The interactive effects of ketamine and magnesium upon depressive-like pathology

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Abstract

Approximately one third of patients with major depressive disorders (MDD) are resistant to current treatment and the majority of cases relapse at some points during therapy. This has resulted in novel treatments being adopted, including sub-anaesthetic doses of ketamine, which affects aberrant neuroplastic circuits; glutamatergic signaling and the production of brain derived neurotrophic factor (BDNF). Ketamine rapidly relieves depressive symptoms in treatment resistant MDD patients with effects that last for up to two weeks even after one administration. However, it is also a drug with abusive potential and can have marked side effects. Hence, we conducted studies aimed at enhancing the anti-depressant-like effects of ketamine (allowing for lower dosing regimens) by co-administering magnesium hydroaspartate (Mg^{2+} normally affects the same receptors as ketamine). To this end, we found that ketamine alone induced rapid anti-depressant-like effects in the forced swim test and influence brain levels of BDNF. However contrary to our hypothesis, magnesium had no effect on these outcomes nor did it enhance the effects of ketamine. Thus, these data do not support the use of magnesium as an adjunct agent and instead suggest further research involving other antidepressant and animal models is required to confirm the present findings.

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Introduction

Background: Depression and treatment approaches

Major Depressive Disorder (MDD) is a recurrent chronic psychiatric condition that affects upwards of 121 million people worldwide annually (Tizabi et al, 2013). In the United States, more than 30,000 clinically depressed patients commit suicide each year (Tizabi et al, 2013). The lifetime prevalence of major depressive disorder within Canada was recently reported to be approximately 12 % (Patten et al, 2006) and the suicide rate at 11.7 per 100,000 individuals (Kral, 2012). Based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 2000), MDD is a heterogeneous disorder defined by the core symptoms of sustained low mood and/or loss of interest in the environment, together with secondary symptoms including disturbances of psychomotor functioning, cognition, sleep and appetite.

Depression is also comorbid with numerous other chronic diseases and can not only affect quality of life but also the progression of such diseases. Indeed, depression is considered as a risk factor for cardiac morbidity and mortality in patients with coronary heart disease (CHD) (Chapman DP et al, 2005) and also renders individuals susceptible to type 2 diabetes mellitus (Knol et al, 2006). Apart from its significant clinical morbidity and increased mortality associated with suicide, depression is a remarkable economic burden on society and health care system due to impaired work performance, loss of work days and treatment cost (Berto et al, 2000). It is estimated that depression will be the second leading cause of disability in the world by the year 2020 and the leading cause of disability in females and developing countries (Murray et al, 1997).

Despite the high prevalence of depression and its noticeable impact on individuals and public health, little is known about its underlying mechanisms; in part, owing to the complexity of the neurocircuitry involved, the lack of proper biomarkers and the heterogeneity of both symptoms and precipitating factors (Duman RS et al, 2012). Early theories concerning depression have mainly focused on the role of monoamines, largely owing to the clinical efficacy of monoamine targeting antidepressant drugs. These generally fall under the categories of monoamine oxidase inhibitors (MAOIs), tricyclic antidepressant (TCAs), selective serotonin reuptake inhibitor (SSRIs), noradrenaline reuptake inhibitor (NRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs). As indicated, their function is mostly based upon monoamine hypothesis of depression, which posits that depression is the result of decreased monoamine activity in various emotion regulatory brain regions like limbic system and PFC (Berton O et al, 2006 and Krishnan V et al, 2009). Hence, these medications work primarily through the inhibition of degradation or blocking the reuptake of monoamines (Berton O et al, 2006 and Krishnan V et al, 2009).

Patients experience a wide spectrum of physical and emotional symptoms including disturbed sleep and feeding, agitation and psychomotor retardation as well as cognitive and emotional aspects of the disease (e.g., melancholia, anhedonia) (Keers et al, 2010). A polypharmacy approach in depression thus allows for the tailoring of treatment to the individual (i.e., based on the specific symptom clusters exhibited by each patient). For instance, augmentation of SSRI and SNRI treatments with low-dose atypical antipsychotics (e.g., risperidone, aripiprazole) has been shown to not only boost the basic antidepressant response on mood (especially among treatment-resistant and/or suicidal patients) but also to greatly diminish anxiety and neurovegetative symptoms (Reeves et al, 2008 and Trivedi et al, 2006). At the same

time, combining SSRIs with atypical antidepressants such as mirtazapine also greatly alleviates certain neurovegetative features, particularly disturbed sleep (Holm et al, 1999 and Blier et al, 2009). Ultimately, the heterogeneity in both symptom profile and response profile to the various SSRIs probably stems from the complexity of genetic backgrounds in depression, as well as wide variation in depressed patients' history of prior stress.

Unfortunately, the currently used antidepressants (even when used in combination) have several caveats, including a slow onset of action; requiring weeks or months of treatment to produce therapeutic effects (Kavalali ET et al, 2012 and Machado-vieira et al, 2009). This delayed onset of action is a major concern due to the associated increased morbidity and increased vulnerability to suicide in depressed patient (Katz et al, 2004). Moreover, recent estimates indicate that around 30% of depressed patients do not respond to these antidepressant therapies and even in case of responders, residual symptoms are still often present and there is a very high risk of relapse (Pacher et al, 2001 and Ghaemi N, 2008). Remission, the virtual absence of symptoms, is unfortunately, not reached in 60% to 70% of patients (Greden JF, 2001 and Trivedi et al, 2006). Considering this, it is apparent that our current antidepressant medications do not adequately meet the ultimate goal of treatment. Hence, there is a compelling need for novel, more efficacious and faster acting treatments. To this end, recent studies have begun to focus on the possibility of glutamatergic based therapies to be an effective alternative to the more traditional monoamine-acting drugs (Rasmussen et al, 2013 and Machado-vieira et al, 2009 and Zarate et al, 2006).

Targeting the glutamatergic system to treat depression

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and is critically involved in synaptic plasticity, learning and memory (Platt SR, 2007). It is

widely distributed throughout the brain and its levels are strictly regulated, given the potential for toxicity at high concentrations. The triad of glial cell, together with pre-synaptic and post-synaptic neurons, is referred to as a “tripartite synapse” and plays an important role in buffering synaptic and extra-synaptic levels of glutamate (Lapides KA et al, 2013). As well, glutamate is actively removed from synaptic cleft and transported into the glial cell by excitatory amino acid transporters (EAATs) (Lapides KA et al, 2013).

Glutamate exerts its effect through glutamate receptors, which are divided into two broad categories; ionotropic and metabotropic (Meldrum BS, 2000). The ionotropic glutamate receptors are ligand-gated ion channels, which rapidly open in response to agonist-binding resulting in a flux of calcium (Ca^{2+}) and sodium (Na^+). They consist of N-Methyl-D-Aspartate (NMDA) receptors and non-NMDA receptors, including α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors (Tapiero H et al, 2002 and Platt SR et al, 2007). The NMDA receptors are heterotetramers resulting from various combinations of the following subunits: GluN1, GluN2A-D and GluN3. Apart from glutamate, activation of NMDA receptors also requires a co-agonist or membrane depolarization to remove voltage dependent magnesium (Mg^{2+}) block (Waxman EA et al, 2005). In contrast, metabotropic receptors act through intra-cellular second messenger systems involving G-protein coupling. To date, eight metabotropic glutamate receptors have been recognized (mGluR1-8). These are categorized in three groups; group I (mGluR1 and mGluR5), group II (mGluR 2 and mGluR 3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8). (Tapiero H et al, 2002 and Platt SR et al, 2007).

