Antidepressant and Molecular Responses to Ketamine Linked to its Inhibition of Glycogen Synthase Kinase-3 (GSK3)

Steven F. Grieco
University of Miami, sgrieco@med.miami.edu

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ANTIDEPRESSANT AND MOLECULAR RESPONSES TO KETAMINE LINKED TO ITS INHIBITION OF GLYCOGEN SYNTHASE KINASE-3 (GSK3)

By

Steven F. Grieco

A DISSERTATION

Submitted to the Faculty of the University of Miami
in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2016
A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ANTIDEPRESSANT AND MOLECULAR RESPONSES TO KETAMINE LINKED TO ITS INHIBITION OF GLYCOGEN SYNTHASE KINASE-3 (GSK3)

Steven F. Grieco

Approved:

Richard S. Jope, Ph.D.
Professor of Psychiatry and Behavioral Sciences

Eleonore Beurel, Ph.D.
Assistant Professor of Psychiatry and Behavioral Sciences

Mohammad Faghihi, M.D., Ph.D.
Assistant Professor of Psychiatry and Behavioral Sciences

Shaun Brothers, Ph.D.
Assistant Professor of Psychiatry and Behavioral Sciences

Arthur Zelent, Ph.D.
Research Professor of Medicine

Guillermo Prado, Ph.D.
Dean of the Graduate School
Depression is one of the most prevalent and costly health burdens in society. It is caused by a combination of genetic and environmental factors. Current therapies for depression are only effective in some patients and this takes several weeks to occur. Ketamine, however, produces rapid antidepressant effects and is beneficial in classical antidepressant non-responders. The mechanism of action induced by ketamine is not completely known though, and there is a strong interest in discovering the antidepressant aspects of this mechanism which do not contribute to ketamine’s potential as a drug of abuse.

Ketamine is a NMDA receptor antagonist and the antidepressant effects it produces in mouse models are dependent on AMPA receptor activity. The antidepressant effects produced by ketamine in mouse models are also dependent on inhibition of glycogen synthase kinase-3 (GSK3). It is unclear however, how ketamine-induced inhibition of GSK3 results in antidepressant responses. We found that in mouse hippocampus, treatment with an antidepressant does of ketamine (10 mg/kg) increased expression of a cluster of intronic miRNAs within the serotonergic (5HT) 2C receptor (5HTR2C). 24 hr after administration of ketamine, miRNAs 448-3p, 764-5p, 1264-3p, 1298-5p and 1912-3p were
increased 2-11 fold. In GSK3 knockin mice that contain a mutant GSK3 that cannot be inhibited, ketamine administration did not affect miRNA expression from the 5HTR2C miRNA cluster, indicating that GSK3 inhibition is required for their up-regulation by ketamine. GSK3’s role in this pathway was further confirmed since intranasal administration of L803-mts, a specific GSK3 inhibitor, induced up-regulation of the 5HTR2C cluster miRNAs in hippocampus. Intranasal administration of L803-mts also had antidepressant effects in both the learned helplessness model of depression-like behavior and in the novelty suppressed feeding test. Mice that were resistant to depression-like behavior displayed increased expression of the 5HTR2C cluster miRNAs. Administration of an antagonist to one of the 5HTR2C cluster miRNAs, miRNA 448-3p, blocked the antidepressant effect of ketamine in the learned helplessness test.

We also found that administration of an antidepressant dose of ketamine up-regulated expression of Insulin-like growth factor 2 (IGF2) in hippocampus, in a GSK3-dependent manner as well. Intranasal treatment with L803-mts increased expression of IGF2, and in mice with learned helplessness-induced reductions in GSK3 inhibition, ketamine treatment increased GSK3 inhibition and increased expression of IGF2. Resilient mice were also found to have increased expression of IGF2 after the learned helplessness test. Administration of an antagonist to IGF2 blocked the antidepressant effect of ketamine in the learned helplessness test.

Herein are described two novel outcomes, the up-regulation of the 5HTR2C cluster miRNAs and IGF2, as a result of GSK3 inhibition by an antidepressant dose of ketamine that may contribute to the behavioral responses to ketamine.
ACKNOWLEDGEMENTS

I would like to thank everyone who has helped me to succeed in graduate school in my years in Miami.

First, I would like to thank my first mentor, Dr. Dunaief, that I worked with when I was a very young technician, and who encouraged me to do research.

Next I would like to thank Dr. Richard Jope and Dr. Eleonore Beurel who took me into their lab, encouraged me, and constantly mentored me in a manner that was truly impressive.

I would also like to thank Darlah Michelle Lopez-Rodriguez who became my best friend in Miami.

I would like to thank Jerald Collins for his many supportive talks with me. Finally I would like to thank Fran Grieco, for her unconditional love and support, throughout my entire education.
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PUBLICATION NOTE

**Chapters 3:**
Versions of some material in this chapter have been published or are under review in:
World Journal of Biological Psychiatry

**Chapters 4:**
Versions of some material in this chapter have been published or are under review in:
Progress in Neuro-Psychopharmacology & Biological Psychiatry
1 Chapter – Introduction

1.1 Overview of depression

1.1.1 Prevalence of depression

Major depressive disorder is the 11th highest contributor in the world to disability-adjusted life years, a measure of the numbers of years lost due to illness, disability or death (Murray et al. 2012). In the United States major depressive disorder is the 5th highest contributor to disability-adjusted life years (Murray et al. 2013). Therefore depression is a highly debilitating disease and can in many cases even cause death due to suicide (Wong and Licinio 2001). In fact suicide is the 3rd leading cause of death for people 15-24 years of age in the United States (Center for Disease Control and Prevention 2014). In the United States military, a particularly susceptible population, death by suicide has even outnumbered deaths by combat in certain years (Lineberry and O’Connor 2012). In the United States population as a whole, major depression is one of the most common diseases of any medical specialty and affects up to 20% of all Americans at some point in their lifetime (Kessler et al. 2005). Due to this high societal burden, major depression is estimated to take a very significant toll on the economy (Olchanski et al. 2013; Wang, Simon, and Kessler 2003). Unfortunately, the field of psychiatry has not seen a change in the morbidity or mortality caused by depression in many decades, even as biomedical research in this area has steadily increased (Insel 2012).

1.1.2 Causes of depression

Some of the first antidepressant drugs found to be efficacious for major depression were the anti-tuberculosis drug iproniazide, and the anti-histamine drug imipramine...
Studies on these drugs, iproniazide and imipramine, eventually led to the formulation of the monoaminergic hypothesis (Schildkraut 1995). This hypothesis states that the underlying cause of depression is low amounts of the monoaminergic neurotransmitters noradrenaline and serotonin in relevant brain areas (Hirschfeld 2000). This prompted investigators to research drug interventions that would increase the levels of these neurotransmitters in the brain (Feighner 1983). Later, the monoaminergic hypothesis was supported by the findings that monoamine oxidase inhibitors, tricyclic antidepressants and selective serotonin reuptake inhibitors all increase serotonin levels in the brain and are the most effective and widely used antidepressants (Duman et al. 1997).

### 1.1.3 Treatments for depression

The classical antidepressant drugs, though being the best treatments available, have had only modest success thus far (Nestler et al. 2002). This is because many patients with major depressive disorder are treatment resistant to antidepressants (Berton and Nestler 2006; Fekadu et al. 2009; Little 2009; Mathew 2008). In fact most patients will not respond to first-line classical antidepressants (Connolly and Thase 2011). Sometimes after many years of trials with a variety of medications, a patient will find one drug that they respond well to, but often residual symptoms will still be present and this predicts the possibility of a relapse (Keller 2004; Trivedi et al. 2006). Treatment refractory depression can ultimately require a polypharmacy approach which increases the chance of responsiveness, but is also associated with many unwanted side-effects (Shelton et al. 2010).

Another major problem with currently prescribed classical antidepressants is that although their biochemical actions are nearly immediate, their antidepressant actions take
two to four weeks to occur in responsive patients. This delay can significantly prolong the effort to find an effective treatment, given that many attempts to find a drug that works with a particular patient are often necessary (Rush et al. 2006; Insel and Wang 2009; Fava et al. 2006; Nierenberg et al. 2006; Adell et al. 2005). For antidepressants targeting the serotonergic system, a delayed response in patients may be caused by a slow and gradual desensitization of the serotonin receptor 1A (5HT1A) autoreceptor, which functions to decrease serotonin release (Stahl 1994). Another possible explanation of the delayed response to classical antidepressants, is that it may require much time for these drugs to alter the expression of genes necessary to repair depression-relevant signaling networks (Tanis and Duman 2007). Given that patients endure much suffering during the course of finding an efficacious treatment, there is a consensus in the field of psychiatry that rapid acting antidepressants that also function well in non-responders are strongly needed (Machado-Vieira et al. 2008).

1.2 Ketamine as an antidepressant

1.2.1 Rapidity and responsiveness

Ketamine is a synthetic derivative of phenylcyclidine (PCP), and has a moderate affinity for N-methyl-D-Aspartate (NMDA) receptors (Mathew et al. 2012). It has been safely used as a dissociative anesthetic since the 1960’s, has a plasma half-life of 1-3 hr, and is approved for use in most patient groups (Clements, Nimmo, and Grant 1982; Domino 2010; Lanning and Harmel 1975). In fact it has been administered safely to several million patients without severe adverse effects (Reich and Silvay 1989). In recent breakthrough clinical findings, it was discovered that sub-anesthetic doses of ketamine have rapid antidepressant effects in patients with depression (Correll and Futter 2006;
Berman et al. 2000; Zarate et al. 2006; Krystal 2007; Newport et al. 2015; Fond et al. 2014; Martinowich et al. 2013). Importantly, the antidepressant responses to ketamine 24 hr after treatment are similar to results obtained 6-8 weeks after classical antidepressant administration (Entsuah and Thase 2001; Thase et al. 2005). Measures of suicide ideation are also reduced 24 hr after ketamine treatment (Price et al. 2009; Aan Het Rot et al. 2012; De Maricourt et al. 2014; Thakurta, Das, et al. 2012; Ballard et al. 2014; DiazGranados et al. 2010; Larkin and Beautrais 2011). In addition, ketamine is also effective as an antidepressant in patients with bipolar depression (Diazgranados et al. 2010; Zarate, Brutsche, et al. 2012; Permoda-Osip et al. 2015). Importantly, sub-anesthetic ketamine treatment is often effective in non-responders who have previously failed to respond to several other antidepressants (Krystal, Sanacora, and Duman 2013). In the field of psychiatry, ketamine, due to its profoundly rapid and robust antidepressant effects, has been called “arguably the most important discovery in half a century” (Duman and Aghajanian 2012). This is in part because before these recent findings with ketamine, there had been no breakthrough drug for depression in many decades (Spedding et al. 2005). Some of the factors that contribute to the difficulty of developing new antidepressant drugs have been technical, organizational, and also scientific, since the pathophysiology underlying depression is highly complex (Berton and Nestler 2006; Roth, Sheffler, and Kroeze 2004; Agid et al. 2007; Pangalos, Schechter, and Hurko 2007; Northoff 2013).

