Differential Effects of NMDA Receptor Antagonism on Spine Density

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmacology and Toxicology
University of Toronto

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2013

Abstract

Recent studies have demonstrated that an acute, low dose of ketamine, a non-competitive NMDA receptor antagonist, provides rapid and sustained antidepressant effects in patients with major depressive disorder. Studies in rodents have shown that the antidepressant properties of ketamine are due to an increase in dendritic spine density in the cortex. Our goal was to determine whether these effects are specific to ketamine and whether they are dependent on dose, drug regimen and brain region. We observed that the effects of ketamine on spine density were dependent on dose and drug regimen and were also brain region specific. In addition, MK-801, another NMDA receptor antagonist, did not demonstrate the same effects on spine density as ketamine. Furthermore, genetic NMDA receptor hypofunction significantly reduced spine density. Our studies demonstrate that while acute ketamine treatment leads to an increase in cortical spine density, chronic administration has opposite and potentially detrimental effects.
Acknowledgments

I would like to thank the following people for helping in the completion of this thesis:

• Firstly, I would like to thank my supervisor and mentor, Dr. Amy Ramsey, for her support and invaluable guidance and advice throughout the completion of this thesis.

• I also want to thank my co-supervisor, Dr. Denis Grant, for his guidance and suggestions.

• I would like to thank Dr. Albert Wong (advisor) and Dr. Ali Salahpour for their helpful comments and advice.

• I would also like to thank members of the Ramsey and Salahpour lab for their beneficial suggestions and comments during my studies.

• Finally, I would like to thank my family and friends for their constant support and encouragement throughout my graduate studies.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4E-BP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AC</td>
<td>Aversive control</td>
</tr>
<tr>
<td>ACPC</td>
<td>1-aminocyclopropanecarboxylic acid</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>AP-7</td>
<td>2-amino-7-phosphonoheptanoic acid</td>
</tr>
<tr>
<td>BDI</td>
<td>Beck Depression Inventory</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BPRS</td>
<td>Brief Psychiatric Rating Scale</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CGP 37849</td>
<td>(E,2R)-2-amino-4-methyl-5-phosphonopent-3-enoic acid</td>
</tr>
<tr>
<td>CP-101, 606</td>
<td>Traxoprodil</td>
</tr>
<tr>
<td></td>
<td>(1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino-1-propanol</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CUS</td>
<td>Chronic unpredictable stress</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GluR1</td>
<td>Glutamate receptor 1</td>
</tr>
<tr>
<td>Glx</td>
<td>Ratio of glutamate, glutamine and GABA</td>
</tr>
<tr>
<td>HDRS</td>
<td>Hamilton Depression Rating Scale</td>
</tr>
<tr>
<td>LH</td>
<td>Learned helplessness</td>
</tr>
<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MST</td>
<td>Magnetic seizure therapy</td>
</tr>
<tr>
<td>N200</td>
<td>Neurofilament 200</td>
</tr>
<tr>
<td>N2O</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>NAC</td>
<td>Nonaversive control</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide</td>
</tr>
<tr>
<td>NIH</td>
<td>Novelty induced hypophagia</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NR1</td>
<td>Glutamate (NMDA) receptor subunit zeta-1 (GRIN1)</td>
</tr>
<tr>
<td>NR2A</td>
<td>Glutamate (NMDA) receptor subunit epsilon-2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NMDA receptor subtype 2A</td>
<td>Glutamate (NMDA) receptor subunit epsilon-2</td>
</tr>
<tr>
<td>NR2B</td>
<td>NMDA receptor subtype 2B</td>
</tr>
<tr>
<td>NSFT</td>
<td>Novelty suppressed feeding test</td>
</tr>
<tr>
<td>pAKT</td>
<td>Phospho-AKT</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>pERK</td>
<td>Phospho-ERK</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>rTMS</td>
<td>Repetitive transcranial magnetic stimulation</td>
</tr>
<tr>
<td>S6K</td>
<td>Ribosomal protein S6 kinase beta-1</td>
</tr>
<tr>
<td>SAP102</td>
<td>Synapse-associated protein 102</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>SPT</td>
<td>Sucrose preference test</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TST</td>
<td>Tail suspension test</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analog scale</td>
</tr>
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</table>
Chapter 1
Introduction

1 Overview

1.1 Purpose of Study

Major depressive disorder (MDD) is a prevalent and devastating psychiatric disorder, but current treatment has a delayed onset and is ineffective in many patients. Current antidepressants mainly target the monoaminergic system, but the delayed onset of action of these drugs indicate that the therapeutic effect occurs downstream of the target (Connolly and Thase, 2012). Recent studies demonstrate that the glutamatergic system is involved in the manifestation of depressive symptoms, and clinical trials are beginning to evaluate drugs targeting this neurotransmitter system (Connolly and Thase, 2012). Ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, is one of the drugs currently being evaluated as an antidepressant in clinical trials. Early evidence shows that ketamine has a rapid onset of action, unlike traditional antidepressants, and its antidepressant effects are sustained for up to two weeks (Berman et al., 2000, Zarate et al., 2006). However, ketamine is a controlled substance with psychotomimetic effects in psychiatric patients and healthy individuals, and the long-term effects of acute or chronic ketamine treatment remain unknown.

A recent study by the Duman laboratory attributed the antidepressant effects of ketamine to its ability to increase spine density of cortical neurons. They showed, in an animal model of depression, that a reduction in cortical spines could be reversed with a single acute, low dose of ketamine. Increased spine density was correlated to antidepressant behavioural effects (Li et al., 2010). Because activation of NMDA receptors promotes spine maintenance, it was surprising that ketamine, as an NMDA receptor antagonist, would have a positive effect on spine density. In fact, our laboratory has demonstrated that mice treated subchronically with MK-801, another non-competitive NMDA receptor antagonist, showed a reduction in dendritic spine density in the striatum (Ramsey et al., 2011). Thus, these two NMDA receptors antagonists, which bind at the same site on the receptor, have opposing effects on spine density. This study aimed to reconcile these seemingly incompatible findings by exploring the differences in the effects of ketamine and MK-801 on spine density.
1.2 Research Objective

The purpose of this study was to compare the effect of acute and subchronic NMDA receptor antagonism on dendritic spines in different neuronal subtypes. More specifically, we determined the effect of different dose regimens of ketamine and MK-801, two NMDA receptor antagonists, on spine density in layer V pyramidal neurons in the cortex and medium spiny neurons in the striatum. In completing these studies, we hoped to understand the biological basis for the antidepressant effects of ketamine, and to determine whether the beneficial effects would be limited to ketamine or would extend to other NMDA receptor antagonists.

1.3 Hypotheses and Rationale

Prior studies on the effects of NMDA receptor antagonism on spine density used chronic administration to show that NMDA receptor antagonists reduce spine density (Elsworth et al., 2011, Ramsey et al., 2011, Velázquez-Zamora et al., 2011). The only study to examine acute effects of NMDA receptor antagonism was the Li et al study, where ketamine was shown to increase spine density (Li et al., 2010). Therefore, we hypothesized that acute and chronic ketamine treatment would have opposite effects on spine density. We predicted that, while acute ketamine treatment would increase spine density, repeated drug treatment would result in a reduction in spine density. This prediction was based on our earlier observations that chronic, genetic reduction in NMDA receptors caused a loss of spines in the striatum (Ramsey et al., 2011). Therefore, transient or acute antagonism may have opposite effects on spine density than sustained or chronic antagonism. We also hypothesized that MK-801 would act in a similar manner to ketamine with respect to spine density because it is another NMDA receptor antagonist that binds at the same site as ketamine. Finally, we hypothesized that chronic genetic reduction in NMDA receptors would have the same effect as subchronic NMDA receptor antagonism, which is to decrease spine density. Previous studies using a genetic mouse model, the NR1-KD mouse, demonstrated an age-dependent loss of spines in the striatum. We hypothesize that similar reductions would be seen in cortical neurons in an age-dependent manner.
2 Background

2.1 Major Depressive Disorder (MDD)

Description

Major depressive disorder (MDD) is a significant health problem affecting approximately 16% of the population worldwide (Kessler et al., 2007). MDD is characterized by at least one depressive episode occurring for more than two weeks. A depressive episode consists of at least five of the following symptoms: depressed mood, anhedonia, changes in weight or appetite, disturbance in sleep patterns, psychomotor impairment, fatigue, feelings of worthlessness, decreased concentration or thoughts of death or suicide (American Psychiatric Association, 2000). The course of the disease is variable, but the likelihood of experiencing another major depressive episode increases with each episode. Since MDD is associated with high mortality, effective antidepressant treatment with a rapid onset is a necessity (American Psychiatric Association, 2000).

Current Treatment

The first antidepressant drugs were discovered serendipitously in the 1950s with the discovery that iproniazid, an antitubercular drug, and imipramine, an antihistamine, had positive effects on mood when given to depressed patients (Deverteuil and Lehmann, 1958, Azima, 1959, Slattery et al., 2004). From this starting point, many improvements have been made to develop first-line treatments that are used today, with the common target of the monoaminergic system. However, major setbacks still exist in current antidepressant treatment, such as delay of onset and treatment resistance.

Drugs affecting the monoaminergic system

The monoamine hypothesis of depression postulates that the physiological basis of depression is a reduced amount of monoamines, namely serotonin, norepinephrine and dopamine, in the central nervous system (Delgado, 2000). The principal and immediate function of current antidepressants is to increase monoamine levels in the brain; however, the effect of these drugs is delayed for weeks. Evidence also demonstrates that depletion of monoamines does not cause depression in healthy volunteers; it also does not worsen depressive symptoms in MDD patients.
This data suggests these drugs must alter a downstream effector in the brain leading to antidepressant effects, and targeting the effector directly may yield more rapid results (Machado-Vieira et al., 2009a). The following is an overview of current antidepressant treatment.

I. Selective Serotonin Reuptake Inhibitors (SSRIs)

Selective serotonin reuptake inhibitors (SSRIs) are typically the first line of treatment for MDD. The first SSRI, fluoxetine, was introduced in 1987 and was well tolerated due to its few adverse effects and safety with respect to overdose potential (Connolly and Thase, 2012). SSRIs were created as a more selective drug with fewer adverse effects compared to tricyclic antidepressants (TCAs), the previous first line of treatment for MDD. As the name suggests, SSRIs function by inhibiting the serotonin reuptake pump and therefore increasing the amount of serotonin at the synapse (Vaswani et al., 2003). SSRIs bind to a specific site on the transporter and act as negative allosteric modulators, decreasing the transporter’s affinity for serotonin (Stahl, 1998). Before SSRIs were developed, the standard antidepressant treatment was TCAs. A meta-analysis found that there was no significant difference in efficacy between SSRIs and TCAs, with the exception of an inpatient group, where TCAs showed higher efficacy (Anderson, 2000). SSRIs had an approximately 4-5% lower drop out rate due to side effects compared to TCAs, with the main adverse effects of SSRIs being nausea, anxiety, headache, movement disorders and sexual dysfunction (Anderson, 2000, Vaswani et al., 2003). However, these are less severe than TCAs and the risk of death by overdose of SSRIs is very low. Although the efficacy of SSRIs and TCAs is very similar, the manageable and few side effects and the low risk of overdose favours SSRIs as the current first line of treatment for MDD. Nonetheless, both treatment options still lack a rapid onset of therapeutic action.

II. Monoamine Oxidase Inhibitors (MAOIs)

The first antidepressant discovered, iproniazid, was originally developed as a treatment for tuberculosis, but was found to have antidepressant effects (Deverteuil and Lehmann, 1958, Nutt, 2002). It was later determined that iproniazid is a monoamine oxidase inhibitor (MAOI). Monoamine oxidase is an enzyme located in the outer mitochondrial membrane, and it keeps cytoplasmic levels of monoamines low by catalyzing their deamination (Owens, 1996, Youdim et al., 2006). The first generation MAOIs were irreversible and non-selective MAO-A and -B
inhibitors (Owens, 1996, Nutt, 2002). They function by increasing levels of 5-HT and norepinephrine in the cytoplasm, which is then packaged into vesicles for synaptic release (Owens, 1996). First generation MAOIs were beneficial at elevating mood, but these inhibitors had potentially life threatening side effects (Nutt, 2002). For example, patients taking these MAOIs could not consume foods high in tyramine, such as cheese, as this could lead to serious cardiovascular problems (Nutt, 2007). MAOIs also have significant overdose toxicity (Nutt, 2007). These adverse effects make MAOIs a second or third line of treatment (Nierenberg et al., 2008). However, it has since been discovered that there are different subtypes of MAOs and now selective MAO-A and MAO-B inhibitors are used for treatment of different disorders (Nutt, 2002). A transdermal patch of selegiline, an MAO-B inhibitor, was approved for treatment of MDD; however, it has shown only modest efficacy in MDD patients (Amsterdam, 2003, Nierenberg et al., 2008).

III. Tricyclic Antidepressants (TCAs)

The first tricyclic antidepressant, imipramine, was found by chance to elevate mood and was then used as a treatment for MDD (Azima, 1959, Hollister, 1981). TCAs exert their effect by preventing the reuptake of both serotonin and norepinephrine, but also act as antagonists on α₁-adrenoceptors, histamine (H₁), muscarinic cholinergic and serotonin (5-HT₂) receptors (Nutt, 2007). TCAs therefore have a more heterogeneous mechanism of action than antidepressants that have since been developed, which leads to their many extrapyramidal side effects. Some side effects of TCAs include tremor, delirium and dyskinesia, as well as memory impairment and sedation (Lejoyeux et al., 1992). These side effects become particularly troublesome when the drug is prescribed chronically. Due to these adverse effects, there are frequent issues of dosing and compliance when prescribing TCAs. In one study, it was found that many patients receiving TCAs were treated with a low dose, which is not always effective (Keller et al., 1982). Compliance may also be a problem due to adverse effects, as the compliance decreases with increased incidence and severity of side effects (Christensen, 1978, Lejoyeux et al., 1992). Due to extrapyramidal effects, TCAs are no longer the first line of treatment for MDD since the development of SSRIs.
Treatment Resistance

One major obstacle in the treatment of MDD is treatment resistance, where patients have failed to show response or remission to an adequate treatment with antidepressants, including adequate dose, duration and compliance (Vieta and Colom, 2011). Treatment resistance is a major obstacle in MDD, as approximately 50% of patients do not respond to their first or second antidepressant trial, and chance of remission decreases with subsequent antidepressant trials (Fava, 2003, Gaynes et al., 2008). Various algorithms have been developed in an attempt to better treat patients quickly and effectively starting with pharmacological treatments that have been most successful (Adli et al., 2006, Gaynes et al., 2008). If these are insufficient, the dose is elevated or a different antidepressant is tried depending on the algorithm. Eventually, more drastic approaches are needed, which usually involve electroconvulsive therapy (ECT), but other strategies, such as magnetic seizure therapy (MST), are currently being studied (Adli et al., 2006).

I. Electroconvulsive Therapy (ECT)

Electroconvulsive therapy is an effective method of treating depressive symptoms, but is typically administered only after pharmacological intervention has failed to provide adequate therapeutic benefit. It is uncommon as the first line of treatment due to the general risks of anesthesia, as well as the controversy surrounding its use (Carney and Geddes, 2003, Read and Bentall, 2010, Allan and Ebmeier, 2011). ECT consists of stimulating the brain with electric current to induce a seizure and usually requires 2-3 sessions per week and between 6-12 sessions overall (Allan and Ebmeier, 2011). Although the exact mechanism of action has not been established, studies have investigated how electrical stimulation of the brain leads to antidepressant effects. ECT has been shown to increase brain-derived neurotrophic factor (BDNF), a molecule important for growth and differentiation in the brain, which has also been shown to be upregulated with antidepressants (Bocchio-Chiavetto et al., 2006). ECT also leads to an increase in post-synaptic 5-HT(1A) receptor signaling, as do many antidepressants (Savitz et al., 2009). It is therefore likely that many different mechanisms play a role in the antidepressant effects of ECT.