Exciting emerging evidence indicates that the non-competitive NMDA glutamate receptor antagonist, ketamine, which is widely used for its anesthetic and analgesic properties,

could be a useful treatment for depression (Zarate CA Jr et al, 2006 ; Berman et al, 2000 and Larkin GL et al, 2011). When used at high doses, ketamine has anaesthetic properties by blocking the NMDA receptor at the same site that the hallucinogenic drug, PCP, binds (Li et al, 2011). However, when given at substantially lower sub-anaesthetic doses, a single injection of ketamine was found to promote fast-acting (within hours) antidepressant and anti-suicidal effects (Berman et al., 2000; Zarate et al., 2006; Liebreinz et al. 2007; Price et al. 2009; Ibrahim et al. 2012). Importantly, ketamine was rapidly effective in patients that were previously treatment-resistant (Liebreinz et al, 2007; Machado-Vieira et al., 2009; Mathew et al. 2010). Furthermore, the antidepressant effects following a single ketamine dose have been reported to persist for several days to even weeks (Correll and Furrer 2006; Liebreinz et al, 2007; Irwin SA et al, 2010; Mathew et al, 2010; Zarate CA Jr et al, 2006 and Price RB et al, 2009). Hence, ketamine is particularly unique in its clinical profile, making it of potentially enormous therapeutic significance. Although the safety of long-term treatment in depressed patients has yet to be fully evaluated, there are indications that chronic low-dose ketamine may be tolerable, feasible and effective (Liebreinz M et al, 2009; aan het Rot et al. 2010 and Murrough 2013).

Despite some important recent advances in our understanding of the various molecular and cellular sequelae following low-dose ketamine treatment (to be discussed shortly), the precise mechanisms underlying ketamine's antidepressant effects are still largely unknown. Emerging studies examining ketamine's mode(s) of action therefore hold the potential to shed much-needed new light on the pathological underpinnings of depression. This, in turn, would be expected to lead to more specific therapeutic targets and the development of safer and more effective drugs. We will next turn to review the available evidence regarding the biological underpinnings of ketamine's behavioural effects.

Molecular and cellular studies in rodent models indicated that ketamine rapidly (within hours) increases synaptogenesis and thus, synaptic plasticity involved in learning and memory (Liu et al, 2012). Such synaptogenic changes would be expected to influence the cognitive processing of emotionally relevant stimuli and might even alter the retrieval of emotionally laden memories. It could also be the case that ketamine could facilitate the processing of environmental stimuli, so as to increase the ability to extract benefit from positive stimuli. Indeed, there are many clinical and pre-clinical studies indicating that major depressive disorder is associated with impairments in synaptic plasticity and neurogenesis, which are often accompanied by some degree of aberrant cognitive processing (Schloesser et al. 2008; Sawyer et al, 2012 and Czéh B et al, 2007).

Synaptic plasticity refers to dynamic changes both in the number of synapses and also the synaptic strength for signal transmission among neurons (Engert F et al, 1999). One of the best-known mechanisms involved in synaptic plasticity is long-term potentiation (LTP), which has a prominent role in learning and memory (Kessels HW et al, 2009). In LTP, the glutamatergic AMPA receptors become sufficiently excited leading to an influx of Na^+ , which consequently leads to the depolarization of postsynaptic cell (excitatory post synaptic potential, EPSP). The EPSP releases the Mg^{2+} that normally blocks the NMDA receptor, thereby allowing Ca^{2+} to enter the cell (Escobar L, 2007). Then Ca^{+2} activates protein kinases, namely calcium/calmodulin dependent protein kinase II (CaMK II) and protein kinase C (PKC). This results in the phosphorylation of AMPA receptors, which increases their sensitivity and the number of postsynaptic AMPA receptors (Escobar L, 2007). In late of phase of LTP, activated PKC and CaMK II stimulate the intra-cellular messenger enzyme, extracellular signal regulated kinase (ERK). This leads to a cascade of activation of nuclear proteins to induce protein synthesis and

synaptic plasticity (Escobar L, 2007). Certainly some of ketamine's actions could be attributable to modifications in LTP or related processes through its effects on the NMDA receptor.

Besides the NMDA blockage, the activating effects of ketamine on the AMPA receptor are also essential for its antidepressant effects (Murck H, 2013). Indeed, following ketamine administration, up-regulation of AMPA receptors has been reported to increase sensitivity to glutamate (Kavalali ET et al, 2012). Other studies have also shown that an increased hippocampal AMPA/NMDA receptor density ratio was associated with low dose ketamine treatment (Tizabi Y et al, 2012). Moreover, the antidepressant-like effects of ketamine in both the learned helplessness (LH) paradigm and the tail suspension test (TST) in rodents was blocked by subcutaneous treatment with 2,3-dihydroxy-6-nitro-7-sulfoamoylbenzo(f)quinoxaline (NBQX), an AMPA receptor antagonist (Koike H et al, 2011).

Importantly, AMPA receptors can promote the expression and release of brain derived neurotrophic factor (BDNF) (Lauterborn et al, 2000), which is a key regulator of synaptic plasticity and has been repeatedly linked to depression (Brunoni AR et al, 2008 and Hashimoto K et al, 2004). Indeed, following NMDA receptor blockade, AMPA receptor activation results in calcium influx through L-type voltage gated calcium channel, which promotes BDNF release from synaptic vesicles (Hoeffer CA et al, 2010).

BDNF and magnesium: Antidepressant effects

BDNF levels were reduced in MDD patients (Yoshimura et al, 2012) and post-mortem studies have reported reduced BDNF expression in hippocampus and prefrontal cortex (PFC) of depressed suicides (Krishnan et al. 2007 and Yu H et al, 2011). Furthermore, infusion of BDNF itself into the brain produced antidepressant-like effects in rodent behavioral models of depression such as forced swim test (Shirayama et al, 2002). Interestingly, rodents exposed to a

forced swim test displayed a reduction of hippocampal BDNF levels and this effect was reversed with antidepressant treatment (Russo-Neustadt et al, 2001). Current antidepressant medication like SSRIs has been shown to increase BDNF expression in hippocampus and PFC of rats (Ignácio et al, 2014). SSRIs increase synaptic serotonin levels and phosphorylated cAMP response element (pCREB), which leads to an overall rise in expressed BDNF levels (Ignácio ZM et al, 2014).

Likewise, it has been reported that ketamine-mediated NMDA receptor blockade increased hippocampal BDNF levels and that this effect might contribute to its anti-depressant effects (Garcia et al, 2008; Autry et al, 2011 and Machado-vieira et al, 2009). Further support for a role of BDNF in the actions of ketamine comes from a recent study showing that BDNF inhibition rendered animals insensitive to the antidepressant-like effects of the drug (R.-J. Liu et al, 2012).

In terms of downstream signalling pathways, BDNF binds to its receptor, tropomyosin related kinase B (TrkB), leading to the recruitment and engagement of several intracellular signalling factors involved in synaptogenesis and dendritic remodelling (Kavalali ET et al, 2012). These include phosphoinositide-3-kinase (PI3K), followed by AKT or protein kinase B (PKB) and then mammalian target of rapamycin (mTOR) activation (Li N et al. 2010). Particular attention has been devoted recently to the potential role of mTOR in mediating the effects of antidepressant agents, including ketamine (Dwyer JM et al, 2013). In fact, our own recent findings indicated that mTOR was involved in the elevation of hippocampal neurogenesis and anti-depressant-like effects in the forced swim test induced by administration of the trophic cytokine, erythropoietin (Osborn M et al, 2013).

mTOR is a ubiquitously expressed serine/threonine kinase that regulates the initiation of protein translation and controls protein synthesis required for synaptogenesis (Klann E et al, 2004). mTOR acts by stimulating p70 ribosomal protein S6 kinase (p70S6) and inhibiting the 4E binding proteins (4E-BPs). This signalling cascade ultimately regulates the activity of eukaryotic elongation factor 2 (eEF2) and eukaryotic translation initiation factor 4E (eIF-4E), which initiate synaptic protein synthesis (Li N et al, 2010) and up-regulate Arc, GluR1, PSD95 and synapsin I, which are all markers of synaptic plasticity (Duman RS et al. 2012).

Within the brain, there are two forms of neurotransmission; evoked neurotransmission in which neurotransmitter is released in response to an action potential and spontaneous neurotransmission which is independent of action potentials (Kavalali ET et al, 2012). Spontaneous neurotransmission happens when a synaptic vesicle will fuse with the presynaptic membrane. It is hypothesized that low dose ketamine exerts rapid antidepressant like effects by blockade of spontaneous glutamate release and the adverse psychotomimetic side effects observed with higher doses of ketamine are more due to its effects on evoked neurotransmission (Monteggia L et al, 2013; Kavalali ET et al, 2011 and Kavalali ET et al, 2012).