The prevailing theory regarding ketamine’s mechanism of action in depression is that it antagonizes NMDA receptors and this leads to an increase in \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling (Sanacora et al. 2008). Blockade of the NMDA receptors on \( \gamma \)-aminobutyric (GABA)-ergic cortical interneurons,
is then thought to release inhibition of pyramidal neurons. This leads to increases in glutamatergic synaptic neurotransmission (Moghaddam et al. 1997; Homayoun and Moghaddam 2007; Chowdhury et al. 2012). Glutamate levels are altered in patients with mood disorders, implicating it in the pathophysiology of depression (Altamura et al. 1993; Auer et al. 2000; Block et al. 2009; Hasler et al. 2007; Kim et al. 1982; Mitani et al. 2006; Sanacora et al. 2004; Skolnick, Popik, and Trullas 2009; Tokita, Yamaji, and Hashimoto 2012; Hashimoto, Sawa, and Iyo 2007). Glutamate is also thought to be involved in the action of classical antidepressants (Hashimoto 2009, 2011b; Javitt 2004; Paul and Skolnick 2003; Sanacora et al. 2003; Zarate et al. 2003). Classical antidepressants typically dampen glutamatergic signaling (Musazzi et al. 2013). Ketamine, however, increased glutamatergic signaling in prefrontal cortex (Lorrain et al. 2003; Chowdhury et al. 2016). Though ketamine has rapid actions even in non-responders, its mechanism of action merits further investigation (Hashimoto 2011a; Krystal 2010; Murrough 2012).

In pre-clinical studies a variety of ketamine doses have been used to induce antidepressant effects (Hayase, Yamamoto, and Yamamoto 2006; Kos et al. 2006; Popik et al. 2008; Browne and Lucki 2013; Mantovani et al. 2003). Both single and multiple treatments of ketamine have been effective at improving measures of depression-like behaviors in rodents (Yilmaz et al. 2002; Engin, Treit, and Dickson 2009; Parise et al. 2013; Garcia et al. 2008; Gideons, Kavalali, and Monteggia 2014). Although, the antidepressant effects of a single ketamine treatment in rodents is not thought to be very long-lasting (Bechtholt-Gompf et al. 2011; Popik et al. 2008).
1.2.2 Limitations of ketamine

Ketamine has much promise as an antidepressant treatment, but it is not without complications (Abdallah et al., 2015). A potential concern for the use of ketamine as an antidepressant is that its effects are short-lived and therefore multiple infusions are necessary to prevent relapse. Initially there was concern that multiple infusions could eventually cause cognitive impairments or a loss of sensitivity to the drug (Krystal et al. 2005; Trujillo, Zamora, and Warmoth 2008; Blier, Zigman, and Blier 2012). Therefore, various non-ketamine based methods, such as add-on drugs or electroconvulsive therapy, have been used with ketamine infusions in order to prevent relapse, yet many have not been successful (Ibrahim et al. 2012; Mathew et al. 2010; Kranaster et al. 2011; A. D. Krystal et al. 2003; Loo et al. 2012; Okamoto et al. 2010; Ostroff, Gonzales, and Sanacora 2005; Wang et al. 2012; Abdallah et al. 2012; Järventausta et al. 2013). Alternative routes of administering ketamine have also been used in order to improve antidepressant outcomes. It is unclear however, if these ketamine administration routes, such as oral, intramuscular, or sublingual, are more effective than the general ketamine infusion approach (De Gioannis and De Leo 2014; Irwin and Iglewicz 2010; Paslakis et al. 2010; McNulty and Hahn 2012; Irwin et al. 2013; Chilukuri et al. 2014; Cusin et al. 2012; Glue et al. 2011; Harihar, Dasari, and Srinivas 2013; Zanicotti, Perez, and Glue 2012; Lara, Bisol, and Munari 2013; Aroni et al. 2009). Intranasal administration of ketamine has also recently been a safe and effective method of delivery (Carr et al. 2004; Lapidus et al. 2014). However, multiple treatments of ketamine by infusion is thought to be safe and effective and is now the status quo (aan het Rot et al. 2010; Diamond et al. 2014; Rasmussen et al. 2013; Messer et al. 2010; Murrough et al. 2013; Liebrenz, Stohler, and Borgeat 2009; Segmiller et al. 2013).
In fact repeat treatments of ketamine by infusion yielded as high as a 71% response-rate without dissociative symptoms in classical antidepressant non-responders (Murrough, Perez, et al. 2013; Thakurta et al. 2012; Murrough, Iosifescu, et al. 2013). Nonetheless, an idealized treatment protocol in depressive patients is still needed, and it has even been suggested that the basis for the existing clinical procedures is somewhat arbitrary (Henderson 2016).

Ketamine also has much potential for abuse (Sanacora and Schatzberg 2015). Indeed, it has been used heavily as a recreational street-drug (Chen et al. 2009; Clatts, Goldsamt, and Yi 2005; Grov et al. 2006; Halkitis, Palamar, and Mukherjee 2007; Kelly, Parsons, and Wells 2006; Leong et al. 2005; Ng et al. 2010; Pantalone et al. 2010; De Luca et al. 2012). There are many social and psychological problems associated with the illicit abuse of ketamine (Copeland and Dillon 2003). For example, recreational use of ketamine can induce cognitive problems that are dissociative and psychotic in nature (Morgan et al. 2012; Morgan, Muetzelfeldt, and Curran 2009, 2010; Morgan, Rees, and Curran 2008; Curran and Monaghan 2001; Morgan, Monaghan, and Curran 2004). It should be noted however, that ketamine abusers typically use much higher amounts of the drug than is used for the treatment of depression (Morgan and Curran 2012). Though the potential for abuse is certainly present, the argument has been made that this potential should not preclude further in depth studies of ketamine as an antidepressant (Nutt, King, and Nichols 2013).

The potential for increased psychotic and dissociative symptoms is also of concern when ketamine is used in the clinic for depression, though its effects on the cognition of healthy subjects has been studied fairly well (Krystal et al. 1999; Morgan, Mofeez, et al. 2004; Parwani et al. 2005; Malhotra et al. 1996; Umbricht et al. 2000). However, there are
some reports that ketamine, even when used at sub-anesthetic doses, can produce psychotic
effects in patients with mood disorders (Krystal et al. 1994; Adler et al. 1998; Lahti et al.
2001; Newcomer et al. 1999; Krystal, D’Souza, et al. 2003). In fact, ketamine is used as an
experimental model for schizophrenia in rodents (Gunduz-Bruce 2009; Anticevic et al.
2012; Lahti et al. 1995). Some groups think that ketamine treatment-induced
psychomimetic symptoms may be caused by a particular ketamine enantiomer, which when
removed from ketamine infusions could result in antidepressant effects without the
psychomimetic effects (White et al. 1980; Paslakis et al. 2010; Paul et al. 2009; Yang et al.
2015). Another possible explanation for these psychomimetic effects is that they are caused
by ketamine’s high promiscuity and affinity for many other neurotransmitter receptors, one
of which may cause the unwanted side-effects (Kapur and Seeman 2002; Hirota and
Lambert 1996; Chen, Shu, and Bayliss 2009; Tso et al. 2004). Complicating the picture
even further is the finding that the antidepressant effects of ketamine are correlated with
its psychomimetic effects, suggesting that these outcomes may be necessarily linked (Sos
et al. 2013; Luckenbaugh et al. 2014). Other reports regarding the mental side effects of
sub-anesthetic ketamine doses used for depression treatment are more optimistic, though
this sentiment is not shared by all practitioners and requires further study (Perry et al. 2007;
Wan et al. 2015; Niciu et al. 2013). Thus, the discovery of drugs that produce ketamine’s
antidepressant effects without psychomimetic effects is of high interest (Dolgin 2013).

1.2.3 Other NMDA antagonists

Ketamine is a NMDA receptor antagonist and inhibition of NMDA receptors is
thought to be a reasonable approach for developing rapid antidepressants (Zarate, Charney,
and Manji 2007). Ketamine also rapidly increases AMPA receptor activity (Maeng et al. 2008; El Iskandrani et al. 2015). Therefore, ketamine’s mode of action results in an increased AMPA/NMDA activity ratio, an effect that rapidly induces antidepressant effects (Tizabi et al. 2012; Akinfiresoye and Tizabi 2013; Andreasen et al. 2013; Du et al. 2006). Importantly, ketamine’s effect on the activity of these receptors and the resultant antidepressant effects of this, may not require time-consuming alterations in gene expression characteristic of the actions of classical antidepressants (Duman and Voleti 2012).

There are several examples of other NMDA antagonists that induce antidepressant effects, further supporting the hypothesis that NMDA receptors have a primary role in ketamine’s antidepressant effects. The NMDA antagonist Ro 25-6981, for example, has had antidepressant effects in preclinical studies (Li et al. 2010, 2011). Ro 25-6981-like drugs were also effective in patients for treating depression, but were associated with some side-effects (Ibrahim, Diaz Granados, et al. 2012; Preskorn et al. 2008). The NMDA antagonist memantine also showed some antidepressant effects preclinically (Quan et al. 2011; Réus et al. 2010; Gideons, Kavalali, and Monteggia 2014). But memantine had limited antidepressant effects in patients, though some studies were successful (Anand et al. 2012; Kollmar et al. 2008; Zarate, Singh, Quiroz, et al. 2006; Lenze et al. 2012; Stevens et al. 2013; Muhonen, Lahti, et al. 2008; Muhonen et al. 2008). Some other NMDA antagonists, such as MK-801 and GLYX-13, were successful at producing antidepressant effects in animal models as well (Trullas and Skolnick 1990; Burgdorf et al. 2013). Another NMDA antagonist, lanicemine, has kinetics similar to ketamine, but surprisingly has much less antidepressant efficacy. The reason for this difference is not clear (Iadarola et al. 2015;
Mealing et al. 1999; Sanacora et al. 2014; Zarate et al. 2013). Since NMDA antagonism increases AMPA activity, it is also thought that the administration of AMPA potentiators may be a valid treatment for depression (Bleakman, Alt, and Witkin 2007). Indeed, several AMPA potentiators have been reported to induce antidepressant effects in rodent models (Li et al. 2001).

1.2.4 BDNF and ketamine

Another effect of ketamine is the up-regulation of brain-derived neurotrophic factor (BDNF). In humans a deficiency in BDNF activity may contribute to depression (Duman 2002; Hashimoto et al. 2004; Autry and Monteggia 2012; Gatt et al. 2009; Chen et al. 2001; Dwivedi et al. 2003; Karege et al. 2002; Shimizu et al. 2003; Karege et al. 2005; Monteleone et al. 2008; Kim et al. 2007). Antidepressants, however, can restore BDNF levels in depressed patients (Chen et al. 2001; Duman and Li 2012; Sen, Duman, and Sanacora 2008; Shimizu et al. 2003; Duman and Monteggia 2006). Ketamine also increases BDNF in patients (Duncan et al. 2013). In people carrying the Val66Met BDNF mutation, ketamine is less effective, indicating that BDNF is important for ketamine’s antidepressant effect (Laje et al. 2012). BDNF levels in patients are sometimes correlated with ketamine responsiveness, though this is not always the case (Machado-Vieira, Yuan, et al. 2009; Haile et al. 2014).