Although ECT is not the first line of treatment, it has been shown to be very effective in treatment resistant patients. Two different meta-analyses showed that ECT is significantly more
effective at producing antidepressant effects than simulated ECT or pharmacological agents (UK ECT Review Group, 2003, Pagnin et al., 2004). Patients with non-psychotic depression have shown remission rates as high as 83% following ECT, but patients who have failed to respond to previous antidepressant treatments have a lower remission rate of 48% with ECT (Petrides et al., 2001, Heijnen et al., 2010). Although ECT is effective, it does have serious adverse effects, such as retrograde amnesia (O'Connor et al., 2008). In a smaller study with 24 patients, there was a significant impairment in visual and visuospatial memory during and within the week after ECT (Falconer et al., 2010). Furthermore, objective measures show short-term (<6 months) memory loss (Fraser et al., 2008). ECT has been proven to be effective in treatment resistant MDD, but will likely remain a last resort for treatment due to the risks involved and cognitive deficits that develop. An alternative to ECT, a procedure involving transcranial magnetic stimulation, is currently being studied.

II. Magnetic Seizure Therapy (MST)

Due to the negative cognitive effects associated with ECT, other options with a similar therapeutic approach have been investigated. Magnetic seizure therapy (MST) is similar to ECT as it also involves induction of a seizure; however, MST produces a seizure that is highly focused on specific brain regions and does not diffuse to deeper brain regions, such as the hippocampus, potentially avoiding the negative cognitive side effects of ECT (Lisanby, 2002, Hoy and Fitzgerald, 2010b). This method consists of a coil placed on the head used to deliver repetitive transcranial magnetic stimulation (rTMS). The high frequency magnetic field passes through the skull and induces focal brain activity alterations leading to a generalized seizure (Hoy and Fitzgerald, 2010b, a, Allan and Ebmeier, 2011). MST allows for greater control over the exact location and intensity of the stimulation (Lisanby et al., 2001a). Currently, MST is not used clinically as further studies are needed to prove its safety and efficacy.

The first study investigating the feasibility of MST was performed on nonhuman primates and found that rTMS was capable of inducing seizures under general anesthesia; therefore further studies were required to determine safety parameters (Lisanby et al., 2001a). MST was first attempted with a single patient with treatment resistant depression who underwent 3 MST treatments a week for 4 treatments(Lisanby et al., 2001b). Following the fourth treatment the patient’s HDRS score had decreased from 20 to 13. The MST treatments were followed by 8
ECT treatments, which further reduced the score to 6. This study proved the feasibility of the treatment method and points out that MST can be further refined by adjusting the site and frequency of stimulation (Lisanby et al., 2001b). To date, the only controlled study conducted investigating the efficacy of MST randomized 23 patients to receive rTMS and 22 patients underwent ECT. 16.7% of patients who received rTMS achieved remission, as opposed to 59.1% who received ECT. This study concluded that ECT was more effective at short-term treatment of depressive symptoms; however, due to the limited sample size, further studies are needed to determine efficacy.

2.2 Animal Models of MDD

To further investigate novel targets for treatment of MDD, animal models of the disease are necessary. While some models offer the ability to screen antidepressant compounds with high predictive value, other models will allow us to further our understanding of the neurological deficits involved in MDD. Animal models of MDD have been established in an attempt to improve our understanding of the disease; however, this has been challenging due to the fact that the complex genetic and environmental factors are still not completely understood. Therefore, many animal models of depression focus on recapitulating depressive symptoms in animals and attempting to alleviate these behaviours (Nestler et al., 2002).

Behavioural Assays

Many behavioural assays have been developed as a method to model depressive symptoms in animals; however, since MDD is a heterogeneous disease with both genetic and environmental factors it has been difficult to develop a model with different types of validity. The best animal models are those that have all three types of validity. Predictive validity signifies that the model will react to antidepressant treatment in a similar manner to humans (Nestler and Hyman, 2010). Construct validity indicates that the manifestation and cause of the disease in the animal model replicates the origin and symptoms of the disease in humans (Nestler and Hyman, 2010). Finally, face validity signifies that the animal model replicates the phenotypic aspects of the human disease, such as behavioural, biochemical or molecular deficits (Nestler and Hyman, 2010). Many of these assays have predictive validity for the efficacy of antidepressants, while others have face or construct validity. Predictive validity is essential in behavioural assays as a tool to screen new antidepressants but the behaviour is not necessarily caused by neurological
deficits observed in the human disease. Models with face and construct validity may lead to the discovery of new therapeutic targets.

I. Forced Swim Test (FST)

The forced swim test (FST) is a behavioural assay that models behavioural despair and a lack of coping ability. The original FST, developed using rats, consisted of a plexiglass cylinder filled with 15 cm of 25°C water (Porsolt et al., 1977). The rat is placed in the cylinder and monitored for 15 minutes. This procedure is repeated 24 hours later for a 5-minute period and the immobility time is measured (Porsolt et al., 1977). The second study by this group using this method demonstrated that during the first trial the rat was very active for the first 2-3 minutes, whereas after 5-6 minutes the rat was immobile for approximately 80% of the time (Porsolt et al., 1978). The rats were immobile for approximately 75% of the second trial following a short burst of initial activity (Porsolt et al., 1978). Many different antidepressants have behavioural effects in this assay, including TCAs, MAOIs and atypical antidepressants, as well as psychostimulant and anxiolytic drugs. All classes of antidepressants tested demonstrated a significant decrease in immobility time in the FST, while most also caused a reduction in open field activity, indicating that the decrease in immobility is not due to an increase in overall activity (Porsolt et al., 1978). The psychostimulants, on the other hand, decreased immobility time but also led to an increase in overall activity in the open field test (Porsolt et al., 1978). The FST has since been modified to be more sensitive to different types of antidepressants, such as SSRIs (Petit-Demouliere et al., 2005, Yan et al., 2010). Therefore, the FST test has proven to have very good predictive validity in distinguishing the efficacy of antidepressants.

The predictive validity of the FST test has been consistently proven; however, the notion that this test mimics depression or behavioural despair in rodents has been questioned (West, 1990, Cryan et al., 2005, Nestler and Hyman, 2010). It has been suggested that the immobility in the FST does not reflect an inability to cope with stress but rather a possible survival adaptation allowing them to remain afloat for a longer period of time (Nishimura et al., 1988) or conserve energy (Hawkins et al., 1978). Concerns have also been raised that treating wild-type mice with a single dose of antidepressants and testing hours later does not reflect the human disease, in which there are genetic and environmental factors that have neurobiological implications, or the method of current treatment, where chronic drug administration is necessary and the effects are
not immediate (Nestler and Hyman, 2010). Therefore, the FST has been shown to be an effective model to predict the efficacy of current antidepressants; however, there is a lack of face and construct validity that hinders its ability to be utilized for other purposes.

II. Tail Suspension Test (TST)

The tail suspension test (TST) was developed with a rationale and pattern similar to the FST, as both tests place rodents in inescapable, stressful environments, where mice alternate between an active and immobile phase (Steru et al., 1985). The TST consists of suspending a mouse by its tail to a hook connected to a recording device by a piece of adhesive tape. The hook is 350 mm from the ground and the mouse is 150 mm away from the nearest object. The recording device measures body movements over a period of 6 minutes, making it easy to quantify periods of immobility. In control animals, the mice are immediately active and trying to escape, but after a period of time remain more immobile. To test the effect of different drugs, mice are injected with the drug 30 minutes prior to the experiment. All TCAs and atypical antidepressants are successful at reducing immobility time, most in a dose dependent manner, whereas other drugs either do not increase immobility, or do so due to an increase in overall activity (Steru et al., 1985). Steru et al argues that the TST differs from the FST test in three regards: it does not require immersion in water that can lead to hypothermia, the recording of immobility time does not rely on human observation, and it is more sensitive to lower drug doses (Steru et al., 1985). The author, therefore, suggests that the TST may be a more practical behavioural test for the study of antidepressant action. Many of the criticisms of the FST have also been raised regarding the TST. The lack of construct and face validity limits the usefulness of the model beyond its ability to screen for antidepressants (Nestler and Hyman, 2010, Yan et al., 2010).

III. Sucrose Preference Test (SPT)

The sucrose preference test was developed as a model of anhedonia in rodents, one of the core symptoms of MDD. The SPT is rather simple and consists of giving the rodent a choice between water and a sucrose solution. A reduction in sucrose intake indicates a loss of interest in pleasurable activities (anhedonia) and therefore offers face validity to the model. There are a few parameters to consider when administering the sucrose preference test. The concentration must be adjusted depending on the animals and what is being tested. If the sucrose concentration is too high, rats might prefer the sucrose solution even after being exposed to stress (Overstreet,
Another consideration is how to record the sucrose consumption, whether it be intake (ml/kg) or preference (sucrose intake/total intake)(Overstreet, 2012). The SPT not only has face validity, but it also has predictive validity, as sucrose preference is restored upon treatment with antidepressants (Willner et al., 1987, Muscat et al., 1992). The main issues of the test would be to prove that lack of consumption is not due to overall reduced thirst or an aversion to sweet tastes (Willner, 1997). Therefore, the drinking of regular water should also be monitored. The SPT is typically used for models of depression with induced depressive symptoms.

IV. Novelty Induced Hypophagia (NIH)

Novelty induced hypophagia (NIH) refers to the observation that there is a reduction in feeding when animals are exposed to a novel environment (Dulawa and Hen, 2005). It has been used as a model of anxiety, but due to the fact that anxiety and depression are highly inter-related in humans and that most classes of antidepressants also reduce anxiety, NIH can arguably be used as a model of at least some symptoms of MDD (Helmuth, 2003, Dulawa and Hen, 2005, Andrisano et al., 2012). To test the effect of antidepressants on NIH, the antidepressant is administered daily; on day 23, the mice are singly housed. On day 25, the mice are trained to consume sweetened condensed milk for 3 days (30 minutes/day) and on day 28, a stereological pipette containing milk is placed in the home cage and the latency to drink is measured, as is the volume consumed, every 5 minutes. On day 29, mice are placed in the novel environment, which consists of a similar cage with the pipette of milk, but with no shavings, brighter lighting and a white piece of paper placed under the cage. The latency to drink and volume are measured. Chronic antidepressant treatment should lead to a reduced latency to feed in the novel environment due to anxiolytic effects; however, subchronic or acute treatment should not reduce the latency to feed (Dulawa and Hen, 2005).

The NIH test has demonstrated high predictive validity, as well as construct validity, as it is a clear test of anxiety and antidepressant and anxiolytic medications reduce the anxious behaviour (Stephens, 1973, Dulawa et al., 2004). In addition, the assay follows the time course of antidepressant action in patients with MDD. Antidepressants are only effective at reducing the latency to feed if they are administered chronically, whereas anxiolytic drugs with a quicker onset of action are effective with only acute treatment (Soubrie et al., 1975, Santarelli, 2003, Dulawa and Hen, 2005). Evidence demonstrates that the NIH assay is a reliable MDD
behavioural assay that follows the time course of human treatment, as well as demonstrates behaviours similar to the human disease.

V. Marble Burying

The marble burying test is another behavioural test of anxiety, but as mentioned above antidepressant drugs are effective at treating anxiety. Depression and anxiety are highly inter-related making it difficult to compartmentalize the two and indicating that the study of one may be linked to the neurobiology of the other (Dulawa and Hen, 2005). The marble burying test consists of placing the mouse in a cage containing 5 cm of sawdust and 20 evenly spaced clean, glass marbles. After 30 minutes, the number of marbles that are at least 2/3 buried are counted (Njung'e and Handley, 1991b). The act of burying the marbles signifies anxious behaviour and therefore treatment with anxiolytic and antidepressant drugs should reduce the number of marbles buried. Many different types of antidepressant drugs have been tested acutely and the marble burying test shows high sensitivity to the major classes of antidepressants (Borsini et al., 2002). Most antidepressants, such as SSRIs and TCAs, have been shown to reduce marble burying behaviour and therefore to reduce anxiety in mice (Njung'e and Handley, 1991a, Ichimaru et al., 1995, Borsini et al., 2002). Although the effects of these antidepressants on marble burying behaviour show predictive validity, they do not follow the same time course as the human disease with respect to onset of therapeutic effect. However, this test is effective at determining anxiolytic effects of drugs and further testing on the effect of chronic drug treatment is required (Borsini et al., 2002).

VI. Learned Helplessness (LH)

Much like the FST and TST test, learned helplessness (LH) involves short-term exposure of normal animals to stress, which differs from the human disease which develops over time and has a genetic and environmental component (Nestler and Hyman, 2010). The LH test originated by exploring the idea of controllability of a situation and how this would affect behaviour. The LH test consists of separating animals into two groups, a no aversive control group (NAC) and an aversive control group (AC). The pre-exposure phase requires that the AC group receive shocks but are able to escape, whereas the NAC group is unable to escape from the shocks. The test phase then consists of both groups receiving shocks with the ability to escape. The AC group will continue to exhibit escape behaviour whereas the NAC group will not attempt to
escape or the latency to escape will be significantly greater than the AC group. A third group, or naïve group, is also used and this group is not exposed to the pretreatment in order to ensure that the AC group is not learning escape behaviour in the pretreatment (Pryce et al., 2011).

A similar test has been performed on humans, where, in the test phase, subjects received either escapable or inescapable noxious stimulus or a solvable or insolvable cognitive task in the pre-trial, while receiving an escapable noxious stimulus or solvable cognitive task in the test phase. Like in the animal models, the subjects who received the inescapable or unsolvable pre-trial stimulus, did significantly worse in the test phase (Pryce et al., 2011).

Unlike FST and TST, the LH test exhibits construct validity as there are changes in emotion, motivation and cognition due to uncontrollable stress. There is also face validity, as the animals lack goal-oriented behaviour when faced with controllable adverse events. However, antidepressants function acutely in this model, which differs from the human disease.