There is also a regional differentiation of ketamine's effect. Ketamine increases BDNF expression in PFC, hippocampus and amygdala (Murck H, 2013; Zhou W et al, 2013 and Autry A et al, 2012). It is notable that the rise in amygdala-BDNF may be related to its potential side effects including psychotic characteristics (Murck H, 2013).

Despite its beneficial antidepressant effects, ketamine's psychotomimetic effects and abuse potential limits its widespread use. Indeed, the drug has been used recreationally for its hallucinogenic properties (in this regard, it has many similarity to PCP and LSD)(Malhotra et al. 1996 and De Luca et al, 2012). Likewise, high doses of ketamine and other NMDA receptors

have been associated with neurotoxic effects in animal models(Olney et al., 1991). Due to these limitations, several strategies have been proposed in terms of developing more selective NMDA receptor antagonists with reduced side effects, like Ro-25-6891 (Duman RS et al, 2012).

An alternative potential treatment strategy might involve the co-administration of agents that can synergize with ketamine in the promotion of an anti-depressant response. This would allow for even lower doses and a less frequent ketamine-dosing regimen. In this regard, we posit that **magnesium, (Mg^{2+})**, the fourth most abundant ion in the human body could be a potential co-factor that might augment the effects of ketamine. Indeed, **Mg^{2+}** is normally present as a channel blocker on the NMDA receptor, upon which ketamine exerts its antagonist actions. Moreover, there are reports indicating that **Mg^{2+}** is involved in the pathophysiology of mood disorders (Poleszak E et al, 2005).

Mg^{2+} is a co-factor of more than 300 enzymes involved in different enzymatic reaction including energy metabolism and utilization of adenosine-5'-triphosphate (ATP) (McLearn RM, 1994). The vast majority of **Mg^{2+}** is intracellular and is mostly bound to nucleic acids (RNA and DNA), with a small extracellular fraction found mainly in the bloodstream (Elin RJ, 1994). **Mg^{2+}** is an essential mineral for many physiological functions and its deficiency has serious effect on various organs. The symptoms of **Mg^{2+}** deficiency include cardiac arrhythmias, hyperexcitability, headaches (Ianello S et al, 2001)and depression (Hashizume N et al, 1990). Interestingly, it was reported that patients with treatment resistant depression had exceptionally low CNS **Mg^{2+}** levels (Murck H, 2013). Remarkably, magnesium depletion was also associated with depressive-like behaviors in rodents (Poleszak E et al, 2004 and Singewald N et al. 2004). Intravenous magnesium has been also used in the treatment of acute migraine and headaches (Gertsch E et al, 2014 and Choi H et al, 2014). It is also an established treatment for controlling

seizures in pregnant women with eclampsia (Danforth's obstetrics and gynecology, 2003 edition).

Administration of the Mg^{2+} organic salt, magnesium hydroaspartate, had anti-depressant-like effects, including a reduction of immobility time in a forced swim test and reduced escape deficits in a learned helplessness model of depression (Poleszak E et al, 2004 and Decollogne S et al, 1997). As well, the magnesium treatment also diminished signs of anxiety in an open field arena and elevated plus maze, as well as a novelty suppressed feeding test (Zarate et al., 2013). It is also notable that when magnesium was jointly administered with an SSRI, it reinforced the antidepressant like outcomes (Poleszak E et al, 2005 and Poleszak E et al, 2007). In fact, elevated intracerebral Mg^{2+} levels were reported with several existing antidepressant agents when administered to depressed patients (Iosifescu DV et al, 2008 and Abukhdeir AM et al, 2003). Moreover, a recent report indicated that intravenous magnesium hydroaspartate treatment was efficacious for treatment resistant depression (Eby GA et al, 2010).

Although the exact mechanisms through which magnesium can influence behavioural outcomes remain to be determined, one likely possibility is the regulation of BDNF and synaptic plasticity. Indeed, elevations of extracellular Mg^{2+} levels promote synaptogenesis and synaptic plasticity (Zarate C et al, 2013 and Abumaria N et al, 2011). Also, like ketamine, Mg^{2+} is a potent antagonist of NMDA receptor (in particular NR2B), and can promote BDNF expression (Murck H, 2012). Indeed, Mg^{2+} normally functions by blocking the ion channel of the NMDA receptor in a voltage dependant manner, reducing calcium influx but up-regulating NR2B-containing NMDA receptors (Zarate et al, 2013 and Abumaria N et al, 2011). *In vitro*, this blockade occurs at concentrations less than 1mM, which is compatible with the range of Mg^{2+} concentrations normally observed in the plasma and CSF (Morris ME, 1992). Extracellular

Mg^{2+} is also an important regulator of NMDA receptors and deficiency in extracellular concentrations leads to abnormal NMDA receptor function, increased calcium influx, the accumulation of toxic levels of reactive oxygen species and hyperexcitability (Eby GA et al, 2010).

An overview of our proposed model for how magnesium and ketamine could be acting is depicted in Figure 1 (borrowed from Murck H et al., 2013). Magnesium and ketamine can both enhance synaptic strength through phosphorylation and activation of CaMKII (Murck H, 2013). Furthermore, magnesium administration increases phosphorylation of c-AMP response element binding protein (CREB), which in turn, increases BDNF expression (Szewczyk B et al, 2008).

As stated earlier, BDNF is a key regulator of neural plasticity (Murck H.2013) and may underlie many of the actions of ketamine (Kalavali ET et al, 2012). Another similarity between ketamine and magnesium is that they both have suppressive effects on eEF2 phosphorylation, which also influences synaptic protein synthesis (Murck H, 2013).

Ketamine and possibly magnesium are neuroprotectant and there are studies indicating synergistic interaction between magnesium and ketamine at NMDA receptor (Vučković SM et al, 2014 ; Liu HT et al. 2001 and Harrison NL,1985).

In the present thesis we wish to explore the potential additive or synergistic antidepressant-like actions of combined ketamine plus magnesium treatment. We assessed whether these treatments had any interactive behavioural effects in a forced swim test (a reliable antidepressant screening procedure) and whether they might be dose-dependent. Our first experiment involved acute administration of ketamine and magnesium and the second study assessed a more chronic regimen. Locomotor activity was also determined in order to rule out any motoric effects of the treatments. In order to ascertain whether any interactive effects of

ketamine and magnesium might be related to trophic brain changes, we assessed BDNF levels within the hippocampus and PFC (two regions heavily implicated in depression and which are known to show BDNF changes in response to traditional antidepressants).

Figure 1. Proposed pathway of synaptogenesis related to NMDA antagonism and magnesium. H.Murck/Journal of Psychiatric Research 47 (2013) 955-965.

Methods and Materials:

Animals:

These two experiments assessed the potential interactive antidepressant-like effects of ketamine and magnesium combination treatment. For this purpose, mice (CD1, male, 6-8 weeks of age) were acclimatized to the vivarium for a period of 1 week before experimental procedures began. Animals were singly housed in standard polypropylene cages (27 x 21 x 14 cm), and a 12-hour light/dark cycle (light phase: 8:00-20:00 h) was maintained. Water and Teklad Global mouse chow were provided ad libitum and room temperature was maintained at 21 °C. All procedures were approved by the Carleton University Animal Care Committee.

Treatment procedures:

Experiment 1:

Seventy-two mice were randomly divided into 9 groups (n = 8/group): 1. Saline, 2. Ketamine (2.5 mg/kg), 3. Ketamine (5mg/kg), 4. Ketamine (10mg/kg), 5. Magnesium Hydroaspartate (30mg/kg), 6. Ketamine (2.5mg/kg) + Magnesium Hydroaspartate 7. Ketamine (5mg/kg) + Magnesium Hydroaspartate, 8. Ketamine (10mg/kg) + Magnesium Hydroaspartate and 9. Saline (behaviourally naïve). This last group of mice were naïve to the behavioural procedures; thus, these could be considered an outside control, allowing us to eliminate the possibility that any BDNF changes might be related to the forced swim test exposure. Thus, we have a 2 (Magnesium vs Saline) x 4 (Saline vs three different Ketamine doses) ANOVA design, plus an additional outside “pure” control group. All injections were given intraperitoneally (ip) in a volume of 0.2 ml vehicle (saline).