In preclinical studies, ketamine treatments increase BDNF in an AMPA receptor-dependent manner (Yang, Hu, et al. 2013; Zhou et al. 2014; Monteggia and Zarate 2015; Björkholm and Monteggia 2015; Jourdi et al. 2009; Legutko, Li, and Skolnick 2001). Importantly, methods that impair BDNF up-regulation block the antidepressant effect of
ketamine in rodents, suggesting that BDNF is necessary for these effects (Taliaz et al. 2010; Lepack et al. 2015; Lindholm et al. 2012). Ketamine can also restore BDNF levels in stressed animal that have depression-like behavior and reduced BDNF expression (Zhang et al. 2015; Réus et al. 2015). Ketamine may increase BDNF levels by de-suppressing its translation (Kavalali and Monteggia 2012; Autry et al. 2011; Monteggia, Gideons, and Kavalali 2013).

1.2.5 mTOR and ketamine

Another mechanism by which ketamine is thought to exert its effects is by activating mechanistic target of rapamycin (mTOR) signaling (Dwyer and Duman 2013). Levels of mTOR have been found to be reduced in postmortem brains of patients with major depression, implicating it in the pathophysiology of the disease (Jernigan et al. 2011). Ketamine increases peripheral phosphorylation (activation) of mTOR in patients. Thus ketamine may counteract decreased mTOR signaling in depressed patients, an effect that could be important for its antidepressant qualities (Denk et al. 2011). In recent years, the mTOR pathway has been suggested to be especially important for the action of fast acting antidepressants (Cryan and O’Leary 2010). This idea is supported by the finding that ketamine, but not some classical antidepressants, activated mTOR in rodent brain (Park et al. 2014). Also in rodent brain, ketamine administration activates mTOR within 30 min, providing further evidence that it may be involved in the rapid antidepressant effects of ketamine (Li et al. 2010).
1.2.6 Inflammation and synaptogenesis and ketamine

Another mechanism by which ketamine is thought to exert its effects is by acting as an anti-inflammatory agent (Loix, De Kock, and Henin 2011). It is well-accepted that the immune system and inflammation are implicated in the etiology of depression and affect the outcome of antidepressant treatments (Cattaneo et al. 2013; Hepgul et al. 2013; Zunszain, Hepgul, and Pariante 2013; Zunszain et al. 2011; Miller, Maletic, and Raison 2009). Ketamine has anti-inflammatory properties and reduces inflammatory cytokines in patients (Dale et al. 2012; Kawasaki et al. 1999). In animal models, ketamine decreases inflammatory cytokines in brain areas such as hippocampus that contribute to depression-like behavior (Wang et al. 2015; Zhu et al. 2015; Lankveld et al. 2005; Li et al. 1997; Taniguchi et al. 2003; Walker et al. 2013; Zunszain et al. 2013). The mechanism behind ketamine’s anti-inflammatory properties could involve reduced NF-κB signaling (Chang et al. 2010; Welters et al. 2010; Wu et al. 2012).

In preclinical studies chronic stress is associated with a host of prefrontal cortex and hippocampal tissue abnormalities, which include reduced volumes, connections, cell numbers and neurogenesis rates (McEwen 2008; Shansky and Morrison 2009; Liu and Aghajanian 2008; Cook and Wellman 2004; Duman 2014b; Samuels and Hen 2011). Antidepressants can restore these stress-induced pathologies (Bessa et al. 2008; Duman and Duman 2015; McAvoy et al. 2015; Banasr, Dwyer, and Duman 2011; Morais et al. 2014). Ketamine treatment has also been found to improve measures of some of these pathologies, such as synaptogenesis and neurogenesis rates (Keilhoff et al. 2004; Belujon and Grace 2014). These morphological changes were induced more quickly by ketamine
than by other antidepressants, however, this did not occur at the rapid rate at which ketamine induces antidepressant behavior (Li et al. 2010, 2011; Duman et al. 2012).

### 1.3 Glycogen synthase kinase-3, depression, and ketamine

#### 1.3.1 GSK3 and depression

Glycogen synthase kinase-3 (GSK3) designates two highly homologous isoforms, GSK3α and GSK3β, that share many functions and were first identified in the 1980’s for the ability to phosphorylate glycogen synthase (Embi et al. 1980; Cohen and Frame 2001). They are now known to phosphorylate over 100 cellular targets (Pilot-Storck et al. 2010). GSK3 is, very unusually, a constitutively partially active kinase and thus must be very tightly regulated within the cell due to its many targets (Doble and Woodgett 2003). GSK3α and GSK3β are activated by phosphorylation on Tyr279 or Tyr216 respectively (Hughes et al. 1993; Wang et al. 1994). The most well-studied mechanism of GSK3 regulation is the inhibitory phosphorylation of GSK3 on a N-terminal serine, serine-9 of GSK3β and serine-21 of GSK3α (Plyte et al. 1992; Sutherland et al. 1993). This modification impedes GSK3’s ability to phosphorylate most of its substrates by acting as a pseudosubstrate (Kockeritz et al. 2006). There are a number of kinases whose activities result in this kind of inhibition. For example, protein kinase A, protein kinase C, and Akt are all able to phosphorylate GSK3’s inhibitory serine (Goode et al. 1992; Sutherland et al. 1993; Fang et al. 2000). The importance of GSK3 inhibition can be studied very specifically by using GSK3 knockin mice in which the serine-9 of GSK3β and serine-21 of GSK3α are mutated to alanines, thus preventing all inhibitory serine-phosphorylation (McManus et al. 2005). GSK3 knockin mice reproduce normally and do not display any
gross behavioral or physical phenotypes (McManus et al. 2005), and are physiologically relevant since they express GSK3 in brain at levels that are identical to wild-type mice (Polter et al. 2010). GSK3β knockout (KO) mice however are not viable, whereas GSK3α KO are (Hoeflich et al. 2000; MacAulay et al. 2007).

Increasing evidence has linked abnormally active GSK3 to mood disorders. One piece of evidence for this relationship comes from studies of lithium whose mood stabilizing effects were first recognized in guinea pigs (Cade 1949). Lithium was later found to be a GSK3 inhibitor (Klein and Melton 1996; Stambolic et al. 1996). It is also the most effective drug for suicide prevention (Cipriani et al. 2005; Li and Jope 2010, Cipriani et al. 2013; Beurel and Jope 2014). Patients treated with lithium have increased inhibition of GSK3 in the periphery (Li et al. 2007). Also linking GSK3 to mood disorders is that in patients with major depression, GSK3 polymorphisms have been linked to the onset of the disease (Benedetti et al. 2004; Saus et al. 2010), to response outcomes with antidepressants (Adli et al. 2007; Tsai et al. 2008), and to cortical volume (Inkster et al. 2009). Further, in postmortem cortical samples from patients with major depression, GSK3 activity is increased relative to controls (Karege et al. 2007, 2012). Assessing GSK3 activity after death can be difficult though, since its phosphorylation levels rapidly decline (Li et al. 2005; Karege et al. 2012). Therefore the involvement of GSK3 in depression and its potential as a drug target for antidepressants are strong (Gould 2006; Gould et al. 2006).

It is not feasible to perform experiments with human subjects to rigorously determine the role of GSK3 in depression. Therefore, rodents are used even though they are imperfect for modeling human depression (Cryan and Mombereau 2004; Matthews et al. 2005; Kalueff et al. 2007; Nestler and Hyman 2010). Many mouse behavioral tests can
be used to assess depression-like phenotypes (Crawley and Paylor 1997). Some of these
tests, which include the forced swim test (Porsolt et al. 1977; Cryan et al. 2002), the tail
suspension test (Porsolt 2000; Crowley et al. 2004; Cryan et al. 2005), and the learned
helplessness test (Chourbaji et al. 2005), have been pivotal for showing that GSK3
inhibition reduces depression-like behavior.

Lithium treatment of mice reduces depressive-like behaviors in the forced swim
test (O’Brien et al. 2004), the tail suspension test (Pan et al. 2011), and the learned
helplessness test (Beurel et al. 2011). Due to these exciting results and others, a realization
of the therapeutic potential of pharmacological inhibition of GSK3 led to the testing of
many more promising GSK3 inhibitors (Eldar-Finkelman and Martinez 2011). Various
GSK3 inhibitors such as AR-A014418, L803-mts, TDZD-8 and NP031115 were all shown
to have anti-depressive effects on rodent depression-like behaviors (Beaulieu, Zhang, et al.
2008; Gould et al. 2004; Kaidanovich-Beilin et al. 2004; Rosa et al. 2008). Additionally,
other drugs used to treat mood disorders have been shown to inhibit GSK3. For example,
the mood stabilizer valproate reduces depressive-like behaviors in the forced swim test and
the learned helplessness test and inhibits GSK3 (Kobayashi et al. 2012; Xing et al. 2011;
De Sarno, Li, and Jope 2002). The classical antidepressant fluoxetine also increases serine-
phosphorylation of GSK3 in brain and improves multiple measures of depressive-like

Manipulations of GSK3 cellular dosing also provide further evidence for GSK3’s
critical role in mood-related behaviors. Mice that are heterozygous GSK3β KO’s have
decreased depression-like behavior in the forced swim test and the tail suspension test
(Beaulieu, Marion, et al. 2008; O’Brien et al. 2004), and knockdown of GSK3 using
injections of lentivirus-expressing short-hairpin RNA decreases depression-like behaviors in the forced swim test and the tail suspension test (Omata et al. 2011). Conversely, mice with constitutively active or over-expressed GSK3 have increased susceptibility to depression-like behaviors (Polter et al. 2010; Wilkinson et al. 2011; O’Brien et al. 2011). Therefore, it is now recognized that numerous treatments that improve mood-related behaviors also increase inhibition of GSK3 (Beaulieu 2012).

1.3.2 Ketamine and GSK3

Lithium has been reported to increase the efficacy of classical antidepressants in non-responder patients (Bauer et al. 2010, 2013; Chang, Sato, and Han 2013; Lam et al. 2009). In pre-clinical studies augmentation of ketamine treatments with other GSK3 inhibitors, including lithium, results in synergistic antidepressant effects (Ghasemi, Raza, and Dehpour 2010; Liu et al. 2013; Chiu et al. 2014; Scheuing et al. 2015). Augmentation of ketamine with lithium also reduces the psychomimetic effects of ketamine in rodents (Chan et al. 2012). An interesting molecular finding in depressed patients that may also point to synergy between ketamine and GSK3 inhibitors, was that ketamine treatment increased GSK3 inhibition in the periphery, an effect that is also achieved with lithium treatment (Yang et al. 2013).

In animal models both ketamine and lithium treatments result in GSK3 inhibition in brain (Beurel et al. 2011; Zhou et al. 2014). Several other NMDA receptor antagonists such as memantine, MK-801 and 7-chlorokynurenic acid also increase GSK3 inhibition in brain (De Sarno et al. 2006; Seo et al. 2007; Yu et al. 2011; Zhu et al. 2013). Conversely, NMDA treatments can activate GSK3, and stress activates NMDA receptors (Luo et al.
Other NMDA antagonists besides ketamine and lithium, such as memantine and MK-801, have antidepressant effects as well (Quan et al. 2011; Réus et al. 2010; Trullas and Skolnick 1990). In fact, ketamine’s antidepressant effects are dependent on GSK3 inhibition (Beurel et al. 2011). Taken together, these findings suggest that ketamine’s antidepressant effects are likely mediated at least in part by NMDA antagonism-mediated inhibition of GSK3.