**Induction of Depression in Animal Models**

The chronic mild stress model of depression aims to develop depressive symptoms over time in order to better mimic the human disease (Yan et al., 2010). More specifically, it creates a model of anhedonia, a loss of response to pleasurable events, which is a key feature of MDD. The first chronic stress model demonstrated that mice exposed to 3 weeks of chronic stressors, such as footshock, isolation, tail pinch and swim in ice water, had reduced basal activity in an open field test and failed to show an activation response to acute stress, demonstrating a lack of interest, which is a feature of MDD (Katz et al., 1981). A similar model, with less severe chronic stressors, such as food and water deprivation, change in temperature, tilted cages and changes to light/dark cycles, was used to demonstrate that chronic stress leads to a decrease in sucrose consumption, which is reversed after 2-4 weeks of desmethylimipramine treatment, therefore demonstrating predictive validity (Willner et al., 1987). Further studies have provided evidence of the predictive validity of chronic mild stress, demonstrating that the anhedonic effects can be reversed with chronic antidepressants (Sampson et al., 1991, Muscat et al., 1992). The anhedonic effects of chronic mild stress were also reversed with competitive and non-competitive NMDA receptor antagonists, indicating that the glutamatergic neurotransmitter system plays a role in the behaviour (Papp and Moryl, 1994).
In addition to predictive validity, chronic mild stress also possesses face and construct validity. Face validity has been established in this model as it was shown that treatment is not effective until anhedonia has been established in the animal and the time course of treatment corresponds to that of the human disease (Willner et al., 1992). Furthermore, antidepressants have no effect on control animals, which is also true for healthy individuals (Willner et al., 1992). Face validity was also established by the fact that chronic mild stress produces other characteristics of depression, such as decreased sexual and investigative behaviour, reduced locomotor activity and disruptions in sleep patterns (Willner, 1997). Finally, chronic mild stress also demonstrates construct validity. It has demonstrated that following chronic mild stress, over time, these animals develop anhedonia, a core symptom of depression (Willner, 1997). Induced chronic mild stress is the animal model of MDD that most closely represents the disease in humans. It occurs over a period of time and mimics symptoms seen in MDD. Treatment of these symptoms in animals requires chronic antidepressant administration and the effects of these drugs are not seen acutely, similar to antidepressant treatment in humans.

These animal models of MDD have varying uses in the development of antidepressants and in our understanding of the disease. While some models are valuable for their predictive ability, others will allow us to investigate the cause of depressive symptoms. All of these animal models can be useful in determining the role of glutamate in the manifestation of depressive symptoms and in establishing the efficacy of glutamatergic modulating drugs in MDD.

2.3 NMDA Receptor Biology
Glutamate is the major excitatory neurotransmitter in the brain and there are two main classes of glutamate receptors, metabotropic and ionotopic. Within the ionotopic class, there are three main receptors: N-methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA) receptors and kainate receptors. NMDA receptors have been studied more extensively with respect to their role in MDD and current research is focused on potential antidepressants that specifically target these receptors. The following is a brief summary of NMDA receptor biology.

**NMDA Receptor Structure and Composition**

The NMDA receptor is both a voltage-dependent and ligand gated ion channel. It is composed of a combination of multiple subunits, which include the obligatory NR1 subunit, the NR2 (A,
B, C and D) subunits and the NR3 (A and B) subunits (Laurie and Seeburg, 1994, Monyer et al., 1994, Sun et al., 1998). The NMDA receptor is a heterotetramer ion channel composed of multiple NR1 subunits and at least one NR2 or NR3 subunit. The composition of the NMDA receptor varies depending on the brain region and the developmental time point (Yamakura and Shimoji, 1999). The NMDA receptor contains various binding sites, most importantly, glutamate and glycine binding sites. Glutamate and its co-agonist, glycine, must bind to activate the receptor. Along with the glutamate and glycine sites, the NMDA receptor contains several modulatory binding sites. The NMDA receptor contains a Zn$^{2+}$ modulatory binding site, where the Zn$^{2+}$ ion both voltage dependently and independently inhibits the NMDA receptor (Dingledine et al., 1999). The receptor also contains a polyamine binding site, which can act as either inhibitory or excitatory depending on the polyamine (Williams et al., 1989). In addition to these binding sites located on the surface of NMDA receptor subunits, there are binding sites within the ion channel, specifically, a binding site for the Mg$^{2+}$ ion and the phencyclidine (PCP) binding site (Javitt, 2007). The location of the PCP binding site requires that the local membrane potential be depolarized so that the channel is open in order for PCP to bind. The drug PCP shares a common binding site with other non-competitive NMDA receptor antagonists, such as ketamine and MK-801 (Javitt, 2007).

**Figure 1. NMDA receptor structure.** Adapted from CNSforum.com 2002 (http://www.cnsforum.com/imagebank/item/hrl_rcpt_sys_NMDA/default.aspx)
NMERA Eceptor Function

The NMDA receptor is an ion channel located on the post-synaptic membrane at the synapse. Following binding of glutamate and glycine, the cell membrane must be depolarized in order to remove the Mg$^{2+}$ ion block from within the channel. This is essential to allow the influx of Na$^+$ and Ca$^{2+}$ ions and the efflux of K$^+$ ions (Javitt, 2007). NMDA receptors play an important role at the synapse. They are essential in the regulation of spine dynamics and synaptic plasticity (Matus, 2000, Debanne et al., 2003). Activity-dependent activation of NMDA receptors leads to an influx of Ca$^{2+}$ ions, which subsequently activates many downstream signaling cascades. The influx of Ca$^{2+}$ can lead to the activation of the cAMP pathway, whose downstream effects include phosphorylation of NMDA receptor subunits targeting them to the cell membrane as well as positive modulation of the NMDA receptor ion channel (Scott et al., 2003). In addition to the cAMP pathway, Ca$^{2+}$ influx leads to activation of a CaMKII dependent signaling pathway. CaMKII activation leads to the phosphorylation of AMPA receptors and recruitment of these receptors to the cell surface, increasing the strength of the synapse. It also leads to increased NMDA receptor subunit cell surface expression (Lau and Zukin, 2007). Overall, these calcium dependent pathways lead to increased strength of the synapse and synaptic transmission.

Genetic Model of NMDA Receptor Hypofunction

Many genetic mouse models have been generated to understand the role of NMDA receptors in various aspects of neuron physiology and neurobiology (Mori and Mishina, 2003). In mice, null mutants of the NR2 subunits are viable, because other NR2 subunits can be expressed to compensate for their loss (Kadotani et al., 1996). However, null mutation of the NR1 subunit causes perinatal lethality, demonstrating the essential nature of this subunit in the composition of the NMDA receptor (Forrest et al., 1994). Partial-loss-of-function mutations have been generated that are viable. These include point mutations and hypomorphic mutations to reduce NR1 subunit levels. NR1 knockdown mice (NR1-KD), which have a global reduction in NR1 and NMDA receptors, are viable and have been useful to study the consequences of NMDA receptor hypofunction (Mohn et al., 1999). They were generated using homologous recombination in embryonic stem cells (Mohn et al., 1999). A neomycin resistance gene was inserted into the Grin1 gene, thereby altering its ability to be expressed (Mohn et al., 1999). This insertion caused a global knockdown of the NR1 subunit of the NMDA receptor, producing only
about 10% of the normal amount of protein (Mohn et al., 1999). Many biochemical and behavioural experiments have been performed on these mice. Behavioural abnormalities of NR1-KD mice include deficits in sociability and sensorimotor gating as well as increased locomotor activity (Mohn et al., 1999, Duncan et al., 2004). Spine density studies performed on these mice have demonstrated that they have significant age-dependent reductions in spine density in medium spiny neurons of the striatum (Ramsey et al., 2011). These mice represent an excellent model to study the effect of NMDA receptor hypofunction on dendritic spine density, which will be discussed in the following section.

2.4 Dendritic Spines

Dendritic spines are the sites of excitatory transmission in the brain. They are dynamic postsynaptic structures whose number and morphology reflect the strength of the synapse and synaptic transmission at that location. The glutamatergic system, particularly NMDA receptors, plays a role in regulating spine density in an activity-dependent manner. Spine density is altered in many neurological disorders and therefore it is important to study and understand the status and implication of spine density changes in these diseases.

**Spine Structure and Function**

Dendritic spines are protrusions located along the length of the dendrite and are the site of excitatory synapse formation in the brain. The formation and maturation of spines is constantly fluctuating depending on neuronal activity (Hotulainen and Hoogenraad, 2010). Synaptic activation leads to sustained and mature dendritic spines with increased spine head volume, while lack of neuronal activity will lead to loss of dendritic spines (Nimchinsky et al., 2002). Immature spines typically consist of thin filapodia-like structures, while mature spines are mushroom-shaped with distinct head and neck compartments. The larger spine head allows...
for more signal input, while the neck compartmentalizes the spine and controls what reaches the rest of the neuron (Halpain et al., 2005). Spines contain the postsynaptic density, which is comprised of a network of proteins including AMPA and NMDA receptors as well as signaling and adhesion molecules (Hotulainen and Hoogenraad, 2010). The cytoskeleton of dendritic spines is mainly composed of filamentous actin (Landis and Reese, 1983). Spines are dynamic structures; the shape and size of the spine is mainly controlled through signaling cascades that alter actin dynamics.

**NMDA Receptors and Dendritic Spines**

NMDA receptors play a role in controlling the number and maturation of dendritic spines. Numerous studies have demonstrated that NMDA receptor hypofunction leads to reductions in dendritic spines (Ultanir et al., 2007, Roberts et al., 2009, Brigman et al., 2010, Ramsey et al., 2011). Alterations in spine density are due to changes in actin dynamics either directly through interactions with NMDA receptors at the postsynaptic neuron or through changes caused by the NMDA receptor dependent influx of Ca\(^{2+}\). Many downstream pathways can lead to alterations in spine density through changes in actin dynamics. This process can involve proteins that interact directly with NMDA receptors to regulate actin remodeling (Tolias et al., 2005). Actin dynamics can also be altered through the role of Rho GTPases (Saneyoshi et al., 2010). This large network of proteins facilitates either the promotion of spine growth and maintenance or causes spine retraction and disassembly (van Galen and Ramakers, 2005). NMDA receptors, as well as other synaptic proteins, play a significant role in activation of these signaling pathways and therefore in actin dynamics and spine regulation.

**Spine Dysregulation in Neurological Disorders**

Evidence shows that changes in spine density are observed in many neurological disorders. A postmortem study has revealed that there is a significant reduction in dendritic spines in the dorsolateral prefrontal cortex in patients with MDD (Kang et al., 2012). Further postmortem studies are needed to determine whether there are changes in other brain regions. Dendritic spine density is also reduced in postmortem brains of patients with schizophrenia and Alzheimer’s disease. Postmortem studies of patients with schizophrenia revealed that reductions in spine density correspond with reductions in gray matter loss in certain brain regions (Penzes et al., 2011). Interestingly, these brain regions with reduced spine density are strongly associated
with behaviours observed in schizophrenia patients (Penzes et al., 2011). More specifically, there was a significant 23% reduction in spine density in the deep layer 3 dorsolateral prefrontal cortex neurons in schizophrenic patients compared to controls (Glantz and Lewis, 2000). Another study demonstrated that schizophrenia patients had a significant reduction in spines forming synapses in the hippocampus (Kolomeets et al., 2005). Postmortem studies of patients with Alzheimer’s disease have demonstrated significant loss of spines in the hippocampus and frontal cortex, brain regions that have been implicated in the disease (Penzes et al., 2011). Specifically, one study found a significant decrease of 35% in spine density in biopsied cortical samples of Alzheimer’s disease patients and a 42% reduction in spine density in autopsied brain samples (DeKosky and Scheff, 1990). In addition, one postmortem study demonstrated that patients with mild Alzheimer’s disease had a 55% reduction in total synapse number in the hippocampus compared to control samples (Scheff et al., 2007). Spine loss has been shown to be correlated with cognitive impairment and dendritic spine loss worsens with worsening symptoms of the disease (Penzes et al., 2011). These studies demonstrate that changes in dendritic spine density are implicated in many neurological disorders and changes in spine density are correlated with changes in behaviour and cognitive impairment (Penzes et al., 2011).

Reductions in spine density have been associated with many disorders; however, an increase in spine density can also be detrimental. Postmortem studies of patients with autism spectrum disorders indicated an increase in spine density by approximately 20% in the cortex (Hutsler and Zhang, 2010). The increase in spine density is hypothesized to be due to a lack of synaptic pruning. Increased spine density in autism spectrum disorder is inversely correlated with cognitive function (Penzes et al., 2011).

It is evident that changes in dendritic spine density play a role in many neurological disorders. Several studies have investigated the effect of antidepressant treatment on dendritic spine density in rodents. Chronic fluoxetine treatment led to increased dendritic spine density in the rat cortex (Ampuero et al., 2010). Interestingly, changes in spine density were only observed after four weeks of treatment, providing supporting evidence that the antidepressant effects of fluoxetine have the same time scale as changes in spine density. Another study demonstrated that acute and subchronic combination antidepressant therapy of rolipram and imipramine also led to significant increases in spine density in the hippocampus (Marchetti et al., 2010). Recent studies have also demonstrated that drugs affecting the cognitive and neurological symptoms of
these disorders correspondingly influence spine density in relevant brain regions. For example, administration of L-methionine, a drug which exacerbates schizophrenic symptoms in patients, causes reduced spine density in mice; however co-administration with the anticonvulsant valproate prevents this reduction (Tueting et al., 2010). In addition, PCP administration in rats, a model of schizophrenia, decreases dendritic spine density in layer II/III pyramidal neurons of the cortex; this is reversed by acute and chronic antipsychotic (olanzapine) drug treatment (Elsworth et al., 2011). One study demonstrated that PBT2, a drug in clinical trial stages for Alzheimer’s disease, increases hippocampal spine density in a mouse model of the disease that has spine density deficits (Adlard et al., 2011). Overall, these studies demonstrate that drugs that help alleviate symptoms of neurological disorders also lead to increased spine density in mouse models. These observations are notable in the study of antidepressants and their mechanism of action. Since reductions in spine density have also been detected in MDD brains, it is important to determine whether antidepressants exert their therapeutic effect through changes in spine density.

2.5 Role of the Glutamatergic System in MDD

With the limitations of current antidepressant treatment options, including delayed onset of action and treatment resistance, researchers have enlarged their focus to include the glutamatergic neurotransmitter system, as evidence has suggested it may be altered in the disease state. Although there are no current antidepressants on the market that specifically target the glutamatergic system, clinical trials are underway and have demonstrated promising results with the use of glutamatergic modulating drugs in the rapid treatment of depressive symptoms (Connolly and Thase, 2012). The following data is further evidence suggesting that drugs targeting the glutamate neurotransmitter system should be explored as a therapeutic option.

Postmortem studies implicating the glutamatergic system in MDD

Emerging postmortem evidence has revealed that the glutamatergic neurotransmitter system is altered in patients that suffered from MDD. Significant changes in NMDA receptor subunits have been reported. These studies indicate that there is an increase in NR2A protein levels in the amygdala, a reduction in NR2A and NR2B protein levels in the frontal cortex and no change in the striatum (Meador-Woodruff et al., 2001, Feyissa et al., 2009, Karolewicz et al., 2009). Levels of the NR1 subunit were unchanged in the prefrontal cortex, however there is evidence
of a decrease in NR1 mRNA expression in the hippocampus (Law and Deakin, 2001). This data suggests that although there may be differences in how the glutamatergic system is altered between brain regions, it is evident that this neurotransmitter system is modified in the disease state.

These postmortem studies also focused on scaffolding proteins at the synapse, which are used to anchor the glutamate receptors to the synaptic membrane. These studies revealed an increased level of PSD95 in the amygdala, while showing a reduction in the prefrontal cortex, which corresponds to the relative changes in NMDA receptor subunit levels (Feyissa et al., 2009, Karolewicz et al., 2009). There was a significant decrease in mRNA expression of SAP-102, which along with other proteins determines the localization of NMDA receptors at excitatory synapses (Kristiansen and Meador-Woodruff, 2005). The evidence from these postmortem studies strongly implicates a role of the glutamatergic neurotransmitter system in MDD. The glutamate system appears altered and disturbed in many brain regions that have been implicated in the disease state of MDD.