Behavioural procedures:

A 2-day forced swim test (FST) procedure was used according to the methods of Hunsberger & Duman, (Hunsberger et al, 2007). The test involved two separate exposures to a 2500 mL beaker filled 2/3 with $23 \pm 1^{\circ}$ C water. Importantly, mice cannot touch the bottom of the beaker. On the first experimental day, all animals were forced to swim for 15 minutes (pre-test). At 24 hours following this pre-test, mice were injected with either of three doses of ketamine or saline and then subjected to the second FST one hour later. Based on the rapid kinetics of Magnesium Hydroaspartate, it was administered 0.5 hour after the ketamine/saline injection (hence 0.5 hours before the FST).

Mice were placed in the beaker for 15 minutes and their behavior was videotaped. After each swim, mice were lightly towel dried and introduced back into their home-cage, which was then placed for 15 min on a heating pad set to medium heat. The water in the beaker was changed between each test. Each FST session was video recorded and later scored by a person blind to the groups. Time spent swimming or climbing (pedaling or circular movements around the beaker or active attempts to climb the beaker wall) was considered as mobility and immobility was defined as lack of movement beyond those movements necessary to maintain balance.

In order to assess whether the drug treatments might be affecting motor activity (which could confound FST results), home-cage activity was assessed for the 10 minutes immediately prior to the second FST. Undisturbed locomotion was continuously evaluated in the home cage using a Micromax beam-beam apparatus (AccuScan, CA) equipped with 16 photocells interfaced into a digital analyzer. The number of light beams crossed by the mice was recorded to give an index of the overall activity level (Poleszak et al, 2004).

Immediately after FST, all animals were rapidly decapitated; the brain of each animal was collected. The hippocampus and PFC were carefully microdissected from 0.5 mm coronal tissue slices. All tissues were placed on dry ice followed by storage at -80° C until assayed.

Pilot study:

Unfortunately, we failed to detect any basic effects of ketamine itself on FST performance in Experiment 1. This was surprising given the previous reports demonstrating significant antidepressant-like effects at these 5 and 10 mg/kg doses (Liu et al, 2012; Ghasemi M et al, 2010; Ma XC et al, 2013 and Maeng S et al, 2008). Hence, we conducted a small pilot study (n=8/group; ketamine vs saline) using a 15-mg/kg dose, which did significantly reduce immobility time, relative to saline treated mice. Thus, the dose of ketamine used in Experiment 2 (15 mg/kg) differs from that of the Experiment 1 (5 and 10 mg/kg). As well, we have also adjusted our FST procedures from Experiment 1, in which we used two 15 min trials, to one 6-minute trial used in Experiment 2. Once again our initial procedure was based on procedures used by Hunsberger and Duman 2007 (Hunsberger and Duman 2007), however, others have used the 6 min procedure (Liu et al, 2012; Ghasemi M et al, 2010; Ma XC et al, 2013 and Maeng M et al, 2008). As well, we successfully utilised the 6 min trial FST in our pilot study.

Experiment 2:

This experiment examined the effects of a single ketamine injection prior to chronic administration of magnesium hydroaspartate. Thirty two mice were randomly divided into two groups. (n=16/group): 1. Saline, and 2. Magnesium hydroaspartate. In this experiment, the

treatments were administered intraperitoneally once daily for 14 days. Then each group of mice was sub-divided into 2 groups (n=8/group); one receiving Ketamine (15mg/kg) and the other Saline on test day. Injection side were randomized and sterile procedures used to reduce the chances of any infection/inflammation.

Behavioral procedure:

On Test day, which was 24 hours after the last magnesium injection, mice were injected either by ketamine or saline. One hour later animals were subjected to the FST, as was the case for Experiment 1. The FST was performed according to the methods of Liu et al., 2012. Briefly mice were placed in a 2500 mL beaker filled 2/3 with $22 \pm 1^{\circ}$ C water. They were allowed to swim for 6 minutes during which time their activity was videotaped. Immobility was defined as the absence of active, escape-oriented behaviors such as swimming, jumping, rearing and sniffing and was assessed during the last 240 seconds of the test. The recorded videos were later scored by a person blind to the treatment groups. Also, in order to assess whether the drug treatments might be affecting motor activity (which could confound FST results), the open field test was used, as described by (Carola V et al, 2002). A 50 cm³ open black Plexiglas arena floor was divided into 36 squares (8×8 cm) then, each animal was randomly placed in one of four corners and their locomotor activity was recorded for a period of 10 minutes. The number of lines crossed by each mouse was counted by a person blind to study. The open field test was also used to assess anxiety like behaviors in mice, as previously described by (Litteljohn D et al. 2010). The open black Plexiglas arena was divided into pre-defined zones (outer, middle, and inner/center). The amount of time spent in each zone was measured by a person blind to study

groups. Decreased time spent in the center zone is an index of anxiety-like behavior. The arena was cleaned with 10% EtOH between trials.

Immediately after the FST, all animals were rapidly decapitated and brains collected. The hippocampus and PFC were carefully microdissected from 0.5 mm coronal tissue slices. All tissues were placed on dry ice followed by storage at -80°C until assayed.

BDNF analyses:

Prior to analysis, samples were removed from the freezer and weighed. Lysis buffer (100mM PIPES (PH 7), 500mM NaCl, 0.2% Triton X-100, 0.1% NaN₃, 2% BSA, 2mM EDTA.Na₂.2H₂O, 200 μM PMSF (frozen in iso-propanol), 10 μM leupeptin (frozen separately in deionized water), 0.3 μM pepstatin (frozen separately in DMSO)) was then pipetted into each tube containing hippocampal or PFC samples (2mL). Samples were then homogenized and sonicated (Virtis Virsonic, Virtis Company, Gardiner, NY with a microtip at power level 4 and pulses at 1 s intervals for 15s). Then an additional 2mL of lysis buffer were added to the samples and they were again sonicated. Samples were split and one half of each sample was spiked (artificially increasing the concentration of BDNF by 250pg/mL to determine percent recovery). Samples were centrifuged for 30 minutes at 16,000 $\times$ g at 4°C . Supernatants were removed and frozen at -80°C until analysis.

BDNF expression was determined by Western blot analysis. Protein concentration in the supernatants of tissue extracts was determined using a BCA protein assay kit (Pierce biotechnology, Inc.) A quantity of 30-50 μg total proteins was loaded onto a 4-15% gradient polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membrane and probed with the following primary anti-body: BDNF (1:1000, Santa Cruz biotechnology, Inc.).

β -actin (1:2000; Sigma-Aldrich) was used as an internal control. Secondary antibodies were horseradish peroxidase conjugated to anti-mouse IgG (Sigma-Aldrich, USA). The membrane were developed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL). Densitometric analysis was performed using for the quantification of the immunoblotting using the Scion Image Software (Scion Corporation).

Statistical analyses:

Statistical analyses were performed using SPSS. Differences among experimental groups in the FST and in the assessment of BDNF levels will be determined by ANOVA. Independent t-test was used when two groups were compared. Where there was an interaction effect, two-way ANOVA's were then followed by Fisher's planned comparisons ($p < 0.05$). Data is presented in the form of mean $\pm$ standard error mean (mean $\pm$ SEM). All data was analyzed using the statistical software SPSS (version 19.0) and differences were considered statistically significant when $p < 0.05$.

Results:

Experiment 1:

Spontaneous home-cage locomotor activity: Home cage activity was not influenced by the ketamine or magnesium treatments (Figure2.) ($F_{(3,64)} = 2.157$, $P=0.103$). This finding suggests that any prospective difference in forced swim behavior is not likely to be related to any changes in motor functioning. Also, there was no significant difference between the outside control group (i.e., behaviorally naïve group) and saline group ($t(14) = -1.3381$, $P=0.2$). (Figure3). This implies that the first forced swim test did not affect locomotor activity (LMA) results.

Fig2. *Spontaneous home-cage locomotor activity.* Total home-cage locomotors activity (LMA) expressed as total number of infrared beam breaks over 10 minutes. All data is expressed as mean $\pm$ SEM. No significant difference was apparent among the saline treated mice and those that received the three different ketamine doses. (P -value=0.103).

Fig3. *Spontaneous home-cage locomotor activity.* Total home-cage locomotor activity expressed as total number of infrared beam breaks expressed over 10 minutes. All data is expressed as mean $\pm$ SEM. No significant difference among the two groups. (P-value=0.2).