It is unclear how NMDA antagonism-mediated inhibition of GSK3 results in antidepressant effects. However, antidepressant responses induced by ketamine in mouse models are dependent on AMPA receptor activity. This was found by using the AMPA receptor blocker 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione NBQX, that inhibits the antidepressant effects of ketamine (Li et al. 2010; Machado-Vieira et al. 2009; Maeng et al. 2008; Autry et al. 2011; Koike, Iijima, and Chaki 2011; Maeng and Zarate 2007). Also, the antidepressant effects of lithium were dependent on AMPA receptor signaling (Gould et al. 2008). These results suggest that there may be a link between NMDA antagonism, GSK3 inhibition and AMPA receptor activity.

One possible link could be depression of the synapse, a process dependent on AMPA receptor internalization that is increased by stress and contributes to depression (Holderbach et al. 2007; Marsden 2013). Activation of NMDA receptors increases the internalization of AMPA receptors and the formation of synaptic depression. Interestingly, GSK3 activity also promotes synaptic depression (Bradley et al. 2012; Peineau et al. 2007, 2009; Liu et al. 2013; 2014). Conversely, synaptic depression is impaired by GSK3 inhibition (Peineau et al. 2007; Peineau et al. 2009). It is unclear though how GSK3 inhibition prevents these synaptic effects. Postsynaptic density protein 95 (PSD-95), a
synaptic protein which plays a role in regulating AMPA receptor internalization, can be regulated by its phosphorylation levels at Thr-19. GSK3-mediated phosphorylation of PSD-95 on Thr-19 increases internalization of AMPA receptors and its subunits such as GluR1 (Nelson et al. 2013). Also, sub-anesthetic and antidepressant doses of ketamine can block or prevent synaptic depression that is induced by stress (Li et al. 2011; Izumi and Zorumski 2014). Therefore, ketamine-mediated antagonism of NMDA receptors leads to inhibition of GSK3 which may in turn rapidly reduce AMPA receptor internalization and depression-like behavior.

GSK3 inhibition also increases long-term synaptic potentiation (Bradley et al. 2012; Zhu et al. 2007). Therefore, inhibition of GSK3 by ketamine, may rapidly promote AMPA receptor activity that could lead to antidepressant effects and long-term synaptic potentiation. Since it is thought that multiple molecular events contribute to ketamine’s rapid antidepressant effects, and ketamine has restorative and sustained antidepressant effects as well, it could be that GSK3 inhibition is the keystone of these multiple mechanisms.
Chapter – Materials and Methods

2.1 Methods

2.1.1 Mice

Male, adult (8-10 weeks old) C57BL/6 wild-type and homozygous GSK3α/β21A/21A/9A/9A knockin mice (McManus et al. 2005) were used. GSK3 knockin mice develop and reproduce normally with no overt phenotype (McManus et al. 2005; Eom and Jope 2009; Polter et al. 2010). Mice were housed in groups of 3-5 in standard cages in light and temperature controlled rooms and were treated in accordance with National Institutes of Health and the University of Miami Institutional Animal Care and Use Committee regulations. Mice were treated intraperitoneally (i.p.) with vehicle, ketamine (10 mg/kg), 2,3-dihydroxyl-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2, 3-dione (NBQX, 10 mg/kg; Tocris) or fluoxetine (20 mg/kg; National Institute of Mental Health Chemical Synthesis and Drug Supply Program). The specific GSK3 inhibitor L803-mts, a substrate-competitive peptide inhibitor (Plotkin et al. 2003), was dissolved in DDX1 vehicle (128 mM NaCl, 8 mM citric acid, 17 mM Na2HPO4, 0.0005% benzalkonium chloride), using a protocol (60 µg/mouse; intranasal, 24 hr pretreatment) that was effective in previous studies (Kaidanovich-Beilin et al. 2004; Beurel et al. 2013). For miRNA antagonism experiments, 2 and 4 hr after ketamine was administered (10 mg/kg; i.p.), mice received a miR448-3p mirVANA inhibitor (5 mg/kg hsa-miR-448 in DDX1; intranasal; 5 µL/nostril in each nostril; ThermoFisher Ambion #MH10520), which was designed to specifically bind to and inhibit endogenous miRNA-448. Mice were treated with IGF2 siRNA 2 hr after ketamine treatment (10 mg/kg; i.p.) and again 2 hr before exposure to escapable foot shocks
(10 µg/mouse/treatment in DDX1; intranasal; 5 µL/nostril; #J-043709-09, GE Healthcare Dharmacon, Inc). Scrambled siRNA (AM4642; Ambion) was used for control mice.

### 2.1.2 Western blotting

The hippocampus was rapidly dissected in ice-cold phosphate-buffered saline and stored at -80°C after being snap-frozen. Brain regions were homogenized in lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. Tissue lysates were centrifuged at 14000 rpm for 10 min at 4°C to remove the insoluble fraction. The Bradford protein assay was used to determine the concentration of protein in the supernatants. Hippocampal proteins (5-20 µg) were resolved with SDS-PAGE, transferred to nitrocellulose membranes and then immunoblotted. Antibodies used were to phospho-Ser9-GSK3β (#9336, Cell Signaling Technology), total GSK3β (#610202, BD Transduction Laboratories), phospho-Ser21-GSK3α (#9316, Cell Signaling Technology) and total GSK3α/β (clone 4G-1E, #05-412Millipore). The membranes were rebotted with β-actin (#A5441, Sigma Aldrich) to ensure equal protein loading.

### 2.1.3 Quantitative real-time polymerase chain reaction

Total RNA from mouse hippocampus and prefrontal cortex was isolated by TRIzol extraction according to the manufacturer’s instructions (Invitrogen). RNA was converted to cDNA using ImProvII reverse transcriptase (Promega) according to the manufacturer’s
instructions. Quantitative changes in the mRNA levels were determined by real-time PCR using Taqman gene expression assays for 5HTR2C (00434127) and 18S (4332641) according to the manufacturer’s instructions. qRT-PCR was performed using SYBR Green master mix, according to the manufacturer’s instructions (Applied Biosystems), and the primers described below. Quantification of cDNAs was made using the $2^{-\Delta\Delta Ct}$ method. The primers for IGF2 were: (Forward-TGTGCTGCATCGCTGCTTAC; Reverse-CGGTCCGAACAGACAAACTGA). The primers used for GAPDH, which was used as an internal control, were: (Forward-AGGTCGGTGTGAACGGATTTG ; Reverse-TGTAGACCATGTAGTTGAGGTCA).

Low molecular weight RNA fractions were isolated from total RNA using the NanoSep 100K spin column (Pall). Low molecular weight RNA fractions were then concentrated using the RNeasy MinElute spin column (Qiagen). Low molecular weight RNAs were converted to cDNA using the Taqman miRNA reverse transcription kit. Quantitative changes in the miRNA levels were determined by real-time PCR using Taqman miRNA expression assays for miRNAs 193a-3p (002250), 448-3p (001029), 764-5p (002031), 1264-3p (464426), 1298-5p (002861), 1912-3p (464370), 1941-3p (121130) and for the small nuclear RNA U6 (001973), according to the manufacturer’s instructions. Experiments were performed on a 7900 HT Fast (Applied Biosystems) instrument.

### 2.1.4 Enzyme-linked immunosorbent assay (ELISA)

Proteins from hippocampal extracts were prepared as described above, and ELISAs were performed according to the manufacturer’s instructions (RayBiotech) using 100 μg protein.
2.1.5 Learned helplessness

Inescapable foot shocks were used to induce learned helplessness depression-like behavior in mice as described previously (Polter et al. 2010; Beurel et al. 2013). Mice were placed inside a Modular Shuttle Box (Med Associates Inc., St. Albans, VT, USA), with the gate between the two chambers closed. 180 inescapable foot shocks were delivered at an amplitude of 0.3mA, at random durations of 6-10 sec, with a randomized inter-shock interval of 5-45 sec. Twenty-four hr later, depression-like behavior was tested using the shuttle box by exposing the mice to 30 escapable 0.3mA foot shocks that lasted for a maximum of 24 sec. Latency to escape from these foot shocks was recorded using the MED-PC® Data Acquisition Software. An escape failure was tallied if a mouse did not escape within the 24 sec time limit. Mice with greater than 15 escape failures were defined as having acquired learned helplessness depression-like behavior.

2.1.6 Novelty suppressed feeding

The novelty suppressed feeding test was carried out as previously described (Beurel et al. 2013). Mice were weighed prior to and after a 24 hr period of food deprivation to assure that body weight loss was equal between groups. During testing mice were allowed to explore a novel open field arena with a food pellet placed in the center on a small platform for a maximum of 10 min. Latency to begin feeding on the pellet was recorded. Mice were then returned to their home cages, and latency to feed and total food consumed was monitored during a 5 min period in order to control for potential differences in feeding not due to the novel environment.
2.1.7 **Statistical analysis**

Statistical significance was analyzed with a Student’s *t* test or one-or two way ANOVA with a Bonferroni post hoc test using Prism software, and *p*<.05 was considered significant.
Chapter – Ketamine up-regulates a cluster of intronic miRNAs within the serotonin receptor 2C gene by inhibiting glycogen synthase kinase-3

3.1 Summary

We examined mechanisms that contribute to the rapid antidepressant effect of ketamine in mice that is dependent on glycogen synthase kinase-3 (GSK3) inhibition. We measured serotonergic (5HT)-2C-receptor (5HTR2C) cluster miRNA levels in mouse hippocampus after administering an antidepressant dose of ketamine (10 mg/kg) in wild-type and GSK3 knockin mice, after GSK3 inhibition with L803-mts, and in learned helpless mice. Ketamine up-regulated cluster miRNAs 448-3p, 764-5p, 1264-3p, 1298-5p, and 1912-3p 2- to 11-fold. This up-regulation was abolished in GSK3 knockin mice that express mutant constitutively active GSK3. The GSK3 specific inhibitor L803-mts was antidepressant in the learned helplessness and novelty suppressed feeding depression-like behaviors and up-regulated the 5HTR2C miRNA cluster in mouse hippocampus. After administration of the learned helplessness paradigm mice were divided into cohorts that were resilient (non-depressed) or were susceptible (depressed) to learned helplessness. The resilient, but not depressed, mice displayed increased hippocampal levels of miRNAs 448-3p and 1264-3p. Administration of an antagonist to miRNA 448-3p diminished the antidepressant effect of ketamine in the learned helplessness paradigm, indicating that up-regulation of miRNA 448-3p provides an antidepressant action. These findings identify a new outcome of GSK3 inhibition by ketamine that may contribute to antidepressant effects.