**Biomarker Studies Implicating the Glutamatergic System in MDD**

The major complication with relying on postmortem studies is the postmortem interval, which can vary between every sample. The length of postmortem interval can alter the amino acid levels in the brain, therefore making postmortem data difficult to interpret (Hashimoto et al., 2007). For this reason, in vivo data is a valuable method to investigate changes that occur in MDD. Several studies have investigated the peripheral levels of glutamate and other excitatory amino acids in the blood and cerebrospinal fluid (CSF) of MDD patients. One study showed that there is an increased level of glutamine in the CSF of patients with the disease (Levine et al., 2000). Furthermore, increased plasma levels of glutamate, glutamine, glycine and taurine have been shown in MDD patients compared to healthy controls. Additionally, this study indicated that there was a positive correlation between plasma levels of glutamate and alanine and depression severity, as measured by Hamilton Depression Rating Scale (HDRS) scores (Mitani et al., 2006). Some of these findings were confirmed by another group who demonstrated increased plasma levels of glutamate, taurine and lysine in patients with MDD compared to controls (Mauri et al., 1998). While the direct cause of increased levels of glutamate and glutamine is unclear, these studies provide some evidence that the glutamatergic
neurotransmitter system could be altered in patients with MDD. Furthermore specific amino acid plasma levels could be used as an indicator of depression severity, a potentially valuable tool as a biomarker of the disease.

**In Vivo Imaging Implicating the Glutamatergic System in MDD**

Although CSF and plasma biomarker studies are valuable, they lack the ability to identify what is occurring in specific brain regions; in vivo imaging studies can provide this information. In vivo imaging is typically performed using proton magnetic resonance spectroscopy, which can quantify the amount of certain amino acids in the brain, in this case, glutamate and glutamine specifically. An in vivo proton magnetic resonance spectroscopy study, focusing on the anterior cingulate cortex, found a reduction in unresolved glutamine and glutamate, as well as a significant reduction in glutamate alone (Auer et al., 2000). In contrast, another study demonstrated a significant reduction in GABA concentrations and an increase in glutamate in the occipital cortex (Sanacora et al., 2004). This study suggests that there is an altered balance between inhibitory and excitatory neurotransmission, which could be affecting the disease state. Another study corroborating the evidence of altered glutamatergic neurotransmission in MDD reported a significant reduction in the ratio of glutamate, glutamine and GABA, referred to as Glx to creatine (a marker of cell energy metabolism), as well as a reduction in the ratio of glutamine alone to creatine in the hippocampus (Block et al., 2009). The quantification of the glutamate system in various brain regions of depressed patients further emphasizes that there are significant changes in this neurotransmitter system in patients with MDD.

**2.6 Ketamine as a Novel Antidepressant**

Ketamine, a non-competitive NMDA receptor antagonist, has recently been used as an antidepressant in clinical trials. It has demonstrated a rapid onset of antidepressant effects in MDD patients and a single infusion can lead to sustained therapeutic effects (Berman et al., 2000, Zarate et al., 2006). The following evidence outlines the clinical studies performed to date on the antidepressant effects of ketamine.

**Clinical Studies of Ketamine for MDD**

The first clinical trial exploring the use of ketamine as an antidepressant used a small group of 7 patients suffering from MDD. In this randomized, double blind study, two test days were
separated by a one-week period (Berman et al., 2000). Patients received a 40-minute infusion of either saline or ketamine hydrochloride (0.5 mg/kg) on the first treatment day and the opposite on the second treatment day. It was determined that ketamine treatment caused a robust antidepressant effect in these patients 240 min following treatment, as demonstrated by a significant decrease in Hamilton Depression Rating Scale (HDRS) and Beck Depression Inventory (BDI) scores (Berman et al., 2000). It is important to note that the blinding of this study may have been compromised due to psychotomimetic effects, as demonstrated by increased scores on the Visual Analog Scale (VAS) and Brief Psychiatric Rating Scale (BPRS) following ketamine treatment (Berman et al., 2000). These scores returned to baseline by 2 hours following infusion. This study demonstrated that with a single infusion of ketamine, antidepressant effects were immediately evident and persisted for up to two weeks (Berman et al., 2000).

Due to the promising findings of this small trial, a larger clinical trial was conducted with an increased number of patients. These patients were treatment resistant, lacking an improvement of symptoms with at least two antidepressant trials. This study was arranged much like the previous study, as it was also a randomized, double blind crossover trial with patients receiving a 40-minute infusion of either saline or ketamine hydrochloride (0.5 mg/kg) (Zarate et al., 2006). Patients demonstrated antidepressant effects 110 minutes after treatment, which persisted through 7 days, as monitored by HDRS scores at 40, 80, 110 and 230 minutes, as well as 1, 2, 3 and 7 days (Zarate et al., 2006). More specifically, 71% of patients met response criteria after one day, 29% met remission criteria after one day and 35% maintained response criteria for at least one week (Zarate et al., 2006). This clinical trial further corroborates the results of the previous study by demonstrating that with a larger number of patients the immediate and prolonged antidepressant effects of ketamine are still evident (Zarate et al., 2006).

One concern with these clinical trials is the observation that the effects of ketamine are only evident for up to two weeks at the most. A clinical trial was therefore conducted to determine if there are more prolonged antidepressant effects of ketamine with repeated dosing (Rot et al., 2010). All patients in this study were treatment resistant, who had previously participated in a single dose ketamine study with a greater than 50% reduction in symptoms for at least one day, but who had subsequently relapsed (Rot et al., 2010). Patients received a 40-minute infusion of ketamine hydrochloride (0.5 mg/kg) on day 1, 3, 5, 8, 10 and 12. 10 participants received the
first infusion, with nine patients meeting response criteria after 24 hours (Rot et al., 2010). These nine patients continued with the remainder of the drug treatments. After the final infusion, all patients met response criteria, while eight met remission criteria (Rot et al., 2010). Eight of these patients relapsed after, on average, 30 days. Safety was also an important aspect of this study. Patients were monitored for psychotomimetic and dissociative effects, as well as for vital signs and self reported side effects (Rot et al., 2010). This study demonstrated the relative safety and efficacy of repeated ketamine dosing for treatment of depressive symptoms; however, long-term effects are still unknown. In order to address this, cognitive and neuropsychiatric tests should be administered and a follow up study should be completed with a larger sample size.

Several other clinical trials utilizing ketamine to treat major depressive disorder have been performed. They have shown similar results related to psychotomimetic effects and efficacy of ketamine and some have demonstrated that ketamine significantly reduces suicidal ideation, an important observation as the effects of ketamine have a rapid onset (Diazgranados et al., 2010, Larkin and Beautrais, 2011). In conclusion, these clinical trials demonstrate that ketamine is relatively safe and has high efficacy among treatment resistant MDD patients with a rapid onset and prolonged antidepressant effects that have not been shown with current antidepressant treatment.

**Effect of Ketamine on Animal Models of MDD**

Preclinical studies have been performed assessing the efficacy and mechanism of ketamine in the treatment of depressive symptoms in rodent models. An important study by the Duman laboratory was the first to demonstrate that the antidepressant effects of ketamine require increased activation of the mammalian target of rapamycin (mTOR) signaling pathway (Li et al., 2010). This is accompanied by an increase in dendritic spine density in the prefrontal cortex of rats treated with an acute, low dose of ketamine (Li et al., 2010). In this study, rats received a single i.p. injection of 10 mg/kg ketamine hydrochloride. Western blots confirmed that following ketamine treatment there was a significant, transient increase in phosphorylated mTOR (pmTOR), as well as its downstream effectors. There was also an increase in phosphorylated AKT (Protein Kinase B) and phosphorylated ERK (Extracellular-signal Related Kinase), proteins that lie upstream of the mTOR pathway. Pretreatment with NBQX, an AMPA receptor inhibitor, U0126, an ERK inhibitor, and LY294002, an AKT inhibitor, abolished the increased
activation of the mTOR signaling pathway, signifying that ketamine is acting through these upstream proteins to activate the mTOR pathway (Li et al., 2010).

This group also demonstrated a sustained increase in several synaptic proteins, such as synapsin I, PSD95 and GluR1, which was maintained for at least 72 hours following ketamine administration (Li et al., 2010). This coincides with the observed increase in spine density in layer V pyramidal neurons in the prefrontal cortex. Pretreatment with rapamycin, an mTOR inhibitor, eliminated the increase in synapse formation and synaptic protein synthesis seen with ketamine treatment. Ketamine treatment also improved depressive behaviours, as monitored using the FST and learned helplessness in response to inescapable stress, as well as anxiety, observed with the novelty suppressed feeding test (NSFT). These behavioural improvements were reversed when rats were pretreated with rapamycin, U0126 and LY294002, further indicating that the mTOR pathway is necessary for the antidepressant effects of ketamine (Li et al., 2010). Overall, this study has considerably increased our understanding of how ketamine exerts its antidepressant effects. It has proven that antidepressant effects of ketamine require activation of the mTOR signaling pathway, providing researchers with a new potential therapeutic target in the treatment of MDD.

In a follow-up study by the same group, ketamine was tested on a chronic unpredictable stress (CUS) model of depression (Li et al., 2011). Rats were exposed to different stressors for 21 days and CUS-exposed rats demonstrated a reduction in a sucrose preference test (SPT) and an increase in latency to feed in the NSFT. Both behaviours were improved following a single ketamine treatment and the effects in the SPT lasted for up to 7 days. An intracerebroventricular injection of rapamycin reversed the effects of ketamine in these behavioural tests. CUS-exposed rats also exhibited reductions in synaptic proteins and spine density, but these deficits were reversed with a single injection of ketamine. The effects of ketamine on synaptic protein expression were sustained for up to 7 days, which coincides with the effects of ketamine on behavioural tests. Pretreatment with rapamycin reversed the beneficial effect of ketamine on spine density and synaptic protein expression, indicating that the mTOR pathway is necessary for the antidepressant effect of ketamine in CUS-exposed rats (Li et al., 2011).

The antidepressant behavioural effects of ketamine have been investigated in numerous studies. Ketamine has consistently been shown to reduce immobility time in the FST with both acute
and chronic treatment without displaying an affect on locomotor activity (Garcia et al., 2008a, Garcia et al., 2008b, Autry et al., 2011, Tizabi et al., 2012, Yang et al., 2012). Overall, ketamine shows promising effects in animal models of depression and its rapid onset of action is mimicked in clinical trials.

**Actions of Ketamine on NMDA Receptors**

Ketamine acts as a non-competitive antagonist on NMDA receptors, a subtype of glutamate receptors. NMDA receptors are both voltage dependent and ligand gated ion channels consisting of combinations of different subunits: the obligatory NR1, the NR2 (a, b, c or d) and the NR3 (a or b) subunits. Activation of AMPA receptors, and subsequent depolarization of the cell membrane, leads to the removal of the Mg+ ion block within the NMDA receptor channel allowing for the influx of calcium and sodium. Activation of the NMDA receptor also requires binding of glutamate to the NR2 subunit and its co-agonist glycine to the NR1 subunit (Stawski et al., 2010).

Ketamine exerts its effect by binding to the PCP site within the ion channel of the receptor. Ketamine is a chiral molecule with the R- and S-enantiomers possessing different properties, but is typically used as a racemic mixture. It is water soluble but has higher lipid solubility than other anesthetics, like thiopentane; therefore ketamine has high bioavailability (Reich and Silvay, 1989). It has very low protein binding and is a high extraction drug (Wagner and O'Hara, 1997). Ketamine is metabolized by CYP3A4 in the liver (Hijazi and Boulieu, 2002) and is N-demethylated to its metabolite norketamine, which is hydroxylated and conjugated to water-soluble compounds to be excreted in urine (Reich and Silvay, 1989). Ketamine has a lower potency and much shorter half-life compared to PCP, with the half-life of ketamine being 2-3 hours compared to anywhere from 7 to 51 hours for PCP (Clements and Nimmo, 1981, White et al., 1985, Busto et al., 1989).

Ketamine principally acts on NMDA receptors, but has been shown to have other non-specific targets as well. It binds to several different receptors including non-NMDA glutamate, nicotinic and muscarinic cholingergic, and opioid receptors (Hirota and Lambert, 1996). It has also been shown to have inhibitory effects on sodium and calcium channels (Kohrs and Durieux, 1998). Ketamine also causes inhibition of serotonin and dopamine reuptake (Hocking and Cousins, 2003). These actions of ketamine may or may not contribute to its antidepressant effects;
therefore, determining the cause of its antidepressant effect is necessary to determine a target for future drugs.

**Potential Mechanisms of Antidepressant Action of Ketamine**

Several mechanisms of action have been proposed to explain the rapid onset, antidepressant effects of ketamine. These proposed mechanisms provide a starting point to investigating new potential therapeutic targets.

I. Synaptogenesis through mTOR

Accumulating evidence indicates that the mammalian target of rapamycin (mTOR) signaling pathway is involved in and may be the cause of the antidepressant effects of ketamine. mTOR is downstream of both AKT and ERK and is upstream of many different proteins, including ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which are essential for translation of synaptic proteins and can lead to increased dendritic spine density and long lasting synaptic alterations (Hoeffer and Klann, 2010, Duman and Li, 2012, Duman and Voleti, 2012). The Duman laboratory showed that acute ketamine treatment leads to activation of the mTOR signaling pathway, which leads to an increase in synaptic protein synthesis and synaptogenesis (Li et al., 2010). Pretreatment with rapamycin, an mTOR inhibitor abolishes these synaptic changes and prevents the antidepressant behavioural effects seen with ketamine alone on the LH and FST (Li et al., 2010). In a rat model of chronic unpredictable stress, the Duman laboratory demonstrated that intracerebroventricular infusion of ketamine improved antidepressant behaviours in the sucrose preference test and novelty suppressed feeding test, but this was reversed when rats were pretreated with rapamycin (Li et al., 2011). In another study conducted on rats, hippocampal pmTOR was significantly increased following acute ketamine treatment (5, 10 and 15 mg/kg) (Yang et al., 2012). However, a study in mice found that pmTOR was not significantly altered following ketamine administration and that pretreatment with rapamycin did not affect antidepressant behaviours in the FST (Autry et al., 2011). Therefore, although some studies strongly demonstrate a necessary role of mTOR, other studies demonstrate mTOR may not be altered. Further studies are needed to investigate the role of mTOR in ketamine treatment in different models of depression.
The effect of ketamine on mTOR in humans has only begun to be investigated. A case study showed that following a 40-minute infusion of (S)-ketamine, there was a significant increase in p-mTOR in peripheral blood cells, which returned to baseline 24 hours later (Denk et al., 2011). Although further studies are required to determine the effect of ketamine on mTOR in humans, the compelling animal studies suggests that increased mTOR signaling could be the cause of ketamine’s antidepressant effects.

II. Antidepressant effects through BDNF

Brain-derived neurotrophic factor (BDNF) is a growth factor that plays an important role in neuronal plasticity, as well as survival and differentiation of neurons (Huang and Reichardt, 2001, Lu, 2003). Due to the large impact of BDNF on neuronal plasticity, it has been studied with respect to several psychiatric diseases (Autry and Monteggia, 2012). Patients with MDD have consistently shown reduced BDNF levels (Sen et al., 2008) and patients with MDD who had stopped drug treatment three weeks prior to testing, as well as drug naïve MDD patients, have shown lower serum levels of BDNF compared to controls (Karege et al., 2002, Shimizu et al., 2003). Antidepressant treatment in MDD patients has been shown in most cases to increase BDNF levels, however, some have had no effect on BDNF levels (Aydemir et al., 2005, Hellweg R, 2008, Sen et al., 2008, Matrisciano et al., 2009). The effects of ketamine on BDNF in MDD patients have not been extensively studied; however, one study showed that ketamine did not increase BDNF levels in the blood 40, 80, 120 and 230 minutes following infusion (Machado-Vieira et al., 2009b).