Forced swim test: The forced swim test was used as per the method of Hunsberger et al (2007) in order to capture any potential antidepressant-like effects of ketamine and magnesium treatments. The two-way ANOVA revealed no significant Ketamine X Magnesium interaction on immobility time for any of the three ketamine doses. ($F_{(3,64)} = 0.564$, $P=0.641$). (Figure4).

In order to assess possible time-dependent changes in forced swim behaviour, analyses of the 15-min FST paradigm and the relevant immobility time data are presented in (Table1). As was the case for the 15-min immobility time analysis, the multiple separate ANOVAs failed to reveal a significant ketamine X magnesium interaction for immobility at any of the times assessed.

It is important to note, however, that a significant difference between the outside control group (behaviorally naïve group) and saline group was observed. ($t(14) = 2.808$, $P=0.014$)

(Figure5). This raises the possibility that the pre-test FST session did have carryover stressor or learning effects that impacted subsequent test performance.

Fig4. *Immobility in the forced swim test.* Effect of Ketamine and Magnesium treatment on immobility time in the forced swim test. Time spent immobile was determined by assessing the time mouse spent floating while making only the necessary movements required to keep their head above the water. No significant difference among saline (S) or ketamine (K) treated groups at any of the three doses. All data is expressed as mean $\pm$ SEM.

Fig5. *Immobility in the forced swim test.* Effect of Ketamine and Magnesium treatment on immobility time in the forced swim test. A significant difference between Saline group and behaviorally naïve saline group. (*P-value <0.05).

Table1. *Forced swim test data.* Immobility time in the last 3 minutes, last 6 minutes, last 9 minutes, last 12 minutes and total 15 minutes. Data are presented as mean ± SEM.

FST	Saline				Magnesium			
	Saline	Ket2.5	Ket5	Ket10	Saline	Ket2.5	Ket5	Ket10
3min	172.7±2	167.57 ±2.6	160.63±4.9	156.30±6.7	156.33 ±6.2	168.08±4.09	156.25±11	160.65±6.8
6min	340.58±4.5	324.3±7.3	318.41±9.4	316.13±11.6	311.01±8.1	331.31±9.1	298.2±22	318.2±12.7
9min	502.1±8.8	479.97±12.7	462.19±19.9	463.0±13.8	454.93±14	489.6±13.7	450.6±30.4	461.48±20
12min	653.79±15	617.87±24.2	588.27±28.7	600.52±22.1	590.71±23	631.59±15.5	589.5±36.1	591±28.6
15min	735.38±19.4	707.55±37.4	671.58±37.9	665.47±33.6	655±29.9	703.49±21.7	662.38±40.7	650.93 ±41

PFC and Hippocampal BDNF expression:

Western blot analysis of prefrontal cortex BDNF protein levels revealed a significant ketamine X magnesium treatment interaction ($F_{(3,56)} = 3.11$, $P=0.035$). As shown in figure 6 and further confirmed by Fisher's planned comparisons, in mice only exposed to ketamine (i.e., not to the ketamine-plus-magnesium combination), the drug dose-dependently increased BDNF protein expression in prefrontal cortex ($P < 0.05$). In contrast, among mice also receiving magnesium, the highest expressed BDNF is seen with the lowest ketamine dose (2.5mg/kg). (Figure6, Table2). In effect, while ketamine tended to increase PFC BDNF expression in a dose-dependent manner, pre-treatment with magnesium blunted the effects of ketamine at the highest dose.

In contrast to what was observed in the PFC, BDNF levels in the hippocampus were not affected neither by magnesium ($F_{(1,56)} = 2.730$, $P=0.105$) nor ketamine treatment ($F_{(3,56)} = 0.205$, $P=0.892$), nor their interaction ($F_{(3,56)} = 1.0339$, $P=0.387$) (Figure 6).

Also, there was no significant difference between the outside control group (behaviorally naïve group) and saline group with respect to BDNF expression in either the PFC ($t(11) = 0.834$, $P=0.422$) or hippocampus ($t(10) = -0.477$, $P=0.644$). This therefore excludes any potential effect of FST exposure on BDNF expression (at least in the drug-naïve condition).

Fig6. *BDNF western blot*. Prefrontal cortex BDNF on the left side and Hippocampal BDNF on the right side. In PFC, saline group showed a significant dose dependent trend with ketamine. In mice receiving magnesium, the highest BDNF expression is observed with ketamine 2.5 mg/kg. In hippocampus, no significant difference was found. Data are presented as mean $\pm$ SED. (*P-value <0.05).

Table2. *BDNF expression in PFC and hippocampus*. There is a dose dependent increase in PFC BDNF expression as shown. Data are presented as mean $\pm$ SEM.

BDNF	Saline				Magnesium			
	Saline	Ket2.5	Ket5	Ket10	Saline	Ket2.5	Ket5	Ket10
PFC	0.77±0.01	0.84 ±0.1	0.91±0.03	0.94±0.06	0.78 ±0.01	1±0.08	0.97±0.02	0.76±0.02
Hippo	0.88±0.02	1.01±0.09	0.94±0.08	0.95±0.03	1.03±0.08	0.96±0.02	1.04±0.02	1.04±0.04

Pilot Study:

As we were somewhat surprised by the null effect of ketamine (up to a dose of 10 mg/kg) on forced swim immobility, in a small comparison study we assessed the behavioural effect of a 15-mg/kg dose of ketamine. As shown in Figure 7, this higher ketamine dose did significantly reduce forced swim immobility time, relative to saline treated mice ($t(14) = 3.912$, $P = 0.002$). Thus this dose was used for the second experiment.

Fig7. Forced swim test. Effect of Ketamine (15mg/kg) on immobility time in the last four minutes of FST. Data are presented as mean $\pm$ SEM. Mice treated with Ketamine (15mg/kg) spent significantly less time immobile than saline treated mice. ($t(14) = 3.912$, $*P = 0.002$).

Experiment 2:

Locomotor activity in the Open field test: Once again, as shown in figure 8, overall locomotion activity was not influenced by ketamine or magnesium treatment in any of the groups ($F_{(1,32)} = 0.886$, $P\text{-value} = 0.358$), indicating that the treatments were not having general deleterious consequences on motor systems.

Fig8. Locomotor activity in the open field test. Locomotor activity was expressed as the total number of lines passed by mice over 10 minutes in a 50 cm³ open white Plexiglas arena which was divided by 36 squares each 10 cm. All data is expressed as mean ± SEM. No significant difference among groups. ($P\text{-value} = 0.358$).

Anxiety-like behaviour in the open field test:

In the open filed arena, no significant difference was found between the time spent in Center zone ($F_{(1,32)} = 0.143$, $P=0.708$), middle zone ($F_{(1,32)} = 0.148$, $P=0.734$) or outer zone ($F_{(1,32)} = 0.066$, $P=0.798$). (Figure 9)(Table3). Importantly, reduced exploration of the central portion of this arena is typically taking as an index of anxiety-like behavior.

Table3. Anxiety-like behavior in the open field test. Time spent in each zone is shown in different groups. The more time the mice spent in center zone, the less stressed they are. No significant difference among group. Data are presented as mean $\pm$ SEM.

Openfield	Saline		Magnesium	
	Saline	Ketamine 15	Saline	Ketamine 15
Center zone	29.56 $\pm$ 4.0	28.85 $\pm$ 3.5	34.88 $\pm$ 3.5	36.41 $\pm$ 5.7
Middle zone	93.47 $\pm$ 8.9	92.91 $\pm$ 10.72	99.20 $\pm$ 8.9	91.14 $\pm$ 10.29
Outer zone	476 $\pm$ 9.1	478.23 $\pm$ 10.36	465.90 $\pm$ 10.23	472 $\pm$ 8.41

Fig9. Anxiety-like behavior in the open field test. Effect of ketamine and magnesium treatment on anxiety-like behavior in the open field test. Time spent in the center zone ($P=0.708$), middle zone ($P=0.734$) and outer zone ($P=0.785$) were measured in seconds. No significant difference was found in any of the groups. All data is expressed as mean $\pm$ SEM.

