 and GluR2.
Chapter 5: Concluding Remarks

Depression is a widespread and disabling mood disorder affecting up to 20% of the population that takes a heavy toll on health care expenses and is a leading cause of disability (Kessler et al., 2003; Marcus & Olfsen, 2010). Although there are good treatment options, they have several drawbacks. The inadequacies of antidepressants are unpleasant side effects, chronic administration, low efficacy, and a 2-4 week delay of onset of therapeutic benefits in patients that experience any symptom improvement at all. One-third of patients in the United States fail to exhibit remission of symptoms following two or more antidepressant therapies and are defined as treatment-resistant (Covvey et al., 2012). These weaknesses present the need for the development and improvement of antidepressant medications.

For the past 50 years antidepressant drug development has been based on modulating the monoamine system (MAOI, TCAs, and SSRIs). The discovery that ketamine, an NMDA receptor antagonist, has rapid-acting antidepressant effects provides new avenues of research for developing improved antidepressants. There have been promising clinical trials with ketamine having rapid acting antidepressant effects within 24 h and success in treatment resistant patients (Berman et al., 2000; DiazGranados et al., 2010; Larkin & Beautrais, 2011; Machado-Vieira et al., 2009; Mathew et al., 2009; Price et al., 2009; Zarate et al., 2006). However, widespread routine clinical use of ketamine as an antidepressant is not practical because it is not easily self-administered, the long-term harmful effects are unknown, and it has dissociative and psychosomatic
side effects (aan het Rot et al., 2012; Covvey et al., 2012; Murrough & Charney, 2012; Zunszain et al., 2013). Therefore, current studies of the antidepressant properties of ketamine are directed towards understanding the underlying mechanism to develop improved pharmacological therapies without the undesirable side effects. The goal of this thesis was to contribute to the growing knowledge about ketamine’s antidepressant effect and the underlying mechanism of action.

Using animal models, several actions of ketamine have been reported that may contribute to its antidepressant effect. Two of these pathways involved in the rapid antidepressant effect of ketamine are inhibition of GSK3 by serine phosphorylation (Beurel et al., 2011) and increased AMPA receptor activation (Maeng et al., 2008). In this thesis I investigated the inhibition of GSK3 and AMPA receptor activation and trafficking in response to ketamine with the aim of better understanding the antidepressant effect ketamine elicits.

In chapter 3, I showed that inhibition of GSK3 is required for the antidepressant effect of ketamine. Ketamine rapidly, within 30 min, increases levels of phospho-Ser21 GSK3α and phospho-Ser9 GSK3β, indicators of GSK3 inhibition. Beurel et al. (2011) showed that GSK3 inhibition was required for the ketamine antidepressant effect in the learned helplessness mouse model of depression using the GSK3 knockin mouse model in which GSK3 cannot be inhibited by serine phosphorylation due to a single amino acid mutation. Ketamine reduced the number of failures to escape in wild-type mice and this response was abolished in GSK3 knockin mice. I confirmed this result and
extended it to two additional mouse models of depression. Wild-type mice treated with ketamine had a reduced latency to eat in the novelty suppressed feeding test and reduced immobility time in the tail suspension test. This effect was abolished in GSK3 knock mice. Therefore, modulating GSK3 activity required to mediate the ketamine antidepressant action. This evidence provides insight into the mechanism of ketamine’s antidepressant effect and suggests the possibility of using specific GSK3 inhibitors as antidepressants.

In chapter 4, I showed that AMPA receptor activation is required for the ketamine antidepressant response and proposed a mechanism for ketamine’s antidepressant effect involving GSK3 regulating AMPA receptor trafficking. Using the AMPA receptor antagonist NBQX, the antidepressant effect of ketamine was abolished in the novelty suppressed feed and tail suspension tests. Phosphorylation of GSK3 was not affected by NBQX, so AMPA receptor activation is not required for GSK3 inhibition by serine phosphorylation in response to ketamine. However, in response to ketamine there is still a connection between GSK3 and AMPA receptors. The AMPA receptor subunit GluR2 is increased in membranes in response to ketamine in wild-type mice, but not in GSK3 knockin mice, so the increase is dependent on inhibition of GSK3. Phosphorylation of the Thr19 site of PSD-95 is decreased in response to ketamine in wild-type mice, but not in GSK3 knockin mice. This leads to the possible mechanism that in response to ketamine, GSK3 is inhibited, preventing phosphorylation of Thr19 PSD-95 which stabilizes it at the cell surface, leading to
reduced mobilization of AMPA receptors from the membrane. This provides evidence for a possible mechanism for the antidepressant effect of ketamine.

Ketamine is a promising model for better understanding depression and providing rapid antidepressant effects. Research into ketamine’s mechanism will provide potential targets for improved antidepressant development.