Studies using animal models have highlighted the importance of the antidepressant effects of BDNF. Evidence indicates that intracerebroventricular and intrahippocampal infusions of BDNF lead to rapid and sustained antidepressant effects using the FST and LH (Shirayama et al., 2002, Hoshaw et al., 2005). An infusion of BDNF into the midbrain also improved LH and FST in rats (Siuciak et al., 1997). These studies consistently demonstrate that BDNF infusions lead to improved performance on tests for depressive symptoms. On the other hand, the effects of ketamine on BDNF are more variable. Ketamine administration led to an increase in hippocampal BDNF at a dose of 10 and 15 mg/kg in one study, whereas in another this increase was only observed at 15 mg/kg (Garcia et al., 2008b, Yang et al., 2012). The increase in BDNF did not correspond to the doses which showed improvement in the FST as this was seen with 10
mg/kg as well (Garcia et al., 2008b). Some studies demonstrated increases in BDNF levels; however others failed to show any changes. Ketamine treatment in rats that had undergone 40 days of chronic mild stress did not significantly alter hippocampal BDNF levels (Garcia et al., 2009). Another study demonstrated that chronic ketamine treatment in rats, given once daily for 2 weeks at 5, 10 or 15 mg/kg, reduced immobility in the FST, but did not show increased hippocampal BDNF levels (Garcia et al., 2008a). To summarize, evidence shows that BDNF can have antidepressant effects, however, the overall effect of ketamine on BDNF levels is equivocal and requires further investigation.

III. Synaptogenesis through AMPA

AMPA receptors, a type of voltage-dependent glutamate receptor, have been implicated in the antidepressant effect of ketamine and appear to be a necessary component to the actions of ketamine. One potential mechanism for the involvement of AMPA states that since ketamine produces a pre-synaptic release of glutamate, which will preferentially bind AMPA receptors, as NMDA receptors are blocked by ketamine, there will be greater AMPA throughput as opposed to NMDA, increasing synaptic potentiation (Maeng and Zarate, 2007). Treatment of cultured hippocampal neurons demonstrated that certain antidepressants also lead to an increase in AMPA receptor surface expression (Du et al., 2007). One study found that chronic treatment with ketamine led to an increased AMPA/NMDA ratio in the hippocampus of Wistar-Kyoto rats, a putative model of depression (Tizabi et al., 2012).

Several studies investigating the role of AMPA receptors in the antidepressant effects of ketamine found that the beneficial behavioural effects of ketamine were abolished when animals were pretreated with an AMPA receptor antagonist. Ketamine led to a decrease in immobility time in the FST, however, this reduction was attenuated when mice were pretreated with NBQX, an AMPA receptor inhibitor (Maeng et al., 2008). Acute ketamine treatment reduced the number of escape failure in LH in rats; however this was also attenuated by pretreatment with NBQX (Koike et al., 2011). Similarly, ketamine decreased immobility in the TST but this too was attenuated by treatment with NBQX (Koike et al., 2011). These studies suggest that ketamine functions through the AMPA receptor to produce downstream effects and that without activation of this receptor the antidepressant effects of ketamine are abolished.
2.7 Potential Adverse Effects of Ketamine Administration

Ketamine has demonstrated promising antidepressant effects in both animal models of MDD and patients with the disease; however, psychotomimetic effects pose a significant clinical problem. Long-term effects of ketamine treatment are currently unknown, but studies of ketamine in animal models do show negative effects of the drug on the brain. These potential adverse effects need to be considered and further studies should be done in humans to assess the potential negative effects of ketamine.

Psychotomimetic Effects of Ketamine

The psychotomimetic effects of ketamine have been well documented and remain one of the main obstacles in the use of ketamine as an antidepressant. The first evidence of psychotomimetic effects of ketamine were explored due to its similarities to neuropsychiatric diseases such as schizophrenia (Luby et al., 1962, Javitt and Zukin, 1991). Ketamine was found to mimic both positive and negative symptoms of schizophrenia, as well as cognitive deficits in humans, and therefore is used as a pharmacological animal model of the disease (Krystal et al., 1994, Adler et al., 1998, Newcomer et al., 1999, Koenig, 2008, Neill et al., 2010). All clinical trials investigating the antidepressant effect of ketamine have reported psychotomimetic effects, specifically perceptual disturbances, euphoria, confusion, dizziness and dissociative symptoms (Berman et al., 2000, Zarate et al., 2006, Larkin and Beautrais, 2011); however, these psychotomimetic effects usually revert back to baseline within hours. Although the psychotomimetic effects are typically mild and only last for a short period of time, it could potentially pose a major problem if ketamine were to be used in repeated doses as an antidepressant; therefore, it is necessary to find alternate drugs with a similar antidepressant effect or a method to reduce the psychotomimetic effects of ketamine.

Neurotoxic Effects of Ketamine

Although ketamine is currently used in high anesthetic doses, evidence from animal studies indicates that ketamine can lead to neurotoxicity and neuronal death. During development the brain undergoes a growth spurt period, where NMDA receptor-containing neurons are hypersensitive to glutamate and, therefore, to excitatory neurotoxicity (Ikonomidou et al., 2001). One study demonstrated that 4 hours after ketamine administration in rats, there was formation
of vacuoles in cingulated and retrosplenial cerebrocortex neurons (Olney et al., 1989). In an additional study, ketamine, as well as other NMDA receptor antagonists, was shown to have a proapoptotic effect in 7-day old rats. Ketamine was administered in a fashion that led to steady NMDA receptor blockade for 8 hours which caused significant apoptosis in several brain regions, including parietal, frontal and cingulated cortex and the laterodorsal thalamus (Ikonomidou, 1999). Evidence indicates that the effects of ketamine vary depending on dose and gender. Female and male rats received increasing doses of ketamine and while females began showing signs of neurotoxicity at 40 mg/kg with increased severity at high doses, males only began to demonstrate neurotoxicity at 50 mg/kg with reduced severity (Jevtovic-Todorovic et al., 2001). When both genders were exposed to 60 mg/kg ketamine, female rats displayed neurotoxic effects beginning at 2 months, whereas male rats did not display neurotoxic effects until 8 months and were significantly less severe than female neurotoxicity at 8 months (Jevtovic-Todorovic et al., 2001).

The main clinical use of ketamine is as an anesthetic, consequently, it is mainly administered in combination with other drugs. Therefore, the neurotoxic effects of ketamine have been investigated alone, as well as in combination with other drugs that would likely be administered simultaneously. Diazepam is frequently given with ketamine because it is a positive allosteric modulator of the inhibitory GABA<sub>A</sub> receptor. Diazepam prevented the psychotomimetic effects of ketamine when both were administered to rats, and diazepam blocked 50% of the neurotoxic effects of ketamine (Olney et al., 1991). Four barbiturates (GABA<sub>A</sub> agonists) were also tested and were found to completely protect neurons from the neurotoxic effect of ketamine, which cannot be due to general anesthetic properties because halothane, a nonbarbiturate anesthetic, did not have the same effect (Olney et al., 1991). In addition, ketamine produces dose dependent neurotoxicity in posterior cingulated retrosplenial cortex in rats which was increased with nitrous oxide, but neurotoxic effects were decreased when given with GABA agonist anesthetics (Jevtovic-Todorovic et al., 2000). These studies demonstrate that although ketamine produces neurotoxic effects, co-administration with other drugs that inhibit neuron firing will protect the neurons from toxicity. The use of ketamine as an antidepressant, however, does not currently include co-administration with other drugs; therefore, neurotoxicity is still a concern.
Abuse Potential of Ketamine

Ketamine has been used as a recreational drug since the early 1970s and is still being used today, therefore it may not be practical to be used outside of a hospital setting (Wolff and Winstock, 2006). Recreational ketamine can be administered through many routes, but is typically administrated intranasally. It also has been shown to have abuse potential. Ketamine can lead to physical harm both acutely and chronically, as well as both physical and psychological dependence (Nutt et al., 2007). One study compared the residual effects of ketamine in frequent and infrequent ketamine users and determined that frequent ketamine users had lasting memory impairments three days after an acute dose of ketamine (Curran and Monaghan, 2002). Although it is used as a recreational drug and has abuse potential, it still does have some medical uses. Ketamine is used as analgesia in young patients, as a sedative agent for burn victims and for treatment of chronic pain (Aroni et al., 2009). Studies are underway to reevaluate the different medical uses for ketamine, as it has favourable respiratory and hemodynamic properties. Although there is evidence for abuse potential with ketamine use, the advantages and disadvantages of the drug must be weighed to determine if the beneficial effects of the drug outweigh the risks.

2.8 Therapeutic Profile of Other NMDA Receptor Antagonists

NMDA Receptor Antagonists

As previously mentioned, ketamine is currently being investigated as an antidepressant in clinical trials; however, there are setbacks including psychotomimetic effects and abuse potential. In addition, the long-term effects of ketamine on the brain remain unknown. Ketamine binds to the PCP site within the ion channel, as does MK-801, another non-competitive NMDA receptor antagonist. All three drugs have a similar mechanism of action, however, their pharmacological properties are different and could therefore lead to differential effects on spine density, which is true for other NMDA receptor antagonists as well. Though these drugs are not being used for treatment of MDD, their pharmacology and their effect on spine density may offer insight into the mechanism of ketamine and how it affects spine density.
I. Comparing the pharmacology of PCP, ketamine and MK-801

Phencycline (PCP), ketamine and MK-801 are all non-competitive NMDA receptor antagonists. They act by binding to the same specific site within the receptor’s ion channel complex inhibiting the flow of sodium, calcium, and potassium ions through the channel. Due to the binding site’s location within the channel, binding of PCP, ketamine and MK-801 requires that the cell membrane is depolarized and that the receptor is in the open/active position. Although all three drugs share these properties, they also have pharmacological differences. MK-801 is the most potent (approximately 16 times more potent than PCP) and ketamine is the least potent (Sircar et al., 1987). MK-801 also has the shortest half-life of about 2 hours, while ketamine has a half-life of 2-3 hours (Clements et al., 1982, Vezzani et al., 1989). PCP has a significantly longer half-life of anywhere from 7 to 51 hours (Busto et al., 1989). In addition to differences in half-lives, these drugs have varying off rates. MK-801 has the slowest off-rate, followed by PCP, while ketamine has the fastest off-rate (MacDonald et al., 1991). MK-801 has higher specificity than PCP or ketamine, with few off target effects. Due to these differences in pharmacological properties, these three drugs may have differential affects on behaviour, spine density and other properties of the brain.

Table 1. Pharmacological profiles of non-competitive NMDA receptor antagonists

<table>
<thead>
<tr>
<th>Specificity</th>
<th>PCP</th>
<th>Ketamine</th>
<th>MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>NMDA receptor</td>
<td>NMDA receptor</td>
<td>NMDA receptor</td>
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<tr>
<td></td>
<td>NE/DA/5-HT reuptake</td>
<td>Opioid receptor</td>
<td>Opioid receptor</td>
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<td></td>
<td>α receptor</td>
<td>Cholnergic receptor</td>
<td>Cholnergic receptor</td>
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<tr>
<td></td>
<td>Na⁺ and Ca⁺² channels</td>
<td>Na⁺ and Ca⁺² channels</td>
<td>Na⁺ and Ca⁺² channels</td>
</tr>
<tr>
<td></td>
<td>NE/DA/5-HT reuptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-life</td>
<td>7-51 hours</td>
<td>2-3 hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>45-80 μM</td>
<td>0.1 μM</td>
<td>2-15nM</td>
</tr>
<tr>
<td>Off-rate</td>
<td>0.03 s⁻¹</td>
<td>0.2 s⁻¹</td>
<td>0.003 s⁻¹</td>
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</table>

Effect of NMDA Receptor Antagonists on Animal Models of MDD

Many different NMDA receptor antagonists have been investigated using animal models and the results have shown mostly positive outcomes, suggesting that NMDA receptors and the glutamate system may be a good therapeutic target. Inescapable stress, a method used to induce depressive symptoms in animals as a model of depression, leads to impairments in long-term potentiation in the hippocampus, a brain region containing a high number of NMDA receptors (Monaghan et al., 1984, Shors et al., 1989). One group therefore hypothesized that NMDA receptors may be contributing to these depressive behaviours (Trullas and Skolnick, 1990). This study aimed to show the effect of three different NMDA receptor modulators, a competitive antagonist (2-amino-7-phosphonoheptanoic acid (AP-7)), a non-competitive antagonist (dizocilpine (MK-801)) and a partial agonist (1-aminocyclopropanecarboxylic acid (ACPC)), on depressive behaviours. This study demonstrated for the first time that AP-7, MK-801 and ACPC all reduced immobility in a forced swim test compared to controls and ACPC reduced immobility in the tail suspension test, indicating that the glutamate system is playing a role in these behaviours (Trullas and Skolnick, 1990).

Following the previous study, another group demonstrated that MK-801 (0.3 mg/kg) and CGP 37849 (5 mg/kg i.p. and 25 mg/kg p.o.), a competitive NMDA receptor antagonist, gradually reversed deficits observed in the sucrose consumption test (Papp and Moryl, 1994). Rats were trained to consume sucrose then split into two groups with one group subjected to chronic mild stress. The effect and time course of these drugs were similar to that of imipramine (10 mg/kg i.p. or p.o.). The same group reported a similar study where they tested two different glycine receptor partial agonists, ACPC and D-cycloserine (Papp and Moryl, 1996). ACPC led to a dose dependent increase in sucrose intake, which supports the antidepressant properties it showed in the FST and TST mentioned previously (Trullas and Skolnick, 1990). D-cycloserine, however, showed a more modest increase in sucrose consumption at a single dose, but was not as effective as ACPC or imipramine (Papp and Moryl, 1996).

A study by the Duman laboratory, which was previously mentioned, investigated the effect of Ro25-6981, a selective NR2B subunit antagonist (Li et al., 2010). This drug showed similar effects to those of ketamine and led to a transient increase in mTOR signaling, as well as a
significant increase in synaptic proteins. Furthermore, it led to improvements in the FST and NSFT, which were blocked with rapamycin (Li et al., 2010).

Another study further explored the brain regions that may be contributing to these depressive behaviours. Rats were treated either with an i.p. injection or an intrahippocampal injection of ACPC, an NMDA receptor partial agonist, or CGP 37849, a competitive NMDA receptor antagonist (Przegaliński et al., 1997). Both sites of injection led to a significant and dose dependent decrease in immobility time in the forced swim test, indicating that the hippocampus is one brain region contributing to the manifestation of depressive symptoms in these animals. Other studies conducted used other NMDA receptor antagonists or modulators of the glutamatergic system to demonstrate antidepressant properties of these drugs using the FST, as well as other behavioural assays (Layer et al., 1995, Malkesman et al., 2011). In general, these studies demonstrate that with several different drugs altering NMDA receptors, there is a similar pattern of beneficial antidepressant effects that is comparable to a traditional antidepressant.

Clinical Studies of Glutamate Modulating Drugs

I. Glutamate modulating drugs

The discovery that the glutamatergic system may play a role in depression led to several clinical trials using various pharmacological agents to specifically target the glutamatergic system, mainly through the NMDA receptor. The main concerns with the use of ketamine as a therapeutic agent are the psychotomimetic effects of the drug and its abuse potential (Berman et al., 2000, Wolff and Winstock, 2006). Researchers have therefore begun to consider other drugs that modulate the glutamatergic neurotransmitter system (Zarate et al., 2004, Carlos A. Zarate, 2006, Ferguson and Shingleton, 2007, Sanacora et al., 2007).