Forced swim test:

As shown in figure 10, mice treated with ketamine (15mg/kg) spent significantly less time immobile than their saline injected counterparts ($F_{(1,30)} = 5.2$, $P=0.031$). However forced swim immobility was not affected by the magnesium treatment ($F_{(1,30)} = 0.686$, $P=0.415$) nor was there any indication of an interaction between ketamine and magnesium ($F_{(1,30)} = 0.873$, $P=0.359$) (Table 4).

Fig 10. Immobility in the forced swim test. Effect of Ketamine and Magnesium treatment on immobility time in the forced swim test. Mice treated with ketamine (15mg/kg) spent significantly less time immobile than saline group (*P-value <0.05). No significant effect of ketamine and magnesium treatment on immobility time. All data is expressed as mean ± SEM.

Table4. Immobility in the forced swim test. Mice treated with ketamine (15mg/kg) spent significantly less time immobile than saline group. Data are presented as mean ± SEM.

FST	Saline		Magnesium	
	Saline	Ketamine 15	Saline	Ketamine 15
Last4minutes	173.18 ± 10.84	130.30 ± 45.81	149.67 ± 11.42	131.72 ± 12.89

PFC and Hippocampal BDNF expression:

Western blot analysis of hippocampal BDNF protein levels revealed no significant Ketamine X Magnesium treatment interaction ($F_{(1,32)}=0.08$, $p=0.90$). Nor were main effects of

either Magnesium ($F_{(1,32)}=1.43$, $p=0.241$) or Ketamine ($F_{(1,32)}=0.186$, $p=0.669$) (Figure11, Table5).

Western blot analysis of prefrontal cortex (PFC) BDNF protein levels also revealed no significant Ketamine X Magnesium treatment interaction ($F_{(1,32)}=1.244$, $p=0.356$). Nor were any main effects of Magnesium $F_{(1,32)}=0.916$, $p=0.347$ or Ketamine ($F_{(1,32)}=1.46$, $p=0.236$) (Figure11, Table5).

Fig11. BDNF western blot. Prefrontal cortex BDNF on the right side and Hippocampal BDNF on the left side. No significant difference was found among groups either for PFC BDNF or Hippo BDNF.

Table5. BDNF expressed in PFC and hippocampus. No significant differences among groups. Data are presented as mean $\pm$ SEM.

BDNF	Saline		Magnesium	
	Saline	Ketamine 15	Saline	Ketamine1 5
PFC	1.08 $\pm$ 0.04	0.95 $\pm$ 0.06	0.95 $\pm$ 0.05	0.95 $\pm$ 0.08
Hippo	1.01 $\pm$ 0.91	0.98 $\pm$ 0.11	0.92 $\pm$ 0.05	0.88 $\pm$ 0.08

Discussion

Overview

Major depression is a chronic psychiatric disorder, affecting 17-20% of the population worldwide (Kessler et al., 1994). The high prevalence and often debilitating nature of depression, positions the illness as a major social and global economic burden (Kessler et al, 1994). Pharmacotherapy and psychotherapy represent the two main modalities of depression care. Current pharmacotherapies for major depression include selective serotonin and noradrenaline reuptake inhibitors, which have a long delay of action and actually fail to work in many patients (Trivedi et al., 2006). Therefore, there is an emerging need for the development of faster-acting antidepressants, as well as novel non-monoaminergic agents to benefit the large number of treatment-resistant patients.

In this regard, a lot of recent work has been devoted to examining the antidepressant efficacy of compounds targeting the glutamatergic pathway. This research has been spurred on by the finding that the ionotropic glutamate N-methyl-D-aspartate receptor is involved in the etiology of major depression (Garcia et al, 2008; Berman RM et al, 2000; Zarate C et al, 2006). Since then, numerous clinical and infrahuman studies have reported that NMDA receptors antagonists, most notably ketamine, can promote rapid anti-depressant-like behavioural consequences (Garcia et al., 2008; R.-J. Liu et al., 2012; Beurel E et al, 2011; N. Li et al., 2010; (Réus et al., 2011). In fact, the antidepressant effect of ketamine has been observed in a number of different rodent models of depression, including inescapable stress, the forced swim test, the tail suspension test, learned helplessness, and chronic mild stress (Pozzi L et al,2014). Moreover,

a recent meta-analysis of several clinical studies also suggests the effectiveness of single administration of ketamine in rapid treatment of major depressive episodes (McGirr et al., 2014).

Despite the mounting evidence of ketamine's antidepressant potential, and notwithstanding some important recent advances in our understanding of the various molecular and cellular sequelae following low-dose ketamine treatment, the precise mechanisms underlying ketamine's antidepressant effects are still largely unknown. It has been suggested that the antidepressant effect of ketamine may be due to increased expression of brain-derived neurotrophic factor (BDNF), which, in turn, induces synaptic plasticity (Castrén E et al, 2007; Garcia LS et al, 2008). Indeed, NMDA receptors interact with BDNF/Trk B pathway to promote synaptic plasticity. Conversely, blockade of NMDA receptor by ketamine deactivated eukaryotic elongation factor 2 (eEF2) kinase which leads to reduced eEF2 phosphorylation and consequent de-suppression of BDNF translation (Autry et al, 2011; Kavalali ET et al, 2012).

While there remains much to discover about how ketamine promotes antidepressant outcomes, it is important to keep in mind that a major impetus of this line of inquiry has been the considerable concern over the drug's potential adverse effects (tolerance, abuse, side effects) (Garcia et al, 2008; Kavalali et al, 2012; Browne CA et al, 2013). Indeed, the hope is that a better understanding of the molecular and cellular pathways subserving ketamine's antidepressant action will lead to the development of novel glutamatergic drugs that share with ketamine its mode of action/antidepressant potential but not its unwanted effects ((Martinowich K et al, 2013).

In the meantime, further studies are needed (and, in fact, are ongoing) to find the minimum effective and safe dose of ketamine, or to find a favorable ketamine combination

therapy that could permit the down-titrating of drug doses. Poleszak et al. (2007) demonstrated the antidepressant-like effect of magnesium in conjunction with NMDA antagonists (CGP 37849, MK-801, L-701,324) in mice; importantly, these authors suggested that administration of magnesium could be an effective method to reduce the NMDA antagonist dose (Poleszak et al. 2007).

The present study evaluated the effect of different doses of Ketamine, delivered either as monotherapy or in combination with magnesium, on hippocampal and prefrontal cortex BDNF expression and behavioral despair in mice.

The effect of ketamine, magnesium and their joint administration on spontaneous locomotor activity

Spontaneous locomotor activity of mice was assessed in the home cage and open field in the first and second experiments of this thesis. Locomotor activity measurement is commonly used in mice to study sensitivity to the motor-activating or depressing effects of a drug or experimental treatment. In the first experiment, ambulatory counts representing the number of infrared beam interruptions were recorded in a home-cage context. In the open field test, the number of lines crossed by each mouse was counted. Both tests revealed no differential results among groups; indicating that any potential effect in the behavioral despair paradigm (i.e., forced swim test) is likely not due to alterations in locomotor activity. Similarly, previous studies demonstrated that neither ketamine nor magnesium has any effect on locomotor activity in mice (Poleszak et al, 2004; Koike et al., 2011; Poleszak et al, 2005; Ghasemi et al., 2010; Egnin E et al, 2009).

In our second experiment, we also used the open field test to assess exploratory behavior; here, reduced time spent exploring the centre zone of the open field arena is taken to reflect anxiety-like behaviour (Carola V et al, 2002). As was the case for nonspecific motor activity (i.e., beam breaks or line crosses), neither ketamine nor magnesium had exhibited any anti-anxiety effects in the open field test.