3.2 Background

Ketamine produces a rapid antidepressant effect in many patients with major depressive disorder or bipolar disorder that occurs within a time-frame of hours, as opposed to several weeks required for conventional antidepressants (Niciu et al. 2014; Newport et al. 2015;
Scheuing et al. 2015). The mechanism underlying this action of ketamine remains to be established, but it has been reported to depend on the inhibition of glycogen synthase kinase-3 (GSK3)(Beurel et al. 2011). This is of interest because substantial evidence has implicated abnormally active GSK3 as a contributory factor for mood disorders, and inhibition of GSK3 as contributing to therapeutic actions of mood stabilizers and antidepressants (Jope 2011). GSK3 is primarily regulated by phosphorylation of an N-terminal serine in each of the two isoforms, serine-21 in GSK3α and serine-9 in GSK3β (Beurel et al. 2015). Phosphorylation on these sites inhibits the activity of GSK3, and diminished serine-phosphorylation is thought to contribute to hyperactive GSK3 associated with mood disorders. The functional importance of this mechanism of GSK3 inhibition can be studied using GSK3 knockin mice in which serine-9 of GSK3β and serine-21 of GSK3α are mutated to alanines, thus preventing inhibitory serine-phosphorylation. GSK3 knockin mice reproduce normally and do not display any overt behavioral or morphological phenotypes (McManus et al. 2005), and are physiologically relevant because they express GSK3 at levels that are identical to wild-type mice (Polter et al. 2010). Administration of the antidepressants fluoxetine or imipramine to mice increased the inhibitory serine-phosphorylation of GSK3 in mouse brain (Li et al. 2004). Inhibitory serine-phosphorylation of GSK3 in mouse brain also was found to occur rapidly after administration of ketamine, and the rapid antidepressant effect of ketamine in mice is dependent on inhibition of GSK3 (Beurel et al. 2011). Also, GSK3 inhibitors enhanced the antidepressant effects of sub-therapeutic levels of ketamine in mice, suggesting that the agents cooperate via a common mechanism (Ghasemi, Raza, and Dehpour 2010; Liu et al. 2013; Chiu et al. 2014). Furthermore, in patients with depression, ketamine administration
increased inhibitory serine-phosphorylation of GSK3 in lymphocytes (Yang et al. 2013). Taken together, these findings demonstrate that GSK3 is inhibited by ketamine and that this may contribute to the antidepressant action of ketamine.

Although inhibition of GSK3 by ketamine is required for the rapid antidepressant effect in mice, it is unclear how this relieves depression. We were interested in the possibility that it may involve regulation of the serotonin (5HT) 2C receptor (5HTR2C). The 5HTR2C has been linked to modulation of mood-relevant behaviors in rodents and suggested to be linked to depression, but this remains controversial and it is unclear what effect antidepressants have on 5HTR2C expression (Van Oekelen et al. 2003; Chagraoui et al. 2016). The 5HTR2C gene is located on the X-chromosome and the 5HTR2C gene locus hosts five intronic miRNAs, 448-3p, 764-5p, 1264-3p, 1298-5p, and 1912-3p (Eacker et al. 2011; Hinske et al. 2014). These miRNAs share the same transcriptional direction as the 5HTR2C gene, raising the possibility that their expression may be coordinately regulated with the expression of 5HTR2C mRNA. The five intronic miRNAs in the 5HTR2C gene are expressed in human brain (Ziats and Rennert 2014) and in mouse brain, where their expression correlates with 5HTR2C expression (Hinske et al. 2014).

Here we report that administration of an antidepressant dose of ketamine leads to increased expression of the cluster of five intronic miRNAs within the 5HTR2C gene in mouse hippocampus, that this requires ketamine-induced inhibition of GSK3, and that administration of an antagonist to miRNA 448-3p diminished the antidepressant effect of ketamine in the learned helplessness paradigm.
3.3 Results

3.3.1 Ketamine up-regulates 5HTR2C mRNA and a cluster of five miRNAs

Examination of 5HTR2C mRNA expression 24 hr after treatment with a sub-anesthetic, antidepressant dose of ketamine (10 mg/kg; i.p.), revealed a modest, but significant, increase in 5HTR2C mRNA levels (1.5 ± 0.1-fold of control levels) in mouse hippocampus (Figure 1A). We used GSK3 knockin mice, in which the regulatory serines in both isoforms of GSK3 are mutated to alanine to abrogate inhibitory serine-phosphorylation, to test if the regulation of the 5HTR2C mRNA by ketamine requires inhibition of GSK3. This demonstrated that up-regulation of 5HTR2C mRNA induced by ketamine treatment was dependent on inhibition of GSK3 because ketamine treatment did not increase 5HTR2C mRNA levels in the hippocampus of GSK3 knockin mice.

Introns in the 5HTR2C gene code for a cluster of five miRNAs (Hinske et al. 2014), which we examined for changes in expression following administration of ketamine. Treatment with ketamine (10 mg/kg, 24 hr) significantly increased the levels of all five miRNAs in mouse hippocampus, increasing miRNA 764-5p 2-fold, 1912-3p 6-fold, 1264-3p 5-fold, 1298-5p 7-fold, and 448-3p 11-fold (Figure 1A). Two miRNAs not within the 5HTR2C cluster, 193a-3p and 1941-3p, were unaltered or down-regulated by ketamine treatment (Figure 1B), demonstrating selectivity of the response to ketamine. GSK3 knockin mice were used to test if the up-regulation of the 5HTR2C cluster miRNAs by ketamine requires inhibition of GSK3. Without drug treatment, levels of all five 5HTR2C cluster miRNAs were equivalent in the hippocampi of wildtype mice and GSK3 knockin mice except for a lower level of 764-5p in GSK3 knockin mice (Figure 1A). The ketamine treatment-induced increases in all five miRNAs were abolished in GSK3 knockin mice,
Figure 1. Ketamine treatment up-regulates 5HTR2C mRNA and the 5HTR2C cluster miRNAs in mouse hippocampus

Wild-type (n=12-20) and GSK3 knockin mice (n=6-8) were treated with ketamine (10 mg/kg; i.p.) and were sacrificed after 24 hr. (A) Expression levels of 5HTR2C mRNA and 5HTR2C cluster miRNAs (764-5p, 1912-3p, 1264-3p, 1298-5p and 448-3p) in the hippocampus. Data represent Means±SEM, (two-way ANOVA (genotype X treatment); 764-5p: F_{genotype}(1,40)=11.40; 1912-3p: F_{treatment}(1,38)=4.330; 1264-3p: F_{interaction}(1,38)=6.048; 1298-5p: F_{interaction}(1,42)=4.608; 448-3p: F_{interaction}(1,42)=6.347; Bonferroni post-hoc test, *p<0.05, compared to saline-treated wild-type mice, **p<.05, compared to ketamine-treated wild-type mice). (B) Expression levels of miRNAs 193a-3p and 1941-3p in the hippocampus of wild-type mice. Data represent Means±SEM, n=3-4 (Student’s t test; 1941-3p: t(4)=4.901, *p<.05). (C) Expression levels of 5HTR2C mRNA and the 5HTR2C cluster miRNAs (764-5p, 1912-3p, 1264-3p, 1298-5p and 448-3p) in the prefrontal cortex of wild-type mice (Means±SEM). (D). Expression levels of 5HTR2C mRNA and the 5HTR2C cluster miRNAs in hippocampus and prefrontal cortex from untreated wild-type mice (n=4-6)(Means±SEM).
demonstrating the requirement for ketamine-induced inhibition of GSK3 for the miRNAs to be up-regulated. In contrast to the hippocampus, ketamine treatment did not alter 5HTR2C mRNA expression or the levels of the 5HTR2C cluster miRNAs in the prefrontal cortex (Figure 1C). Basal miRNA levels were not significantly different in the hippocampus and the prefrontal cortex (Figure 1D). Thus, ketamine up-regulates the expression of 5HTR2C mRNA and the 5HTR2C cluster of five miRNAs in mouse hippocampus and these responses are dependent on ketamine-induced inhibition of GSK3.

We examined the time-dependence of ketamine-induced up-regulation of the 5HTR2C cluster miRNAs. In the hippocampus, the levels of all five miRNAs did not change 30 min or 3 hr after ketamine administration, but were significantly elevated after 24 hr, and levels returned towards basal levels after 48 hr except for 764-5p, which was still significantly up-regulated at this time (Figure 2A). These results demonstrated that miRNA up-regulation was maximal 24 hr after ketamine administration.
In order to determine if the ketamine-induced up-regulation of the 5HTR2C cluster miRNAs was unique or also was a response to a classical antidepressant, we tested the effect of chronic fluoxetine treatment on the 5HTR2C cluster miRNAs. Expression of the 5HTR2C cluster miRNAs did not significantly change either 1 or 24 hr after acute fluoxetine treatment (Figure 2B) or after two weeks of chronic fluoxetine treatment (Figure 2C).

Ketamine is administered therapeutically to patients with depression. Therefore, we tested if ketamine administration up-regulated the cluster miRNAs in mice that were previously rendered depressed by induction of learned helplessness. Indeed, ketamine administration up-regulated expression levels of all five cluster miRNAs and 5HTR2C mRNA in learned helpless mice to a similar extent as occurred in untreated mice (Figure 3A).
To test if the miRNA responses to ketamine were mediated by AMPA receptors, mice were pretreated with the AMPA receptor antagonist NBQX (10 mg/kg) for 1 hr followed by administration of a sub-anesthetic, antidepressant dose of ketamine (10 mg/kg), and measurements of miRNAs 24 hr after ketamine treatment. Ketamine significantly up-regulated each of the miRNAs and the 5HTR2C mRNA level, but there was not a significant increase in any of these by ketamine after blockade of AMPA receptors by NBQX (Figure 3B), consistent with previous reports that the antidepressant
effect of ketamine is mediated by signaling through AMPA receptors (Malinow et al., 2002; Maeng et al., 2008; Koike et al., 2011).