Riluzole, a drug approved for amyotrophic lateral sclerosis, blocks voltage-dependent Na+ channels and affects the ability of neurons to effectively release glutamate (Prakriya and Mennerick, 2000, Farber et al., 2002). Treatment resistant patients received an increasing dose of riluzole for an average of 5.4 weeks, with most patients completing the 6-week trial (Zarate et al., 2004). All patients who completed the trial met response and remission criteria at week 6 (Zarate et al., 2004). This study demonstrated that a drug altering the glutamatergic system can lead to antidepressant effects in treatment resistant patients and that a larger study should be
conducted to confirm these results. Further studies were conducted to determine if using riluzole as an add-on treatment following a single ketamine infusion would delay the time to relapse. Two studies demonstrated that patients receiving daily riluzole treatments following a single infusion of ketamine did not have a significant delay in the time to relapse, therefore, this combination therapy is not effective at prolonging the antidepressant effects of ketamine (Mathew et al., 2010, Ibrahim et al., 2012a).

Another glutamate modulating drug currently being assessed for use as an antidepressant is memantine, a low affinity noncompetitive NMDA receptor antagonist. It is currently used for treatment of Parkinson’s and Alzheimer’s disease and is well tolerated with few side effects (Parsons et al., 1999, Szakacs et al., 2012). Memantine does not cause the psychotomimetic effects that are seen with ketamine treatment (Parsons et al., 1999). A double-blind placebo controlled study of memantine demonstrated no significant difference over placebo over an 8-week treatment period; however, a later, open-label study of fewer patients demonstrated a significant antidepressant effect of the drug compared to baseline over a 12-week period (Carlos A. Zarate, 2006, Ferguson and Shingleton, 2007). The efficacy of memantine for treatment of depressive symptoms is therefore unclear and a larger study should be conducted.

Clinical trial data examining the antidepressant effects of drugs, other than ketamine, targeting the glutamatergic neurotransmitter system have presented mixed results with some drugs demonstrating more promise than others. Riluzole has proven to have antidepressant effects in a small study, whereas memantine has shown mixed results. These clinical trial results, as well as those of ketamine, establish that the glutamatergic system should be considered a potential therapeutic target and larger studies need to be conducted.

II. NR2B Subunit Antagonists

Ketamine has shown promising results as an antidepressant in clinical trials however, it has several limitations; at antidepressant doses, ketamine has been shown to have psychotomimetic effects and it has the potential for abuse. Other NMDA receptor antagonists have been studied to find a drug with similar, rapid therapeutic benefit, but without the psychotomimetic properties of ketamine. NR2B subunit antagonists are being considered due to the fact that they act directly on the NMDA receptor, like ketamine, and have displayed promising results in animal models of depression.
The first proof of concept clinical trial conducted on an NR2B subunit selective NMDA receptor antagonist examined the effect of the drug CP-101,606 on treatment resistant MDD patients (Preskorn et al., 2008). Patients were treated with paroxetine for the duration of the trial and were given a single infusion of either placebo (15 patients) or CP-101,606 (15 patients). Of the 15 patients who received the drug, 60% met response criteria by 5 days and 33% met remission criteria on day 5. Although the onset of therapeutic effects was not as rapid as ketamine, an onset of 5 days is an improvement from current antidepressants (Vieira et al., 2010).

Another NR2B subunit selective NMDA receptor antagonist study aimed to administer the drug, MK-0657, orally, which would make it a much more feasible and promising antidepressant candidate (Ibrahim et al., 2012b). This study was very small consisting of only 5 patients who completed the double blind, placebo controlled crossover trial. It found that oral administration of MK-0657 led to improvement of depressive symptoms on 2 of 3 scales that were used. This drug did not cause any dissociative or psychotomimetic effects. Due to the limitations in number of patients, this drug requires further testing to determine its antidepressant effects (Ibrahim et al., 2012b).

These clinical trials, although preliminary, are encouraging in that they have demonstrated that some drugs can lead to a more rapid antidepressant effect than current treatment. They demonstrate that further clinical trials need to be conducted to determine the efficacy of these drugs in a larger population. These studies establish that the glutamatergic neurotransmitter system, NMDA receptors in particular, are a valuable target and should be further explored as a potential therapeutic strategy.

3 Summary of Hypotheses

The summary of current literature provides evidence that the glutamatergic system plays a role in the manifestation of depressive symptoms. Current antidepressant treatment is effective at treating MDD patients, however the delay of onset of these drugs is a significant issue. Also, treatment resistance poses a major problem in the treatment of MDD. Ketamine has shown promising results in clinical trials. It has demonstrated a rapid onset of action and sustained therapeutic benefit after a single drug treatment; however, the potential adverse effects of the drug may hinder its clinical use. The use of other drugs targeting the glutamatergic system has been effective in animal models of MDD and further human studies are needed to determine
efficacy in MDD treatment. Evidence has shown that the glutamatergic system clearly plays a role in the development and manifestation of MDD symptoms and should be explored further.

The effects of repeated ketamine administration on the brain have not been extensively studied and previous work in our laboratory has demonstrated that subchronic MK-801 treatment leads to a significant reduction in spine density in the striatum. My thesis research has been to investigate the effects of ketamine on spine density in different brain regions and determine if ketamine and MK-801 have similar effects of synaptogenesis. We have determined the dose-dependent effects of acute and chronic NMDA receptor antagonism on spine density in the cortex and striatum.

Hypothesis

We hypothesize that acute and chronic ketamine treatment will have differential affects on spine density and that while acute ketamine treatment may increase spine density, we predict that repeated drug treatment will result in a reduction in spine density. Furthermore, we hypothesize that MK-801 will act in a similar manner to ketamine with respect to spine density. In addition, we hypothesize that genetic NMDA receptor hypofunction will lead to reductions in cortical spine density.
Chapter 2
Materials and Methods

1 Animals

1.1 Drug treated Wild Type Mice

Mice were obtained from Charles River. Male C57Bl/6 mice aged 11-13 weeks were used in all experiments. All mice were housed in a 12-hour light/dark cycle in accordance with University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee and Canadian Council on Animal Care.

1.2 NR1-KD Mice

The generation of the NR1-KD mouse line is described in Mohn et al 1999. Briefly, a hypomorphic mutation of the Grin1 gene was achieved by homologous recombination in embryonic stem cells (E14Tg2a cell line). The targeting construct inserted the neomycin selection cassette into intron 17 of Grin1, and the polyadenylation sequence of the neomycin cassette produced premature truncation of the Grin1 transcript, thus reducing mature mRNA levels to 10% of normal levels. The mutation has been maintained on two genetic backgrounds, C57Bl/6J and 129X1Sv/J. Previous efforts have determined that the homozygous mutation causes 80% lethality on the C57Bl/6 background, and 100% lethality on the DBA/2J background. Therefore, 129/B6 F1 animals are generated to maintain NR1-KD homozygous mice on a defined genetic background. Male and female NR1-KD mice aged 3, 6, 12 and 24 weeks were used in spine density experiments. All mice were housed in a 12-hour light/dark cycle in accordance with University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee and Canadian Council on Animal Care.

2 Drug Treatment

2.1 Acute Drug Administration

MK-801 was obtained from Sigma-Aldrich and ketamine hydrochloride was obtained from Toronto Research Chemicals. MK-801 and ketamine solutions were prepared fresh daily by
dissolving the drug in phosphate buffered saline solution (PBS) to attain the desired concentration. Mice were given a single dose of MK-801 (0.2 mg/kg), ketamine (10 mg/kg or 20 mg/kg) or saline solution by intraperitoneal injection.

2.2 Subchronic Drug Administration

A single dose of MK-801 (0.2 mg/kg), ketamine (10 mg/kg or 20 mg/kg) or saline solution was administered by intraperitoneal injection daily for 7 days. A stock solution of MK-801 was prepared and stored for one week; this solution was used for each injection of the subchronic treatment. A stock solution of ketamine was prepared; this solution was used for two daily injections and a fresh solution was thus prepared every other day.

3 Locomotor Activity Assay

Locomotor activity was measured using digital activity monitors (Accuscan Instruments). Animals were first injected with MK-801, ketamine or saline solution and immediately placed in a 20 cm x 20 cm x 45 cm plexiglass box. Locomotor activity was monitored and recorded for 90 minutes and total distance travelled (in cm) was recorded in 5-minute intervals. The assay was performed during the light cycle between the hours of 10am and 5pm. Total distance travelled data was analyzed using Microsoft Excel and GraphPad Prism software.

4 Tissue Preparation and Staining

Twenty-four hours following the final drug injection, the mice were anesthetized with 25 mg/kg Avertin and subsequently transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed and post-fixed for 1 hour in PFA. The brains were kept in PBS at 4°C overnight. The following day, the brains were sliced in 150 μm sagittal sections using a Leica VT-1200 vibratome. Sagittal slices were taken starting at the midline and were from the area corresponding to 0 to 1.5 mm from Bregma. Ten slices were collected per brain sample, four of which were stained for medium spiny neurons in the striatum and the remaining six were stained for layer V pyramidal neurons in the cortex.
4.1 Diolistic labeling of fixed brain sections

Sagittal sections were stained by diolistic labeling, in which a lipophilic dye stains neuron plasma membranes and is delivered with a biolistic device (Gan et al., 2000). Neurons were stained using tungsten particles coated with the lipophilic dye 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodide (DiI), which was administered using the Helios BioRad Gene Gun (Gan et al., 2000, Ramsey et al., 2011). The slices that were used for imaging of medium spiny neurons were wrapped in foil to prevent photobleaching and were stored in PBS overnight at 4°C. The slices that were used to image cortical neurons were processed for immunofluorescence as described in section 4.2.

4.2 Immunofluorescent staining of cortical neurons

Cortical slices were first stained by diolistic labeling as described above. In order to selectively label layer V pyramidal neurons, the slices were first incubated in blocking buffer (3% bovine serum albumin (BSA) in Tris-buffered saline and Tween 20 (TBST)) for 30 minutes at room temperature. The slices were then incubated with Neurofilament 200 (N200) primary antibody (1:200 in 3% BSA; Sigma Aldrich) overnight at 4°C (Law and Harrison, 2003). The following day, the slices were washed three times in PBS (15 minutes per wash), then incubated in goat anti-mouse secondary antibody conjugated to Alexa 488 (1:1000 in 3% BSA; Invitrogen) for one hour at room temperature.

Figure 3. Representative image of double labeling of cortical layer V pyramidal neurons with DiI (red) and Neurofilament 200 antibody (green).
5 Dendritic Spine Imaging and Analysis

5.1 Imaging

Brain slices were mounted onto slides with PBS, covered with cover slips and imaged using confocal microscopy. Neurons were imaged using Olympus Fluoview FV 1000 software with IX81 microscope under the 60X water objective lens. Z-stack images of dendritic spines were collected. Dendrites were selected based on the location and morphology of the neuron, as well as the quality of the image and staining. Layer V pyramidal neurons were located by first identifying layer V of the cortex using the N200 antibody staining, then using the DiI labeling to find dendrites that were sufficiently stained. Spine density of layer V pyramidal neurons was determined on secondary basilar dendrites that were a minimum of 50 microns from the cell body. Spine density measurements of medium spiny neurons were taken from secondary dendrites that were a minimum of 50 microns from the cell body. For each treatment group or genotype, multiple images (3-6) of individual neurons were obtained from each animal. Spine density determinations for a single data point were based on the averaged spine density of all neurons from a single animal, and the (n) for a treatment group or genotype represents the number of animals studied.

Figure 4. Representative image of a striatal medium spiny neuron. The small boxes represent the types and sections of dendrites that were selected and imaged.

Figure 5. Representative image of a cortical layer V pyramidal neuron. The large box signifies the area from which dendrites were selected (secondary basilar dendrites) and the smaller box is representative of which section of the dendrite was imaged.
5.2 Analysis

Spine images were analyzed using Nikon NIS Elements software. The length of the dendrite was measured and spines along that length were counted to generate the number of spines/100 microns. Spine density reflects the number of all spine types counted together (mushroom, thin and stubby spines). For each animal, spine density measurements were averaged using Microsoft Excel, and the data collected from all animals in a group were analyzed and graphed using Microsoft Excel and GraphPad Prism. Two-tailed student t tests were performed with GraphPad Prism to determine statistical significance.
Ketamine and MK-801 are two non-competitive NMDA receptor antagonists that act on the same binding site within the NMDA receptor ion channel. Both drugs have been shown to have varying effects on spine density. The Duman laboratory has demonstrated that an acute, low dose of ketamine (10 mg/kg) leads to an increase in spine density in the prefrontal cortex of rats. Conversely, our laboratory has demonstrated that subchronic MK-801 treatment (0.2 mg/kg) leads to reduced spine density in the striatum of mice (Li et al., 2010, Ramsey et al., 2011). We therefore investigated the effects of acute and chronic ketamine and MK-801 on spine density in the cortex and striatum. The dose of 10 mg/kg ketamine was chosen to replicate the study performed by Li et al. in rats and a higher dose of 20 mg/kg was used to determine whether the beneficial effects of ketamine were limited to low dose. The dose of 0.2 mg/kg MK-801 was chosen to replicate previous studies in our laboratory. Notably, doses of 0.2 mg/kg MK-801 and 20 mg/kg ketamine are used to elicit similar behavioural outcomes such as increased motor activity and cognitive impairments (Gilmour et al., 2009). Spine density was investigated in the cortex to replicate the study performed by Li et al. in rats. In addition, spine density has been shown to be reduced in the cortex in a postmortem studies and has been implicated in depressive behaviours (Kang et al., 2012). Spine density was examined in the striatum to replicate previous spine density experiments from our laboratory. The striatum has also been shown to have changes in connectivity in depressed patients (Marchand et al., 2012).

This study aims to reconcile the differences of the effects on spine density between these two NMDA receptor antagonists. In addition, we wanted to determine whether the effect of pharmacological NMDA receptor antagonism had similar effects on spine density as genetic down-regulation of the NMDA receptor. Therefore, another goal of this study was to use the NR1-KD mice to compare the effect of genetic NMDA receptor hypofunction and pharmacological NMDA receptor antagonism on spine density.
1 NMDA receptor antagonism and locomotor activity

Evidence has shown that some NMDA receptor antagonists lead to an increase in locomotor activity. Ketamine causes a modest and brief increase in locomotor activity (Gilmour et al., 2009). Studies have also shown that MK-801 leads to increased locomotor activity (Tricklebank et al., 1989, Ramsey et al., 2011). Therefore, a locomotor activity assay was performed immediately following drug administration to ensure proper dosing. For chronic drug treatment, the locomotor activity assay was performed immediately following drug administration on day 7. Total distance travelled was measured every 5 minutes for 90 minutes. As shown in Figure 6, hyperactivity was observed in mice treated with an acute dose (saline 1644 ± 218.2 cm, n=9 and MK-801 7166 ± 1407 cm, n=9) and chronic dose (saline 2563 ± 293.5 cm, n=8 and MK-801 7940 ± 1108 cm, n=8) of 0.2 mg/kg MK-801, while acute (saline 1996 ± 245.9 cm, n=10, ketamine 10 mg/kg 1473 ± 186 cm, n=9 and ketamine 20 mg/kg 2326 ± 330 cm, n=6) and chronic (saline 1996 ± 272.2 cm, n=6, ketamine 10 mg/kg 2873 ± 337.8 cm, n=6 and ketamine 20 mg/kg 2786 ± 642.9, n=5) ketamine failed to demonstrate an effect on locomotor activity.
Combined results of acute and subchronic treatment demonstrate that acute and subchronic MK-801 treatment leads to a significant increase in locomotor activity, while ketamine had a modest but statistically insignificant effect on locomotor activity. For each experiment, a two-tailed student t-test was performed (± SEM), **p=0.0013 and ***p=0.0003.
2 NMDA receptor antagonism and spine density

Ketamine and MK-801 bind to the same site within the ion channel of the NMDA receptor, but have varying pharmacological properties and therefore may have opposing effects on spine density. We selectively stained medium spiny neurons in the striatum and layer V pyramidal neurons in the cortex following acute and chronic drug treatments to determine the effects of these non-competitive NMDA receptor antagonists on dendritic spine density.