Magnesium-deficient diet induces anxiety-related behavior in mice (Singewald N et al, 2004) and there are studies indicating an anxiolytic-like activity of magnesium in mice (Poleszak E et al, 2004; Poleszak E et al, 2008). This is, however, in contradiction with the results of our present study. Furthermore, animal data regarding the impact of ketamine and its effect on anxiety behaviors is contradictory: In a study conducted by Silvestre JS et al (1997), ketamine was found to produce anxiogenic-like effects in rats, but in another study the drug was shown to promote anxiolytic responses (in the elevated plus maze) (Hayase T et al, 2006). Similarly, the evidence for an anxiolytic-like effect of ketamine in humans is equivocal, and probably is subject to complex dose-and-time effects. For instance, in a study of healthy human volunteers, the participants reported a decrease in anxiety after a low dose of ketamine (0.1 mg/kg IV), but an increase in anxiety at a higher dose (0.5 mg/kg IV) (Krystal et al., 1994). Ketamine is often used as a preoperative agent for painful medical procedures for both adults and children, presumably due to its dissociative anesthetic and anxiolytic properties (Oda A et al, 2000; Karapinar B et al, 2006). However, the potential anti-anxiety effects of ketamine may be completely secondary to sedation and analgesia (Ghai B et al, 2005), which is seen only with doses higher than the sub-anesthetic ones we used in the present study (usually substantially greater than 30 mg/kg).

The effect of ketamine, magnesium and their joint administration on forced swim test (FST)

In our initial experiment, a two day forced swim test (FST) was performed based on the methods of Duman & Hunsberger (Hunsberger et al, 2007). According to this paradigm, a 15-min pre-test FST session on the day before the experiment can be considered a “despair-inducing” stressor. As shown in Figure 5, a significant difference in forced swim immobility between our control group (behaviorally naïve group) and our saline group confirmed the stressor effect of pre-test session. However, we observed no significant effect of ketamine-or magnesium on FST performance (Figure 4). These null effects were rather unexpected, as a majority of published reports have documented potent anti-depressant-like effects of acute ketamine treatment in a variety of mouse and rat FST paradigms (Liu et al, 2012; Beurel et al, 2011; Li et al, 2010; Reus et al, 2011). The reason(s) for the discrepancy between our current and previously published results is not immediately clear, and we entertain a discussion of several possibilities in an ensuing section.

Nonetheless, in our pilot study using a higher dose of ketamine (15 mg/kg) and a different FST paradigm (i.e., no pretest session, 6-min FST), we did find that ketamine significantly reduced immobility time. These positive results, which are of course more closely aligned with what’s been reported previously (though typically with lower ketamine doses), led us to perform a second experiment using the piloted drug and testing parameters (i.e., 15 mg/kg ketamine, 6-min FST without pretest session). We also administered a daily Mg injection for 2 weeks and an acute injection of Ketamine 1 hour before the test. As expected, in this (second) experiment we found a significant effect of Ketamine (15 mg) on FST.

Forced swim test as a rodent model of depression

When studying antidepressant drugs or predicting an antidepressant activity of a new substance, it is necessary to evaluate its neurochemical and behavioral effects in preclinical experiments. However, animal models of depression that have proven sensitive to antidepressants may be oversimplifying a highly complex human illness, and thus they may not be entirely valid (Borsini & Meli, 1988). A proper animal model of depression should satisfy the following criteria: Similarity of etiology, biochemistry, symptomatology and treatment (McKinney WT Jr, et al, 1969). However, depression presents clinically as a complex and highly patient-specific constellation of psychological, neuroendocrine, and somatic symptoms, many of which cannot adequately be seen or tested in animals (Petit-Demouliere B et al, 2005). Therefore, only specific measurable behaviors in animals can be considered relevant to human depression (Holmes PV, 2003); this, of course, makes the generalizability of animal study findings somewhat complicated.

Among all different animal models of depression, the forced swim test, first described by (Porsolt RD et al, 1977;Porsolt RD et al, 1977) remains one of the most widely used tools for screening antidepressants. The procedure entails placing a rat in a cylinder containing room-temperature water for 15 min (pretest session). Then 24 hours later, the animal will be exposed to the similar condition again and the total immobility time during a 5-min period will be assessed (test session) (Porsolt et al, 1977, Porsolt RD et al, 1979; Borsini et al, 1988). The immobility of the rat indicates the state of behavioural despair, since the rat has learned that escape is impossible (Porsolt et al, 1977; Porsolt RD et al, 1977); this immobility is not due to impaired escape performance. All told, the FST is a fast, low cost and reliable model with strong predictive value.

To perform the FST in mice, Porsolt and colleagues (1977) recommended several procedural alterations. Specifically, mice were to be introduced into a cylinder 1 hour after a single injection, and left for 6 min. Forced swim immobility was then quantified and analyzed during the final 4 min (Porsolt et al. 1977). There have since been many modifications of the mice FST parameters to increase the sensitivity, specificity and reliability of detection of antidepressant activity. These modifications addressed any number of testing and experimental parameters, including acute versus chronic administration, interval of observation, test/ retest, and time between treatment and test (Petit-Demouliere et al, 2005). In another modification described by Khisti et al (2000), unlike Porsolt's method for mice (direct immersion of animals after drug injection), animals were subjected to a "pretest session" in order to avoid variations and to maintain consistency in immobility time between different anti-depressant groups (Khisti et al, 2000). This new modified FST included a 15 min pretest session, and 24 hours later drugs were administered, followed by a 6 min session test 1 hour after injection. These authors observed a significant antidepressant effect of the neurosteroid, 3 α -hydroxy-5 α -pregnan-20-one, using this modified FST in mice, which was subsequently successfully applied in a number of studies (e.g., Kadali et al, 2014).

Accordingly, we used the modified FST as our main behavioral test to evaluate antidepressant activity of ketamine and magnesium therapy in mice. The depression model we developed was a modification of Khisti et al. (2000) method involving increasing the duration of test session from 6 min to 15 min. Our experiments demonstrated the efficiency of this new modified FST model in inducing behavioral despair in mice. It will also be recalled that our data do not support a motor-modulatory effect of FST pre-testing. This finding suggests that any

potential significant difference in FST behaviour using this two-trial paradigm is not attributable to disturbed motor function. (Figures 2 and 3).

However, after our first experiment, we noticed non-significant responses of mice to both ketamine and magnesium, no matter the dose or whether the drugs were applied individually or in combination. This was inconsistent with previous FST experiments in mice that established the antidepressant-like effectiveness of 10 mg/kg ketamine (Garcia et al, 2007; Liu et al, 2012; Beurel et al, 2011; Li et al, 2010; Reus et al, 2011). The FST ineffectiveness of ketamine in the present study roused concerns about our new modified FST model. Specifically, our new testing model differed in several important ways from previous FST experiments with ketamine (6 min, without pretest session), and these factors could have rendered the present FST testing paradigm considerably more stressful (two trials of 15-min each) and thus less sensitive to the antidepressant effects of relatively low-dose ketamine (i.e., 10 mg/kg). To investigate this possibility, we performed another pilot experiment using a higher dose of ketamine (15 mg/kg), and using the “less restrictive” FST parameters (6 min without pre-test session). This small study showed significant therapeutic effect of Ketamine 15 mg/kg in animals which confirmed our concerns.

Therefore, we performed another experiment with the standard mouse FST (6 min, without pretest session), and also we increased the ketamine dose to 15 mg/kg – a dose that was previously reported to induce anti-depressant-like outcomes in the mouse FST (Garcia et al., 2008). It is also notable that despite the numerous studies which have documented the antidepressant effectiveness of 10mg/kg ketamine (Liu et al, 2012; Beurel et al, 2011; Li et al, 2010; Reus et al, 2011; Ghasemi et al, 2010), there is a wide range of response in the depression literature regarding the effective subanesthetic ketamine doses (Browne CA et al, 2013; Liu et al,

2012; Beurel et al, 2011; Li et al, 2010). Indeed, some studies have found the minimum effective dose to be as low as 2.5mg/kg (Maeng et al., 2008); whereas other studies failed to detect antidepressant-like FST responses to ketamine with doses lower than 30mg/kg (Koike H et al, 2013; Iijima M et al, 2012; Popik P et al, 2008).