### 3.3.2 Inhibition of GSK3 up-regulates the 5HTR2C cluster miRNAs

The lack of up-regulation of the 5HTR2C cluster of miRNAs by ketamine in GSK3 knockin mice indicated that inhibition of GSK3 is necessary for this response. To test if inhibition of GSK3 is sufficient to up-regulate the expression of this cluster of miRNAs, we evaluated the effects of selectively inhibiting GSK3 on susceptibility to the learned helplessness and novelty suppressed feeding (NSF) depression-like behaviors and on expression of the 5HTR2C miRNA cluster by administering the specific GSK3 inhibitor L803-mts using a protocol (60 µg/mouse; intranasal, 24 hr pretreatment) that was effective in previous studies (Kaidanovich-Beilin et al. 2004; Beurel et al. 2013). In the learned helplessness paradigm, 76% of control mice treated with intranasal administration of vehicle developed learned helplessness, which was reduced to 33% by L803-mts administration (Figure 4A). After exposure to the learned helplessness paradigm, vehicle-treated mice had a significantly increased feeding latency in the NSF test when placed in a novel arena containing a food pellet after a 24 hr period of food deprivation, whereas L803-mts treated mice exposed to the learned helplessness paradigm did not (Figure 4B). Control measurements found that there were no differences in weight loss or appetite (feeding in the home cage) between the groups of mice (Figure 4D). Therefore administration of L803-mts confers mice with protection from these two depression-like behavior models. Administration of L803-mts also increased the levels of the cluster miRNAs to a similar extent as that caused by ketamine administration (Figure 4C). Thus, in vivo inhibition of
GSK3 is sufficient to recapitulate the ketamine-induced protection from two depression-like behaviors and up-regulation of expression of the 5HTR2C cluster of miRNAs.
Figure 4. Inhibition of GSK3 attenuates depression-like behaviors and up-regulates expression of the 5HTR2C miRNA cluster

(A) Escape failures in the learned helplessness test for wild-type mice treated with intranasal administration of vehicle (n=21) or L803-mts (n=12). Each symbol represents results from an individual mouse. The dashed line at 15 escape failures indicates the criteria for learned helplessness (Student’s t test; t(31)=2.958; *p<.05. (B) Latency to feed in the novelty suppressed feeding test with (closed bars) or without (open bars) induction of learned helplessness (LH) in wild-type mice treated intranasally with vehicle (Veh) or L803-mts. Data represent Means±SEM, n=8-10 (two-way ANOVA (LH X treatment); Finteraction(1,32)=4.181 ; Bonferroni post-hoc test, *p<.05, compared to the vehicle-treated control mice, **p<.05, compared to vehicle-treated learned helplessness mice). (C) Expression levels of the 5HTR2C cluster miRNAs (764-5p, 1912-3p, 1264-3p, 1298-5p and 448-3p) in the hippocampus from wild-type mice treated intranasally with vehicle (black bars) or L803-mts (white bars). Data represent Means±SEM, n=4 (Student’s t test; 1912-3p: t(6)=2.547; 1264-3p: t(6)=2.589; 1298-5p: t(6)=2.578; 448-3p: t(6)=2.608; *p<.05. (D) Weight loss after 24 hr food deprivation and home cage food consumption after the novelty suppressed feeding test with or without exposure to learned helplessness in wild-type mice treated intranasally with vehicle or L803-mts (n=10 per group)(Means±SEM).
3.3.3 5HTR2C cluster miRNAs expression and learned helplessness

Since administration of an antidepressant dose of ketamine up-regulated the 5HTR2C cluster miRNAs, we tested if their expression levels were altered by the induction of the learned helplessness depression-like behavior. Mice were exposed to inescapable foot shocks followed 24 hr later by exposure to escapable foot shocks, and failure to escape from 15 out of 30 trials of escapable foot shocks is defined as learned helplessness behavior. Mice were grouped into a resilient group that did not display learned helplessness and a learned helpless group, and mice that were not exposed to foot shocks were used as a control group. Twenty-four hr after the learned helplessness paradigm, mice that were resilient had significant increases in the hippocampal expression of miRNAs 1264-3p (7-fold) and 448-3p (5-fold), but not 764-5p, 1912-3p, or 1298-5p compared to control mice (Figure 5A). Thus, stress-induced up-regulation of 1264-3p and 448-3p correlated with resistance to learned helplessness, suggesting that up-regulation of these two miRNAs may contribute to ketamine’s antidepressant effect. To determine if the antidepressant effect of ketamine in mice is dependent upon up-regulation of one of the 5HTR2C miRNAs, we tested if miRNA interference of 448-3p reduced ketamine’s antidepressant effect in the learned helplessness paradigm because it was up-regulated to the greatest extent by ketamine and was up-regulated in resilient mice. 75% of control mice treated with vehicle developed learned helplessness and this was reduced to only 11% of mice treated with ketamine. However, co-treatment of ketamine plus a miRNA 448-3p antagonist resulted in 58% of mice developing learned helplessness (Figure 5B). Therefore miRNA 448-3p
antagonism blocked ~73% of ketamine’s antidepressant effect on this depression-like behavior.
3.4 Discussion

Ketamine can induce a rapid antidepressant effect in patients with mood disorders and in mice with depression-like behaviors (Niciu et al. 2014; Newport et al. 2015; Scheuing et al. 2015). Although the antidepressant mechanism of action of ketamine has yet to be fully established, several studies have linked it to inhibition of GSK3 (Ghasemi et al., 2010; Beurel et al. 2011; Liu et al. 2013; Yang et al. 2013; Chiu et al. 2014; Zhou et al. 2014). Following up that link, here we found that in mouse hippocampus an antidepressant dose of ketamine up-regulated the expression of a cluster of five miRNAs intronic within the 5HTR2C gene, that this was dependent on inhibition of GSK3 and was matched by
administration of a specific GSK3 inhibitor, and that blocking the most highly up-regulated miRNA, 448-3p, diminished the antidepressant action of ketamine in mice.

Selectivity in the up-regulation of the 5HTR2C cluster miRNAs by ketamine treatment was indicated for three aspects of the response: the miRNA cluster, the hippocampus, and the drug. Two miRNAs, 193a-3p and 1941-3p, outside of the 5HTR2C gene were not up-regulated by ketamine treatment, indicating that the effect of ketamine did not extend to all miRNAs. Brain region selectivity was indicated by the up-regulation of the 5HTR2C miRNA cluster in the hippocampus but not in the prefrontal cortex by ketamine treatment, which is in agreement with several previous studies that emphasized hippocampal responses to ketamine administration (Garcia et al. 2008; Maeng et al. 2008; Autry et al. 2011; Tizabi et al. 2012). Although our measurements were made primarily using hippocampal tissue, these results do not exclude similar effects in other brain regions that may contribute to the response to ketamine. Additionally, the classical serotonin-specific re-uptake inhibitor antidepressant fluoxetine did not up-regulate the expression of the 5HTR2C cluster miRNAs, raising the possibility that this response to ketamine may contribute to its antidepressant capacity in patients non-responsive to classical antidepressants.

Expression of 5HTR2C mRNA was also up-regulated in conjunction with the miRNA cluster by treatment with ketamine. The 5HTR2C has been linked to depression in several studies, although its potential regulatory role remains to be firmly established (Chagraoui et al. 2016). Studies in 5HTR2C null mice (Tecott et al. 1995) and 5HTR2C knockin mice (Bombail et al. 2014) may be complicated if some of the phenotypes result in part by altered expression of intronic miRNAs in the 5HTR2C gene. However, since the ketamine-induced
up-regulation of 5HTR2C mRNA was modest compared with the up-regulation of the miRNAs we did not pursue further studies to determine if it contributed to the antidepressant action of ketamine.

The up-regulated expression of the cluster of miRNAs induced by ketamine was linked to the inhibition of GSK3 induced by ketamine by two findings. First, up-regulation of the miRNA cluster did not occur in GSK3 knockin mice, in which GSK3 cannot be inhibited by ketamine (McManus et al. 2005). Second, inhibition of GSK3 by administration of the specific inhibitor L803-mts was sufficient to diminish susceptibility to learned helplessness, a reduction in susceptibility equivalent to that caused by acute administration of ketamine (Beurel et al. 2011). This extends several previous reports that administration of GSK3 inhibitors had antidepressant effects in rodent models of depression. Mouse immobility time in the forced swim test was reduced by several inhibitors of GSK3, including L803-mts (Kaidanovich-Beilin et al., 2004; Shapira et al., 2007), CHIR99021 (Pan et al., 2011), AR-A014418 (Gould et al., 2004; Rosa et al., 2008), NP031115 (Rosa et al., 2008), SB216763 (Li et al., 2014), and lithium (O’Brien et al., 2004; Shapira et al., 2007; Silva et al., 2008; Can et al., 2011; Pan et al., 2011), but not by SB216763 (Ma et al., 2013). Mouse immobility time in the tail suspension test was reduced by TDZD-8 (Beaulieu et al., 2008b) and lithium (Beaulieu et al., 2008), but not by SB216763 (Ma et al., 2013), and lithium reduced mouse escape failures in the learned helplessness test (Beurel et al., 2011; Beurel et al., 2013). Molecular modifications of GSK3 also indicate its inhibition promotes antidepressant effects (reviewed in Jope, 2011). L803-mts also was sufficient to up-regulate the expression of the 5HTR2C cluster miRNAs in the hippocampus to an extent similar to that of ketamine administration. Thus, inhibition of
GSK3 is both necessary and sufficient to reduce susceptibility to learned helplessness and to increase the expression of the 5HTR2C cluster miRNAs.

The key question of whether the ketamine-induced up-regulation of this cluster of miRNAs contributes to the antidepressant response to ketamine was addressed by several approaches. Ketamine administration up-regulated expression of all five cluster miRNAs in mice that were previously rendered learned helpless, demonstrating that ketamine induces this response not only in untreated mice but also in learned helpless mice, which models the clinical situation better than testing responses in control mice. The peak increase in the expression of the 5HTR2C cluster miRNAs occurred 24 hr after ketamine treatment, which matches the time of the antidepressant effect of ketamine treatment in mice (Maeng et al. 2008; Li et al. 2010; Autry et al. 2011; Beurel et al. 2011; Zanos et al. 2016), raising the possibility that the miRNAs contribute to the antidepressant effect. However, in patients ketamine can produce antidepressant effects at shorter times, suggesting that either the kinetics may differ between humans and mice, or that up-regulated miRNAs do not have a role in human antidepressant responses to ketamine. Although not addressed here, it is also possible that ketamine-induced miRNA up-regulation is related to the recently described prophylactic effect of ketamine against stress-induced depressive-like behaviors in mice (Brachman et al. 2016). Although ketamine treatment up-regulated all five 5HTR2C cluster miRNAs, there were several differences among their responses. Up-regulation of 764-5p by ketamine was lower than the other miRNAs in the cluster, 764-5p was the only miRNA in the 5HTR2C cluster that was significantly lower in GSK3 knockin mouse hippocampus compared with wild-type mice, and 764-5p was the only miRNA in the 5HTR2C cluster that did not return to basal levels.
48 hr after ketamine administration. miRNA 448-3p also differed from the other cluster miRNAs in that it displayed the most robust increase after ketamine administration, and is the only miRNA that is in an intronic and coding region of the 5HTR2C gene. Particularly interesting was the finding that after exposure to learned helplessness only miRNAs 448-3p and 1264-3p were up-regulated in resilient mice that exhibited resistance to learned helplessness but not in mice that developed learned helplessness, raising the possibility that one or both of these miRNAs may play a role in promoting resilience. An antidepressant role for miR448-3p was supported by the finding that antagonizing 448-3p counteracted the antidepressant effect of ketamine in the learned helplessness model.

In summary, this study found that ketamine up-regulates a cluster of intronic miRNAs associated with the 5HTR2C gene locus in mouse hippocampus. This ketamine-induced up-regulation is dependent on GSK3 inhibition, is matched by administration of a GSK3 inhibitor, and is diminished by antagonism of miRNA 448-3p. Thus, in mice ketamine-induced up-regulation of miR448-3p appears to contribute to the antidepressant response.
Chapter – Up-regulation of insulin-like growth factor 2 by ketamine requires glycogen synthase kinase-3 inhibition

4.1 Summary

An antidepressant dose of the rapidly-acting ketamine inhibits glycogen synthase kinase-3 (GSK3) in mouse hippocampus, and this inhibition is required for the antidepressant effect of ketamine in learned helplessness depression-like behavior. Here we report that treatment with an antidepressant dose of ketamine (10 mg/kg) increased expression of insulin-like growth factor 2 (IGF2) in mouse hippocampus, an effect that required ketamine-induced inhibition of GSK3. Ketamine also inhibited hippocampal GSK3 and increased expression of hippocampal IGF2 in mice when administered after the induction of learned helpless. Treatment with the specific GSK3 inhibitor L803-mts was sufficient to up-regulate hippocampal IGF2 expression. Administration of IGF2 siRNA reduced ketamine’s antidepressant effect in the learned helplessness paradigm. Mice subjected to the learned helplessness paradigm were separated into two groups, those that were resilient (non-depressed) and those that were susceptible (depressed). Non-depressed resilient mice displayed higher expression of IGF2 than susceptible mice. These results indicate that IGF2 contributes to ketamine’s antidepressant effect and that IGF2 may impart resilience to depression-like behavior.