2.1 Effect of ketamine administration on spine density

The effects of ketamine on spine density have not been studied at length. The Duman laboratory has demonstrated that a single, low dose of ketamine leads to an increase in dendritic spine density in the cortex of adult rats. However, the effects of ketamine on other brain regions have not been explored. In addition, the effect of long-term ketamine treatment on spine density is unknown, which is a significant problem if the treatment regimen for ketamine in depressed patients entails repeated treatments.

I. Acute ketamine treatment

Acute, low dose ketamine (10 mg/kg, i.p.) in rats was reported to increase spine density when measured 24 hours post-injection (Li et al 2010). In this study, wild type mice were treated with an acute dose of either 10 or 20 mg/kg ketamine and dendritic spine density was measured in the cortex and striatum 24 hours post-injection. As seen in Figure 7, a single, low dose of ketamine led to an increase in spine density in layer V pyramidal neurons of the cortex in mice (saline 105.1 ± 2.707 spines/100 microns, n=7, 10 mg/kg ketamine 125.8 ± 8.634 spines/100 microns, n=6, p=0.0321). Ketamine’s effect at this dose was similar to what was reported in rats by Li et al (2010). However, ketamine did not increase spine density in the cortex when mice were treated with a higher dose of 20 mg/kg ketamine (Saline 105.1 ± 2.707 spines/100 microns, n=7, 20 mg/kg Ketamine 108.2 ± 9.033 spines/100 microns, n=4). In addition, as seen in Figure 8, there is no change in spine density in the striatum following acute ketamine treatment at either of the doses administered (Saline 122.1 ± 5.620 spines/100 microns, n=8, Ketamine 10 mg/kg 125.1 ± 5.235 spines/100 microns, n=7, Ketamine 20 mg/kg 114.2 ± 4.296 spines/100 microns, n=4).
Figure 7. Dose-dependent effect of acute ketamine on cortical spine density. A. Representative images of dendrites from layer V pyramidal neurons of each group. Scale bar: 10 µm. B. Grouped data demonstrate that acute, low dose ketamine administration (10 mg/kg) leads to a significant increase in spine density in the cortex, while a higher dose (20 mg/kg) has no effect. For the saline group, n=7 animals, for ketamine 10 mg/kg, n=6 animals and for ketamine 20 mg/kg, n=4. For each dose of ketamine, a two-tailed student t-test was performed comparing drug treatment to saline (± SEM), *p=0.0321.
Figure 8. Effect of acute ketamine administration on striatal spine density. A. Representative images of dendrites from medium spiny neurons of each group. Scale bar: 10 µm. B. Grouped data demonstrate that acute ketamine administration (10 and 20 mg/kg) had no significant effect on spine density of medium spiny neurons in the striatum. For the saline group, n=9 animals, for ketamine 10 mg/kg, n=8 animals and for ketamine 20 mg/kg, n=5. For each dose of ketamine, a two-tailed student t-test was performed comparing drug treatment to saline (± SEM).
II. Subchronic ketamine treatment

The effect of subchronic ketamine treatment on spine density has yet to be explored, but may be very important if drug regimens require repeated dosing. Therefore, we treated mice once daily for 7 days with an i.p. injection of 10 mg/kg ketamine, 20 mg/kg ketamine, or saline. Repeated 20 mg/kg ketamine treatment led to a significant decrease in spine density in layer V pyramidal neurons in the cortex (Figure 9; saline 114.3 ± 3.821 spines/100 microns, n=6, Ketamine 10 mg/kg 102.9 ± 6.359 spines/100 microns, n=6, Ketamine 10mg/kg 98.39 ± 4.657 spines/100 microns, n=5, *p=0.0254). Interestingly, the effect of increased spine density seen with acute treatment of 10 mg/kg ketamine was absent with repeated doses, as we detected no change in spine density with subchronic administration (Figure 9). Subchronic ketamine treatment at either dose did not significantly change spine density in the striatum (Figure 10; saline 132.3 ± 7.878 spines/100 microns, n=6, Ketamine 10 mg/kg 115.5 ± 7.041 spines/100 microns, n=5, Ketamine 20 mg/kg 121.2 ± 3.312 spines/100 microns, n=4).
Figure 9. Dose-dependent effect of subchronic ketamine on cortical spine density. A. Representative images of secondary basilar dendrites from each treatment group. Scale bar: 10 µm. B. Combined results of subchronic treatments demonstrate that a subchronic 20 mg/kg dose of ketamine leads to a significant reduction in dendritic spine density in layer V pyramidal neurons of the cortex, while a subchronic lower dose has no effect on spine density. For saline and 10 mg/kg ketamine groups, n=6 animals and for 20 mg/kg ketamine group, n=5 animals. For each dose of ketamine, a two-tailed student t-test was performed comparing drug treatment to saline (± SEM), *p=0.0254.
Figure 10. Effect of subchronic ketamine on striatal spine density. A. Representative images of dendrites from each treatment group. Scale bar: 10 μm. B. Combined results of subchronic treatments demonstrate that subchronic 10 mg/kg and 20 mg/kg doses of ketamine have no significant effect on dendritic spine density in medium spiny neurons of the striatum. For the saline group n=6 animals, for the 10 mg/kg ketamine group, n=5 animals and for the 20 mg/kg ketamine group, n=4 animals. For each dose of ketamine, a two-tailed student t-test was performed comparing drug treatment to saline (± SEM).
2.2 Effect of MK-801 administration on spine density

MK-801 acts at the same binding site within the NMDA receptor as ketamine, but MK-801 has different pharmacological properties; therefore, we sought to determine whether these drugs have the same effect on dendritic spine density. Our laboratory has previously shown that subchronic MK-801 treatment led to a significant reduction in spine density in the striatum; however the acute effects of MK-801 on spine density are unknown (Ramsey et al., 2011). We wanted to determine if this effect of reduced spine density would be evident even with acute treatment and if it was specific to a certain brain region.

I. Acute MK-801 Treatment

Mice were treated with a single injection of 0.2 mg/kg MK-801 and spine density was measured 24 hours post-injection. We determined that acute MK-801 led to a significant reduction in spine density in the striatum (Figure 12; saline 127.5 ± 3.127 spines/100 microns, n=4, MK-801 114.5 ± 2.302 spines/100 microns, n=4, p=0.0157). However, MK-801 had no significant effect on spine density in layer V pyramidal neurons of the cortex (Figure 11; Saline 98.83 ± 6.296 spines/100 microns, n=4, MK-801 99.57 ± 4.703 spines/100 microns, n=5).

II. Subchronic MK-801 Treatment

Our laboratory had previously demonstrated that mice treated subchronically with MK-801 for 7 days had a reduction in dendritic spine density in the striatum (Ramsey et al 2011). That previous study was conducted using osmotic mini-pumps to administer the drug. However, once daily injections were performed in my thesis work to determine whether intermittent NMDA receptor antagonism would have the same effect. Mice were treated for 7 days with an injection of 0.2 mg/kg MK-801. Subchronic MK-801 treatment led to a trend toward a reduction in dendritic spine density in medium spiny neurons in the striatum (Figure 14; saline 134.8 ± 5.337 spines/100 microns, n=7, MK-801 118.8 ± 5.648 spines/100 microns, n=6, p=0.0647). Subchronic MK-801 treatment led to a significant reduction in spine density in the cortex (Figure 13; saline 106.3 ± 5.236 spines/100 microns, n=7, MK-801 92.04 ± 7.007 spines/100 microns, n=6).
Figure 11. Effect of acute MK-801 administration on cortical spine density. A. Representative images of secondary basilar dendrites from each treatment group. Scale bar: 10 μm. B. Combined results of acute treatments demonstrate that an acute dose of 0.2 mg/kg MK-801 had no significant effect on dendritic spine density in layer V pyramidal neurons of the cortex. For saline groups, n=4 animals and for the MK-801 group, n=5 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM).
Figure 12. Effect of acute MK-801 on striatal spine density. A. Representative images of dendrites from each treatment group. Scale bar: 10 µm. B. Combined results of acute treatments demonstrate that an acute 0.2 mg/kg dose of MK-801 leads to a significant reduction in dendritic spine density in medium spiny neurons of the striatum. For both groups, n=4 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), *p=0.0157.
Figure 13. Effect of subchronic MK-801 on cortical spine density. A. Representative images of secondary basilar dendrites from each treatment group. Scale bar: 10 µm. B. Combined results of subchronic treatments demonstrates that a subchronic 0.2 mg/kg dose of MK-801 leads to a significant reduction in dendritic spine density in layer V pyramidal neurons of the cortex. For the saline group, n=8 animals and for the MK-801 group, n=7 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), *p=0.0417.
Figure 14. Effect of subchronic MK-801 on striatal spine density. A. Representative images of dendrites from each treatment group. Scale bar: 10 µm. B. Combined results of subchronic treatments demonstrate that a subchronic 0.2 mg/kg dose of MK-801 shows a trend toward a reduction in spine density of medium spiny neurons. For the saline group, n=8 animals and for the MK-801 group, n=7 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), p= 0.0693.

3 Genetic NMDA receptor down-regulation and spine density

Pharmacological and genetic NMDA receptor inhibition may have differential effects on spine density. Pharmacological antagonism is short term and occurs after developmental time periods, whereas genetic NMDA receptor down-regulation is present from birth and can cause compensatory mechanisms to take place, which may affect spine density. Therefore, we explored the effect of NR1 knockdown on spine density in the cortex and compared the results to spine density in the striatum as well as to the effect of pharmacological NMDA receptor antagonism on spine density.

3.1 NR1-KD mice

Using the NR1-KD mice as a genetic model of NMDA receptor hypofunction, we investigated spine density of layer V pyramidal neurons in the cortex of these mice at 3, 6, 12 and 24 weeks of age. We determined that there was no change in cortical spine density at 3 weeks of age (Figure 15; WT 116.2 ± 4.041 spines/100 microns, n=3, NR1-KD 119.4 ± 4.781 spines/100 microns, n=3). We also determined that there was a significant reduction in spine density in these mice at 6 weeks of age (Figure 16; WT 114.4 ± 3.138 spines/100 microns, n=5, NR1-KD 98.10 ± 2.359 spines/100 microns, n=3, p=0.0113) and 12 weeks of age (Figure 17; WT 113.8 ± 5.180 spines/100 microns, n=4, NR1-KD 87.29 ± 2.227 spines/100 microns, n=3, p=0.0090). At 24 weeks of age, there was a trend toward a decrease in spine density in the cortex (Figure 18; WT 107.2 ± 3.720 spines/100 microns, n=3, NR1-KD 89.69 ± 5.697 spines/100 microns, n=4, p=0.0644).
Figure 15. Cortical spine density in NR1-KD mice at 3 weeks of age. A. Representative images of dendrites from each group. Scale bar: 10 µm. B. Combined results demonstrate that NR1-KD mice display no change in dendritic spine density of layer V pyramidal neurons of the cortex at 3 weeks of age. For the WT group, n=3 animals and for the NR1-KD group, n=3 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM).
Figure 16. Cortical spine density in NR1-KD mice at 6 weeks of age. A. Representative images of dendrites from each group. Scale bar: 10 µm. B. Combined results demonstrate that NR1-KD mice display reductions in dendritic spine density of layer V pyramidal neurons of the cortex at 6 weeks of age. For the WT group, n=5 animals and for the NR1-KD group, n=3 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), *p=0.0113.
Figure 17. Cortical spine density in NR1-KD mice at 12 weeks of age. A. Representative images of dendrites from each group. Scale bar: 10 μm. B. Combined results demonstrate that NR1-KD mice display reductions in dendritic spine density of layer V pyramidal neurons of the cortex at 12 weeks of age. For the WT group, n=4 animals and for the NR1-KD group, n=3 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), **p=0.0090.
Figure 18. Cortical spine density in NR1-KD mice at 24 weeks of age. A. Representative images of dendrites from each group. Scale bar: 10 μm. B. Combined results demonstrate that a NR1-KD mice display a trend toward a reduction in dendritic spine density of layer V pyramidal neurons of the cortex at 24 weeks of age. For the WT group, n=3 animals and for the NR1-KD group, n=4 animals. A two-tailed student t-test was performed comparing drug treatment to saline (± SEM), p=0.0644.
## Table 2. Summary of the effects of NMDA receptor antagonism on spine density.

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<th>Regimen</th>
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<th>Effect on Spine density</th>
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Chapter 4
Discussion, Conclusions and
Future Directions

1 Discussion

Major depressive disorder is a debilitating mental illness; however, current treatment can be ineffective in many patients and has a delayed onset of action. Ketamine, a non-competitive NMDA receptor antagonist, is currently being explored as an antidepressant in clinical trials (Connolly and Thase, 2012). The first clinical trial conducted with ketamine as an antidepressant demonstrated that a single 40-minute infusion of ketamine led to rapid and enduring antidepressant effects, while a second clinical trial further confirmed these results with a larger population of treatment resistant patients (Berman et al., 2000, Zarate et al., 2006). Despite the exciting and promising results of these clinical trials, ketamine has several major challenges. Ketamine produces psychotomimetic effects even at the low doses used in these clinical trials and it can also have abuse potential. Furthermore, the long-term effects of ketamine on different brain regions remain unknown.

Although the long-term effects of ketamine on the brain are unknown, several studies have begun to investigate the effect of NMDA receptor antagonism on dendritic spines. A study by the Duman laboratory demonstrated that an acute, low dose of ketamine led to an increase in spine density in layer V pyramidal neurons of the rat cortex (Li et al., 2010). Conversely, our laboratory reported that subchronic MK-801 treatment led to a significant decrease in spine density medium spiny neurons of the mouse striatum (Ramsey et al., 2011). In addition, that study showed that, in a genetic model of NMDA receptor hypofunction, mutant mice have a significant reduction in spine density in the striatum at 6, 12 and 24 weeks of age (Ramsey et al., 2011) (Unpublished). Thus, there were conflicting reports about the effect of NMDA receptor antagonism or hypofunction on spine density in general. Due to the conflicting nature of these results, we aimed to reconcile these differences by performing systematic experiments that would directly compare drug and dosing regimens in the same species and in the same neuron types. Furthermore, we wanted to determine whether there were differential effects on spine
density with pharmacological or genetic intervention. We investigated the effect of acute and subchronic ketamine (10 and 20 mg/kg) and MK-801 (0.2 mg/kg) on spine density in both layer V pyramidal neurons of the cortex and medium spiny neurons of the striatum. Since previous studies had already been performed on striatal neurons of NR1-KD mice, we investigated the effect of genetic NMDAR hypofunction on layer V cortical neurons to complete these studies.