It is not immediately clear what might account for such discrepancy in the research findings. Of course, some of the variability likely stems from the “usual suspects” – various experimental (e.g., time of testing, housing conditions, route of drug administration) and organismic variables (e.g., mouse or rat strain, age at experimentation, rearing and early-life history) that are difficult if not impossible to control across studies. Additionally, it warrants mentioning that ketamine is supplied in different chemical and pharmaceutical preparations; while most are racemic (containing equal parts S (+) and R (-) isomers), enantiomerically pure S(+) and R(-) formulations are also becoming increasingly accessible (Hayley & Littelljohn, 2013). Importantly, the two ketamine enantiomers differ markedly in NMDA receptor binding dynamics and kinetics, with S(+) ketamine being considerably more potent than R(-) ketamine (Vollenweider FX et al, 1997; Pai et al., 2007). Yet, a very recent study by (Zhang et al, 2014) revealed that, in juvenile mice that had been neonatally challenged with dexamethasone (a synthetic corticosteroid), R(-) and not S(+) ketamine (10 mg/kg) induced the more potent and longer-lasting antidepressant-like effects (and without the attendant psychotomimetic side effects seen with the latter). Thus, it would appear that the “potency” of different ketamine preparations depends on the particular variable of interest (e.g., depth of anesthesia or psychotomimetic effects vs. antidepressant action), and its determination more involved than a “simple” consideration of NMDA receptor-ligand interactions (this is an important point to consider when pondering the antidepressant mechanism(s) of ketamine)

In the second experiment we evaluated the effectiveness of combined ketamine-plus-magnesium therapy. Acute ketamine injection (15mg/kg) was again effective in decreasing forced swim immobility time in mice, whereas magnesium showed no effect. This contradicts the work of Poleszak's group, which suggested that magnesium was an effective drug in reducing immobility time in mice (Poleszak E et al, 2004; Poleszak E et al, 2005; Poleszak E et al, 2007). This is certainly not to discount the findings of Poleszak's group, and recent experimental and clinical studies have suggested that the anti-depressant-like mechanism of magnesium involves targeting of the NMDA receptor (Sowa-Kućma et al., 2013; Pochwat et al., 2014). For instance, Pochwat et al. (2014) evaluated the effect of three different doses of Magnesium (10, 15, and 20 mg/kg) in the chronic mild stress (CMS) model of depression in rats. They observed an antidepressant-like effect of magnesium at a dose of 15 mg/kg in sucrose preference test, beginning from the third week of treatment. Moreover, the antidepressant-like behavioural effect of magnesium coincided with reduced brain regional (hippocampus, PFC, amygdala) expression of proteins related to glutamatergic signalling (including GluN1, GluN2A and PSD-95). However this study did not assess BDNF expression.

The effect of ketamine, magnesium and their joint administration on PFC & Hippocampal BDNF expression

The underlying mechanism of rapid antidepressant effect of ketamine is not known. However, it has been suggested that the fast-acting behavioral antidepressant-like effect of ketamine is related to the rapid upregulation of BDNF and the consequent potentiation of synaptic plasticity (Autry et al, 2011). Indeed, acute administration of ketamine was shown to

induce increased hippocampal and PFC BDNF levels, which coincided with improved behavioral responses in rodents (Garcia et al, 2008; Zhou et al., 2013; Yang et al, 2013). Consistently, we found a dose-dependent increase in the levels of BDNF in the PFC (Figure 6); however, this did not correspond with reduced behavioural despair in the FST. As already mentioned, the lack of significant forced swim effects could be related to the specific testing parameters used. From this vantage point, the ketamine-induced rise in PFC BDNF was simply insufficient to overcome the “extra stress” posed by the two-trial, 15-min FST paradigm. Alternatively, the current results could conceivably point to a timing issue, wherein the behavioural effect of ketamine lags behind the PFC BDNF changes, with the latter presumably requiring a certain amount of time to engage or otherwise influence the neural circuitry that is ultimately responsible for regulating behavioural despair.

In contrast to the PFC, we did not find any changes in BDNF levels within the dorsal hippocampus. Though somewhat unexpected, there are certainly precedents for such null effects. For instance, (Garcia et al., 2009) revealed that hippocampal BDNF levels did not change following acute or chronic administration of ketamine. These authors suggested that BDNF protein alterations could occur after stressful situations in some specific hippocampal sub-regions such as the dentate gyrus but not the entire structure. It should be noted, however, that Garcia et al. (2009) found neither a decrease in hippocampal BDNF levels after chronic mild unpredictable stress nor an increase in BDNF expression after ketamine treatment.

Contrary to our hypothesis, we did not observe any therapeutic effect (at least in the FST) of acute magnesium administration nor did it augment the impact of ketamine. Furthermore, our results did not show any effect of chronic Magnesium injection on BDNF protein levels in the PFC or hippocampus. Our study is one of the first to assess the relationship between magnesium

administration and central BDNF expression. However, it should be noted that our results stand in opposition to the recent findings of Slutsky and colleagues (Slutsky et al., 2010) who found that magnesium is, in fact, capable of increasing BDNF expression and various markers of synaptic plasticity.

One of the reasons we did not see an effect of magnesium might be the sub-therapeutic duration of magnesium administration in our experiment. Pochwat et al (2014) found changes in the level of proteins related to glutamatergic system in PFC, hippocampus and amygdala after three weeks of magnesium treatment. We chose a 2-week period based on the Poleszak et al. (2004) study in which they found a reduced immobility time in FST after 2 weeks of magnesium administration. However, neither of these studies assessed BDNF levels.

Also, an in vitro study conducted by (Slutsky et al, 2004) suggested that synaptic plasticity is sensitive only to magnesium levels in the physiological range, and it is the magnesium *deficiency* that has a detrimental effect on synaptic plasticity. Therefore, maintaining magnesium at proper physiological concentrations should be taken into consideration.

Perhaps the most interesting finding of the present investigation was that magnesium appeared to interact with low-dose ketamine (2.5 mg/kg) treatment to influence PFC BDNF protein levels. Importantly, as the 2.5 mg/kg ketamine dose did not on its own significantly raise PFC BDNF levels, it is surely tempting to speculate that pairing magnesium with ketamine could allow for the down-titrating of drug doses whilst maintaining the desired therapeutic effect. Of course, upregulated PFC BDNF protein expression is not synonymous with anti-depressant outcomes (though most would consider it desirable in the context of treating stressor-associated psychiatric pathologies such as depression), and it should be underscored that the presently

described drug-induced BDNF changes occurred in the absence of behavioural improvement in the forced swim test.

One final point warrants consideration here: namely the fact that while magnesium-plus-2.5 mg/kg ketamine appeared to additively increase PFC BDNF expression, increasing the ketamine dose did not lead to further elevations of the trophic factor. In fact, precisely the opposite was noted, as increasing the ketamine dose among mice that had been pre-treated with magnesium led to a normalization or reduction-to-baseline of BDNF levels. This may imply that magnesium induce an inverse ketamine dose response curve, and is actually similar to what was reported by Li et al (2010). In their study, ketamine at lower doses increased mTOR expression, but higher drug doses tended to reverse this effect.

Conclusion

Despite the apparent effectiveness of NMDA receptor antagonists as rapid-onset antidepressants, these agents usually induce severe side effects and this limits the clinical usefulness of such drugs (Willetts et al, 1990). Therefore, it has been suggested that combination therapy of a NMDA receptor antagonist and a non-competitive NMDA receptor blocker can be helpful to augment the treatment efficacy and to reduce the dose of competitive NMDA receptor antagonist (Poleszak et al, 2007). Magnesium as an iatrogenic NMDA receptor antagonist has a proven antidepressant-like activity in the forced swim test (Decollogne et al, 1997; Poleszak et al, 2004). Furthermore, it has been used as a supplementary therapy for mood disorders, reducing significantly the use of lithium, benzodiazepines and neuroleptics in joint therapy (Heiden et al., 1999).

Poleszak et al (2007) revealed effectiveness of joint therapy of magnesium with NMDA receptor antagonists (including CGP 37849, L-701, 324, D-cycloserine, and MK-801) in FST, exhibiting anti-depressant like activity. This study demonstrated that ineffective doses of magnesium given together with ineffective doses of NMDA antagonists produce significant immobility time reductions in the FST (Poleszak et al, 2007). In the present thesis, we observed the effectiveness of magnesium-ketamine joint therapy with regards to brain BDNF expression but not behavioral consequences. Moreover, chronic (2-week) magnesium administration together with a single ketamine injection did not promote any significant FST or BDNF changes. The present results certainly do not support the utility of magnesium treatment in depression. Thus, further studies are clearly needed to ascertain whether magnesium really has any potential as an adjunctive agent with ketamine or even more traditional antidepressants, such fluoxetine or citalopram.

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