4.2 Background

Ketamine treatment induces a rapid antidepressant effect in depressed patients with mood disorders (Newport et al., 2015; Niciu et al., 2014; Scheuing et al., 2015). The mechanism that underlies this action is currently unknown. In mice, ketamine’s antidepressant effect in the learned helplessness model is dependent on its inhibition of
glycogen synthase kinase-3 (GSK3) (Beurel et al., 2011). GSK3 refers to two isoforms that are primarily regulated by inhibitory phosphorylation on ser-21-GSK3α and ser-9-GSK3β, and this inhibitory modulation of both GSK3 isoforms in mouse brain is increased following ketamine administration (Beurel et al., 2015). Ketamine also increased the serine-phosphorylation of GSK3 in lymphocytes of patients with depression (Yang et al., 2013). In GSK3 knockin mice the serines are mutated to alanines to prohibit this major mechanism of GSK3 inhibition, leaving GSK3 constitutively active (McManus et al., 2005). Ketamine treatment does not induce an antidepressant effect in GSK3 knockin mice, demonstrating the necessity for ketamine-induced inhibitory serine-phosphorylation of GSK3 for this effect (Beurel et al., 2011). Further studies showed that administration of GSK3 inhibitors enhanced the antidepressant effects of ketamine (Chiu et al., 2014; Ghasemi et al., 2010; Liu et al., 2013). Thus, ketamine-induced inhibition of GSK3 is linked to its antidepressant action, but it remains unknown how this contributes to the antidepressant effects of ketamine (Zunszain et al., 2013).

Several reports have implicated insulin-like growth factor 2 (IGF2) in regulating depression and actions of antidepressants. IGF2 is one of the most abundant growth factors present in cerebrospinal fluid (Åberg et al., 2015; Tham et al., 1993), and IGF2 is expressed and binds receptors throughout the human hippocampus (Caracausi et al., 2016; Wilczak et al., 2000). Variability in the regulation of IGF2 expression has been associated with depression status in adult monozygotic twins (Córdova-Palomera et al., 2015). In rodents, antidepressant treatments up-regulate IGF2 in the hippocampus (Cline et al., 2012; Lisowski et al., 2013) and other brain regions (Lauterio et al., 1993), and IGF2 is down-regulated in the hippocampus in rodent models of depression-like behaviors (Andrus et al.,
Hippocampal overexpression of IGF2 improved measures of depression induced in rats by chronic restraint stress, including sucrose consumption and immobility time in the forced swim test (Luo et al., 2015). Administration of IGF2 also increased neurogenesis in the hippocampus, a process that may contribute to the action of antidepressants (Bracko et al., 2012; Chen et al., 2007; Ferrón et al., 2015; Kita et al., 2014). In vivo administration of IGF2 in the hippocampus increased the expression of several neurotrophins and growth factors, such as brain-derived neurotrophic factor (BDNF) (Mellott et al., 2014). Thus, diminished IGF2 may contribute to susceptibility to depression and increased IGF2 is linked to antidepressant responses.

Here we report that administration of an antidepressant dose of ketamine up-regulated IGF2 expression in mouse hippocampus, and that this requires inhibition of GSK3. Furthermore, administration of a specific GSK3 inhibitor was sufficient to up-regulate hippocampal IGF2 expression. In mice with GSK3 activation induced by the learned helplessness paradigm, ketamine restored GSK3 inhibition and increased expression of IGF2. Administration of IGF2 siRNA reduced ketamine’s antidepressant effect in the learned helplessness paradigm, and mice that were resilient to learned helplessness displayed higher hippocampal expression of IGF2 than control mice. Thus, increased hippocampal IGF2 contributes to ketamine’s antidepressant effect and may be a regulator of the resilience or susceptibility of mice to depression-like behavior.
4.3 Results

4.3.1 Ketamine treatment increases expression of IGF2 in hippocampus

IGF2 mRNA expression was measured in the hippocampus of male mice 0.5, 12, 24, and 48 hr after administration of a sub-anesthetic, antidepressant dose of ketamine (10 mg/kg; i.p.). There was an up-regulation of IGF2 mRNA 24 hr after ketamine treatment (2.75 ± 0.2-fold of control levels), which then returned to control levels by 48 hr, indicating that ketamine induces a transient increase in IGF2 (one-way ANOVA; F(4,32)=3.070, p<0.05; Bonferroni post-hoc test, *p<.05, compared to saline treated mice; Figure 6A). Female mice had higher basal levels of hippocampal IGF2 mRNA than male mice (Student’s t test; t(22)=2.772, *p<0.05; Figure 6B), but also exhibited an increase in IGF2 mRNA levels 24 hr after ketamine treatment (Student’s t test; t(12)=2.658, *p<0.05; Figure 6C). IGF2 protein levels were also significantly increased in the hippocampus of male mice 24 hr after ketamine administration (Student’s t test; t(8)=2.622, *p<0.05; Figure 6D). Basal hippocampal IGF2 mRNA levels were equivalent in male wild-type and GSK3 knockin mice but 24 hr after ketamine administration there was a lack of change in GSK3 knockin mice (two-way ANOVA (genotype X treatment); Finteraction(1,24)=6.659, p<0.05; Bonferroni post-hoc test, *p<.05, compared to saline treated mice wild-type mice; Figure 6E), demonstrating the necessity for ketamine-induced inhibitory serine-phosphorylation of GSK3 for the up-regulation of IGF2 expression. Furthermore, inhibition of GSK3 was sufficient to up-regulate IGF2 expression, as administration of the selective GSK3 inhibitor L803-mts (60 µg; intranasal; 24 hr) increased IGF2 levels in the hippocampus of male wild-type mice (Student’s t test; t(5)=2.728, *p<0.05; Figure 6F). Ketamine did not increase
IGF2 mRNA expression in prefrontal cortex (Figure 6G), which had nearly the same basal IGF2 mRNA expression as the hippocampus (Figure 6H).
4.3.2 Ketamine, IGF2 and learned helplessness depression-like behavior

We also tested if administration of ketamine inhibits GSK3 and up-regulates IGF2 mRNA in mice after induction of learned helplessness depression-like behavior. Mice were exposed to inescapable foot shocks and 24 hr later learned helplessness depression-like behavior was tested by determining if mice failed to escape from 15 or more trials out of the 30 trials. Two days after escapable shock treatment, to allow normalization of acute effects of the foot shocks, learned helpless mice were treated with ketamine (10 mg/kg; i.p.), and sacrificed 24 hr later. Hippocampal serine-phosphorylated GSK3β and GSK3α levels were lower in learned helpless mice compared to control mice, and the serine-phosphorylation of both GSK3α and GSK3β were restored to control levels by ketamine.
treatment (one-way ANOVA; \( F_{(2,21)}=4.694, \ p<.05; \) Bonferroni post-hoc test, \(*p<.05,\) compared to naïve control mice or depressed saline treated mice; Figure 7A; and one-way ANOVA; \( F_{(2,21)}=7.287, \ p<.05; \) Bonferroni post-hoc test, \(*p<.05,\) compared to naïve control mice or depressed saline treated mice; Figure 7B). Ketamine treatment also up-regulated IGF2 mRNA (2.70 ±0.5-fold of control levels) in learned helpless mice to a similar extent as was observed in mice not exposed to the learned helplessness paradigm (one-way ANOVA; \( F_{(2,23)}=4.706, \ p<0.05; \) Bonferroni post-hoc test, \(*p<.05,\) compared to naïve control mice; Figure 7C).

![Figure 7A: Hippocampus](image)

![Figure 7B: Hippocampus](image)

![Figure 7C: IGF2 mRNA](image)
To determine if the antidepressant effect of ketamine is dependent on IGF2 up-regulation, we tested if IGF2 siRNA reduced ketamine’s antidepressant effect in the

Figure 7. In learned helpless mice ketamine restores GSK3 inhibition and up-regulates IGF2 mRNA
Learned helplessness was induced in male wild-type mice (Depressed) and two days later saline (Sal) or ketamine (Ket; 10 mg/kg; i.p.) was administered followed 24 hr later by measurements in the hippocampus of (A) serine9-phosphorylated GSK3β and total GSK3β (n=6-12), (B) serine21-phosphorylated GSK3α and total GSK3α (n=6-12), and (C) IGF2 mRNA levels (n=7-12). (D) Escape failures of mice treated with vehicle plus a scrambled siRNA (n=11), ketamine plus a scrambled siRNA (n=11), or ketamine plus an IGF2 siRNA (n=13). Each symbol represents an individual mouse. The dashed bar at 15 escape failures indicates the demarcation between mice that are defined as learned helpless (depressed) or not. Graphs show Means±SEM, *p<.05.
learned helplessness paradigm. 100% of vehicle-treated mice developed learned helplessness, and this was reduced to only 36% by ketamine-treatment. Pretreatment with IGF2 siRNA diminished the antidepressant effect of ketamine, resulting in 69% of mice developing learned helplessness, demonstrating that ~60% of ketamine’s antidepressant effect was blocked by IGF2 siRNA administration (Chi-square; $X^2(2)=10.34; p<0.05$; Figure 7D).

The learned helplessness paradigm was used to separate mice into two groups, those that were resilient (non-depressed) and those that were susceptible (depressed) to learned helplessness. Mice were exposed to inescapable shocks, 24 hr later they were tested with escapable shocks, and mice were sacrificed 1 hr later. IGF2 mRNA levels were significantly higher in the hippocampus of non-depressed resilient mice compared to control mice (one-way ANOVA; $F(2, 19)=4.356, p<.05$; Bonferroni post-hoc test, *p<.05, compared to naïve control mice; Figure 8B), raising the possibility that IGF2 contributes to the differential susceptibilities of mice to learned helplessness.
Discussion

Ketamine can induce rapid antidepressant effects in depressed patients with mood disorders (Newport et al., 2015; Niciu et al., 2014; Scheuing et al., 2015) and the antidepressant mechanism of action of ketamine is linked to GSK3 inhibition (Beurel et al., 2011; Chiu et al., 2014; Ghasemi et al., 2010; Liu et al., 2013; Yang et al., 2013; Zhou et al., 2014). Here we found that up-regulation of IG2 expression in mouse hippocampus appears to play an important role in the GSK3 inhibition-dependent antidepressant effect of ketamine in mice. Furthermore, inhibition of GSK3 is sufficient to up-regulate IG2 expression, and differential hippocampal expression of IG2 may distinguish mice that are resilient from those that are susceptible to learned helplessness depression-like behavior.