This research demonstrates that, in agreement with the Duman study, an acute, low dose of ketamine leads to an increase in dendritic spine density in layer V pyramidal neurons of the cortex. However, we found that with a higher dose of ketamine these effects are no longer observed. Our results establish that although ketamine may have a beneficial effect on spine density at a lower dose, these effects are dose-dependent. An increase in dose can eliminate the observed increase in spine density. Since the increase in spine density has been correlated with therapeutic benefit, this has important implications for determining the therapeutic window of ketamine for depression. This study has shown that ketamine has dose dependent effects on spine density. Ketamine has also been shown to have dose dependent effects on behaviour in humans and in animal models. Low doses of ketamine have shown antidepressant effects while higher doses can lead to ataxia and anesthesia (Irifune et al., 1992, Li et al., 2010).

In the Duman study, several doses were tested to determine their effect on the activation of the mTOR signaling pathway. Western blots were used to determine the phosphorylation state of mTOR and its downstream proteins following administration of 1, 5, 10 and 80 mg/kg ketamine (Li et al., 2010). In this study, it was established that the mTOR pathway was activated with 5 and 10 mg/kg ketamine, but not at either lower or higher doses. This data indicates that with increasing doses of ketamine there is a point where the mTOR pathway is no longer activated; however it is unclear at which dose this occurs. It is possible that at 20 mg/kg there is no longer activation of the mTOR signaling pathway and this could explain the lack of increased spine formation at this dose. Further studies will be needed to determine the dose response effect of ketamine on mTOR pathway activation in different brain regions.

The dose dependent effect on spine density can have major consequences in clinical practice as doses are often altered depending on the patient. If the increase in spine density is an indication of its effectiveness as an antidepressant, ketamine will have a very narrow dose range as an antidepressant. In addition, if the administered dose of ketamine is increased, it may lead to
detrimental effects on dendritic spines in different brain regions. Loss of dendritic spines could have a detrimental impact on depressive symptoms and could affect learning and memory, as spine formation has been shown to be important for these functions (Nimchinsky et al., 2002).

Acute ketamine treatment was also proven to have a brain region specific effect on spine density. An acute, low dose of ketamine led to an increase in spine density in layer V pyramidal neurons in the cortex, but the same dose had no effect on spine density in the striatum. Furthermore, while a higher dose of ketamine had no effect in the cortex, there is a trend toward reduced spine density in the striatum. It is evident that ketamine has differential effects on spine density in the cortex and striatum.

Different brain regions are composed of unique neuronal populations and therefore drugs can have distinct effects on individual brain regions. The brain region specificity of ketamine could be due to the composition of the NMDA receptors in these brain regions. It is known that subunit composition is dependent on the brain region and different subunits can infer different properties on the receptor (Yamakura and Shimoji, 1999). In addition, it has been reported that specific populations of neurons are more sensitive to the effects of low doses of NMDA receptor antagonists. Inhibitory interneurons are preferentially affected by NMDA receptor antagonists because a greater proportion of their excitatory postsynaptic potential is due to the opening of NMDA channels (Grunze et al., 1996, Lisman et al., 2008). Therefore, neurons that are strongly inhibited by interneurons will be more disinhibited with low levels of NMDA receptor antagonism (Lisman et al., 2008). Cortical pyramidal neurons may be more strongly inhibited by interneurons than striatal medium spiny neurons. This would result in more cortical excitation and spinogenesis with low levels of NMDA receptor antagonists (Moghaddam et al., 1997, Jackson et al., 2004). This brain region specificity of ketamine could be a beneficial property of the drug as an antidepressant. Further studies are needed to determine the effects on spine density in different brain regions and if these regions are implicated in the manifestation of depressive symptoms.

As previously discussed, this research has demonstrated that acute ketamine treatment has both dose-dependent and brain region specific effects on spine density. While chronic ketamine administration also shares these properties, we have shown that subchronic ketamine treatment produced a different outcome than what was observed with a single dose. Low, repeated doses
of ketamine did not have a significant effect on spine density, while, at a higher dose, ketamine leads to a significant reduction in spine density in layer V pyramidal neurons in the cortex. In addition, the effects of subchronic ketamine treatment on spine density are brain region specific, as there was no significant effect on spine density in the striatum.

The reduction in spine density in the cortex with a higher dose of ketamine follows a similar trend of what is seen with subchronic administration of other NMDA receptor antagonists. Chronic pharmacological NMDA receptor antagonism has been shown to lead to a reduction in dendritic spine density in multiple brain regions. One study demonstrated that hippocampal slices treated with D-APV, a competitive NMDA receptor antagonist, led to a 30% decrease in dendritic spine density (Collin et al., 1997). In addition, another study demonstrated that rats treated with CPP, another competitive NMDA receptor antagonist, for 5 consecutive days had significantly reduced spine density in the prefrontal cortex (Velázquez-Zamora et al., 2011). Chronic treatment with phencyclidine (PCP), a noncompetitive NMDA receptor antagonist with the same binding site as ketamine and MK-801, also led to a significant decrease in spine density in layer II/III and layer V pyramidal neurons in the prefrontal cortex (Elsworth et al., 2011). Overall, many studies of pharmacological NMDA receptor antagonism have demonstrated that sustained inhibition of the NMDA receptor consistently leads to a reduction in dendritic spine density in various brain regions. Reductions of even 10% have been correlated with changes in behaviour and electrophysiology hence it is not unexpected that seemingly small changes would lead to changes in therapeutic benefit or cognitive impairment (Von Bohlen Und Halbach et al., 2006, Velázquez-Zamora et al., 2011).

Our study has shown that the effects on spine density are dependent on dose as well as length of exposure to the drug. These are important considerations if these drugs are to be administered with repeated doses, which is likely the most effective scenario as the antidepressant effects of a single infusion subside after two weeks or less.

The principal objective of this study was to reconcile the differences observed in spine density between ketamine and MK-801. Although acute ketamine treatment led to an increase in spine density in the rat cortex, subchronic MK-801 treatment caused a decrease in spine density in the mouse striatum (Li et al., 2010, Ramsey et al., 2011). Therefore, we investigated the effect of acute and subchronic treatment of drugs on spine density in the cortex and striatum. Though we
found that acute ketamine treatment led to a significant increase in spine density in the cortex, in accordance with the Duman study, we found that acute MK-801 administration had no effect on spine density in this brain region. However, we determined that with a single injection of MK-801, there was a significant reduction in spine density in the striatum, where ketamine showed no effect. These acute treatment results indicate that ketamine and MK-801 are differentially affecting spine density and that these drugs function in a brain region specific manner.

Subchronic treatment with both ketamine and MK-801 further supported the results found with acute drug administration. Subchronic ketamine treatment (20 mg/kg) led to a reduction in spine density in the cortex, but had no significant effect in the striatum. On the other hand, subchronic MK-801 administration caused a trend toward a reduction in spine density in the striatum as well as a significant reduction in the cortex. These studies demonstrate that the effects of ketamine and MK-801 are brain region specific and that although both drugs act on the same binding site, they have differential mechanisms of action with respect to spine density.

Though ketamine and MK-801 act at the same site within the ion channel of the NMDA receptor, both drugs have different pharmacological properties that could affect how they act on different neuronal subtypes. MK-801 has been shown to be approximately 200 times more potent than ketamine (Sircar et al., 1987). In addition, MK-801 has a slightly shorter half-life (2 hours) compared to ketamine (2.5 to 3 hours) (Clements and Nimmo, 1981, Vezzani et al., 1989). MK-801 also has a significantly slower off-rate than ketamine, indicating that it occupies the receptor for a longer period of time before dissociating (MacDonald et al., 1991). Therefore, although these drugs bind at the same site within the receptor, their pharmacological properties could lead to their unique effects on spine density in different brain regions.

Pharmacological intervention has proven that NMDA receptor antagonists can have dose dependent and brain region specific effects on spine density. Furthermore, acute and repeated exposure to these drugs can change how they alter spine density. From numerous studies, as well as the one presented here, it appears that chronic inhibition of the NMDA receptor leads to brain region specific reductions in spine density; therefore, this demonstrates that sustained NMDA receptor inhibition would be detrimental to different brain regions depending on the drug. We therefore investigated the effect of genetic NMDA receptor hypofunction on spine
density in the cortex to determine if spine density changes in this brain region coincided with those of the striatum.

NR1-KD mice were used as a genetic model of NMDA receptor hypofunction. Our laboratory has shown that these mice have significant reductions in spine density in the striatum (Ramsey et al., 2011); therefore we aimed to determine the status of spine density in the cortex. We discovered that with genetic NMDA receptor hypofunction there was no change in spine density at 3 weeks of age. However, there were significant reductions in spine density in layer V pyramidal neurons of the cortex at 6 and 12 weeks of age. This demonstrates that NMDA receptor hypofunction leads to a reduction in dendritic spine density in multiple brain regions at 6 and 12 weeks of age, which is supported by previous studies conducted in genetic models of NMDA receptor hypofunction. One such study demonstrated that genetic deletion of the NR1 subunit in the cortex led to a reduction in spine density in layer II/III pyramidal neurons (Ultanir et al., 2007). Another study demonstrated that deletion of the NR2B subunit in CA1 hippocampal neurons resulted in a reduction in spine density within these neurons (Brigman et al., 2010). In addition, removal of the inhibitory NR3 subunit caused an increase in dendritic spine density in the cortex (Das et al., 1998). These genetic studies further confirm that long-term NMDA receptor hypofunction has detrimental effects on spine density in different brain regions.

2 Conclusions

The goal of this thesis was to address and reconcile the noted differences between the effects of acute and subchronic ketamine and MK-801 treatment on spine density in the cortex and striatum. Using diolistic and immunological staining, we were able to image medium spiny neurons in the striatum and layer V pyramidal neurons in the cortex to address our question. We discovered that ketamine and MK-801 have differential effects on spine density and these effects are brain region specific. An acute, low dose of ketamine led to an increase in spine density in the cortex, while an acute dose of MK-801 led to a reduction in spine density in the striatum.

We also determined that subchronic administration of a higher dose of ketamine leads to a reduction in spine density in the cortex as opposed to the increase that was observed with an acute, low dose. In addition, we demonstrated that acute MK-801 causes a decrease in spine
density in the striatum, while subchronic administration leads to a trend toward a decrease in the striatum and a significant reduction in the cortex. These results clearly demonstrate that MK-801 and ketamine have unique effects on spine density and that these effects are brain region specific. The differences between ketamine and MK-801 are likely attributable to their unique pharmacological profiles while the brain region specificity of these drugs may be due to the distinct composition of the NMDA receptor subunits in these neuronal populations.

In this study, we initially addressed pharmacological inhibition of the NMDA receptor; these analyses were complemented by an examination of the effect of genetic NMDA receptor hypofunction on spine density. In our genetic model of NMDA receptor hypofunction, NR1-KD mice displayed a reduction in spine density at 6 and 12 weeks of age. This follows the same pattern as what was seen in the striatum. This data indicates that without sufficient NMDA receptor activation and input there is a deficit in dendritic spines, which affects multiple brain regions.

Overall, this data proves that the effect of NMDA receptor antagonism on spine density is dependent on the drug. The effects of spine density have also been shown to be dose-dependent and brain-region specific, as well as dependent on the dosing regimen. This study proves that although ketamine may initially lead to an increase in spine density in one brain region, repeated higher doses lead to detrimental effects on spine density, which could be problematic in a clinical setting. A slightly higher dose of ketamine does not produce the same beneficial effects on spine density that are seen with a lower dose. In addition, genetic NMDA receptor hypofunction has been shown to reduce spine density in the cortex. These results indicate that we must be cautious in administering NMDA receptor antagonists in humans as they may have detrimental effects, either short or long term, on spine density.

3 Future Directions

The effect of NMDA receptor antagonism on spine density is variable and, as we have shown, is dependent on the specific drug as well as the dose, drug regimen and brain region. Further studies are needed to elaborate on the mechanism of action of these drugs on changing spine density, as well as evaluating other NMDA receptor antagonists that are being tested as antidepressants in clinical trials.
Investigate signaling pathways that may be involved in the effect of NMDA receptor antagonists on spine density.

Previous studies have shown that the mTOR signaling pathway is involved in the antidepressant effect of ketamine in rats (Li et al., 2010). It was demonstrated that an acute, low-dose of ketamine led to an increase in mTOR signaling and synaptic protein synthesis and that pretreatment with rapamycin, an mTOR inhibitor, attenuated the antidepressant effects of ketamine in the FST, LH and NSFT. In addition, pretreatment with rapamycin attenuated the positive effect of ketamine on cortical spine density. To expand the current study, we could investigate the effect of both drugs on mTOR activity in different brain regions following acute and subchronic treatment. We would investigate whether the effects on spine density are mirrored in the effect of these drugs on mTOR signaling. This would indicate whether the mTOR signaling pathway is responsible for the alterations in spine density changes following drug administration.

Examine the effect of other NMDA receptor antagonists, particularly those being investigated in clinical trials, on spine density in different brain regions.

Due to the psychotomimetic effects and the abuse potential of ketamine, other NMDA receptor antagonists are currently being examined as potential treatment options for MDD. Since this study has demonstrated that NMDA receptor antagonists have unique and dose and brain region specific effects on spine density, it is important to investigate the effect of these other drugs on spine density. Preclinical and clinical studies have begun to examine NMDA receptor subunit selective compounds, such as NR2B receptor antagonist CP-101,606, as well as non-competitive NMDA receptor antagonists (Preskorn et al., 2008). If these drugs are to be used in patients, it is essential to understand their long-term impact on the brain. Future studies could examine the effect of these drugs on spine density and on the signaling pathways that are implicated in spinogenesis.

Determine if the effect of ketamine on spine density and as an antidepressant is age-dependent.

Although ketamine has been shown to be psychotomimetic in adults, these effects are not produced when ketamine is administered in children. For this reason, ketamine is still
commonly used as an anesthetic in children and juveniles. This introduces the question of whether or not ketamine would be a suitable antidepressant in children as it has already been shown to lack psychotomimetic effects and has a good safety profile (Schnabel et al., 2011). However, it also raises the question of whether the antidepressant and psychotomimetic effects work through a common pathway and without the unwanted psychotomimetic effects would we still observe the desired antidepressant properties. Therefore, a proposed study would be to administer ketamine at various ages and evaluate the antidepressant effects using behavioural tests, such as FST, LH and NSFT. It would also be important to evaluate spine density in the cortex and striatum at these ages to determine if changes in spine density correlate with the antidepressant effects and if these changes are age dependent.

**Investigate the effect of ketamine and other NMDA receptor antagonists on other brain regions.**

The brain regions studied in this thesis were chosen specifically to compare two previous studies as well as due to their implications in depressive symptoms; however, there are other brain regions that should be investigated. The hippocampus and amygdala are extensively studied in MDD research as they have been highly implicated in MDD. It would be beneficial to determine the effect of these and other NMDA receptor antagonists on spine density in these brain regions. The effect on these areas of the brain could shed some light on where ketamine is exerting its greatest effect and focus on that brain region.
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Copyright Acknowledgements

Abstract:

1. Ruddy, RM, Ramsey, AJ. The effect of acute and chronic NMDA receptor antagonism on dendritic spine density. Poster session presented at: Southern Ontario Neuroscience Association Annual Meeting. 2012 April 30; Toronto, ON.