The up-regulated expression of IG2 in response to ketamine was linked to GSK3-inhibition by ketamine by two findings. First, up-regulation of IG2 by ketamine did not occur in GSK3 knockin mice, in which GSK3 cannot be inhibited (McManus et al., 2005). Second, administration of the specific GSK3 inhibitor L803-mts was sufficient to increase IG2 expression in the hippocampus to a similar extent as ketamine treatment. These
results demonstrate that inhibition of GSK3 is both necessary and sufficient to up-regulate the expression of IGF2.

Several approaches were used to determine if up-regulation of IGF2 contributes to the antidepressant response to ketamine. Ketamine induced up-regulation of IGF2 expression in the hippocampus coincides with (24 hr) ketamine’s antidepressant actions in mice (Autry et al., 2011; Beurel et al., 2011; Li et al., 2010; Maeng et al., 2008; Zanos et al., 2016). In mice that were first rendered learned helpless and subsequently treated with ketamine, an experimental condition that models the clinical situation, IGF2 expression was increased by ketamine treatment similarly to that occurring in mice treated with ketamine alone without being subjected to learned helplessness. Finally, IGF2 siRNA administration significantly reduced ketamine’s antidepressant efficacy. Taken together these results demonstrate that IGF2 expression is up-regulated by ketamine and contributes to ketamine’s antidepressant effect in mice.

Groups of apparently identical mice that are exposed to stresses that can induce depression-like behaviors are commonly found to exhibit differential responses, with some mice being resilient and others susceptible to developing depression-like behaviors (McEwen et al., 2015). Comparison of IGF2 expression in these two cohorts of mice exposed to the learned helplessness paradigm revealed that it was significantly increased in mice that were resilient to learned helplessness, but not in susceptible mice, suggesting that the level of IGF2 expression may contribute to resilience.

This study found that ketamine up-regulates IGF2 in mouse hippocampus, this was dependent on GSK3-inhibition and was matched by administration of a specific GSK3 inhibitor. Ketamine also induced inhibition of GSK3 and up-regulated IGF2 expression in
learned helpless mice. Administration of IGF2 siRNA significantly reduced ketamine’s antidepressant effects, altogether indicating that up-regulation of IGF2 contributes to ketamine’s antidepressant effect. Furthermore, increased IGF2 expression was linked to resilience in mice. Altogether these findings indicate that IGF2 contributes to ketamine’s antidepressant effects in mice.
5 Chapter - Future Directions and Concluding Remarks

The studies described here found two novel molecular responses (Figure 9), up-regulation of the 5HTR2C miRNA cluster (Figure 1A) and of IGF2 expression (Figure 6 A,D) in mouse hippocampus, after administration of an antidepressant dose of ketamine (10 mg/kg). These findings extend previous studies that emphasize molecular responses to ketamine in the hippocampus for its antidepressant action (Garcia et al. 2008; Maeng et al. 2008; Autry et al. 2011; Tizabi et al. 2012). The other brain tissue that was analyzed in these studies for 5HTR2C cluster miRNAs and IGF2 expression up-regulation after treatment with an antidepressant dose of ketamine was the prefrontal cortex, which may contribute to some aspects of the antidepressant response to ketamine (Abdallah et al. 2016). However, up-regulation of neither the 5HTR2C cluster miRNAs (Figure 1C) nor IGF2 (Figure 6G) occurred in the prefrontal cortex. These findings, however, do not rule out the possibility that other brain regions important for depression could have similar ketamine-induced up-regulation of 5HTR2C cluster miRNAs and IGF2. One future experiment to address this could involve the administration of an antidepressant dose of ketamine to mice, followed by the harvest of various other mood-related brain regions for analysis of differential expression of 5HTR2C cluster miRNAs and IGF2. The basolateral amygdala and the nucleus accumbens are two potential brain regions that may be useful to analyze, since these brain regions may be implicated in the pathogenesis of depression (Duman and Duman 2015). Similarly, the hippocampal 5HTR2C cluster miRNA and IGF2 responses to antidepressant doses of ketamine could be further analyzed for hippocampal sub-region specific up-regulation. In particular the ventral hippocampal regions are thought to be important for the actions of antidepressants (O’Leary and Cryan 2014). The question
of the spatial localization of the 5HTR2C cluster miRNA and IGF2 responses to ketamine could be elegantly determined using in situ hybridization of 5HTR2C cluster miRNAs and IGF2 mRNA in mouse brain sections.

Both 5HTR2C cluster miRNAs (Figure 1D) and IGF2 (Figure 6H) expression were analyzed without treatments to compare relative basal expression levels between the hippocampus and the prefrontal cortex, though no significant differences were observed.
Basal expression levels of 5HTR2C mRNAs and the cluster miRNAs are however, highly expressed in brain compared to other tissues (Hinske et al. 2014). The relative basal expression levels of 5HTR2C cluster miRNAs and IGF2 within the brain could be further analyzed by measuring the molecules in other brain regions such as the amygdala and the nucleus accumbens, or in sub-regions of the hippocampus. This might provide clues about the relative importance of these molecules in those brain regions.

Another important future experiment would be to determine the cell-type-specific expression of 5HTR2C cluster miRNAs and IGF2 within the hippocampus. Two large RNA-Seq studies measured RNAs from the brain, and the 5HTR2C cluster miRNAs were found to be expressed at higher levels in neurons relative to glial cells in untreated animals (Cahoy et al. 2008; Zhang et al. 2014). However both of these studies used developing mice aged postnatal day 1-30, so further validation of these results should be performed on adult mice. Another paper studied cell lines in vitro and determined that the 5HTR2C gene expresses its intronic miRNA cluster primarily in non-neuronal cell lines (Zhang et al. 2013). Therefore, techniques that allow for colocalization of miRNAs with markers of specific cell types could be used to determine the cell types expressing the 5HTR2C miRNA cluster, particularly in response to ketamine.

Cell-type specific expression of IGF2 in the brain should also be elucidated. IGF2 is found in cerebrospinal fluid at high levels (Åberg et al. 2015; Tham et al. 1993). Also, IGF2 is expressed and binds receptors throughout the hippocampus (Caracausi et al. 2016; Wilczak et al. 2000). Further studies could use immunofluorescence techniques to colocalize IGF2 to hippocampal cell subtypes, such as neurons or glia. However, the differential expression patterns of the 5HTR2C miRNAs or IGF2 induced by ketamine
important for its antidepressant responses may not correlate with basal expression levels of these molecules in various tissues or cell types. Therefore, the region-specific and cell-type specific differential expression patterns of 5HTR2C miRNAs and IGF2 should be assessed after treatment with an antidepressant dose of ketamine, in order to determine local expression patterns that are important for ketamine’s antidepressant response.

Sub-anesthetic, antidepressant doses of ketamine have been therapeutic in patients that are not responders to other antidepressants, such as the classical antidepressants, including fluoxetine, also known as Prozac (Krystal et al. 2013). Therefore, in our studies the expression levels of the 5HTR2C cluster miRNAs were measured after chronic or acute treatments with fluoxetine (20 mg/kg). A significant up-regulation of the 5HTR2C miRNA cluster after chronic or acute fluoxetine treatments was not observed (Figure 2B,C). Therefore the hippocampal response of the 5HTR2C cluster miRNAs is specific to ketamine as compared to the classical antidepressant fluoxetine, although future experiments could expand this to test other antidepressants. Although this study did not investigate the effect of fluoxetine on IGF2 up-regulation, this experiment should be performed in order to determine if the IGF2 up-regulation response to ketamine is specific for that drug or if IGF2 up-regulation is common to many antidepressants. In future experiments, drugs that have been found to up-regulate 5HTR2C mRNA in hippocampus, may be good candidates for determining if they would up-regulate the 5HTR2C miRNA cluster as well, since it was found in this study that 5HTR2C mRNA and the miRNA cluster expression levels were correlated (Figure 1A; Figure 3A,B). The correlation between 5HTR2C mRNA and 5HTR2C cluster miRNA expression levels without treatments has also been reported previously in human cortex (Hinske et al. 2014). Imipramine may be
such a drug, as it was found to increase 5HTR2C mRNA in the hippocampus. Several other 5HTR2C mRNA responses to antidepressants have been reported, where some classical antidepressants induced no response or even down-regulated 5HTR2C mRNA (Martin et al. 2014). Our study also found that intranasal administration of the GSK3 inhibitor L803-mts was sufficient to up-regulate the 5HTR2C cluster miRNAs in the hippocampus to a similar extent as ketamine (Figure 4C), and was found to be antidepressant in the learned helplessness test (Figure 5B). Intranasal administration of the GSK3 inhibitor L803-mts was also sufficient to increase expression of IGF2 in hippocampus to the same extent as ketamine (Figure 6F). Therefore it may be that antidepressant drugs that inhibit GSK3 may be good candidates for potentially up-regulating both the 5HTR2C cluster miRNAs and IGF2 in the hippocampus. For example, administration of the mood stabilizer with antidepressant effects, valproate, reduces depression-like behaviors in the forced swim test and inhibits GSK3 in mice (Kobayashi et al. 2012; Xing et al. 2011; De Sarno et al. 2002). Similarly, treatment with the mood stabilizer with antidepressant effects, lithium, as well as ketamine, results in GSK3 inhibition in mouse brain (Beurel et al. 2011; Zhou et al. 2014). Lithium is also interesting because low doses of lithium and ketamine synergize in behaviors measuring depression-like behavior (Scheuing et al. 2015). 5HTR2C cluster miRNAs and IGF2 expression changes in hippocampus after imipramine, valproic acid, or lithium treatments could be addressed in the future. The antidepressants dicholine succinate and desipramine were found previously to increase IGF2 expression in hippocampus (Cline et al. 2012; Lisowski et al. 2013). Altogether, it will be beneficial to try to determine which antidepressants induce similar molecular changes in 5HTR2C cluster miRNAs and IGF2 expression.
Since ketamine is an NMDA receptor antagonist, and NMDA receptor antagonists, such as mementine, MK-801 and 7-chlorokynurenic acid also increase GSK3 inhibition in brain, it would be a good future experiment to determine if other NMDA receptor antagonists, besides ketamine, also up-regulate 5HTR2C cluster miRNAs and IGF2 (De Sarno et al. 2006; Seo et al. 2007; Yu et al. 2011; Zhu et al. 2013). Since treatment with NMDA can activate GSK3, and stress activates NMDA receptors, perhaps similar treatments or exposure to certain stressors would down-regulate the 5HTR2C cluster miRNAs and IGF2 in hippocampus, although none of our studies observed a down-regulation of either the 5HTR2C cluster miRNAs or IGF2 in any of the experimental manipulations (Luo et al. 2003; Zheng and Quirion 2009; Vásquez et al. 2014). In particular, the NMDA antagonists memantine and MK-801 have had antidepressant effects and inhibit GSK3, and thus might be good NMDA receptor antagonist candidates to determine if 5HTR2C cluster miRNAs and IGF2 expression are altered by their administration (Quan et al. 2011; Réus et al. 2010; Trullas and Skolnick 1990). This proposed future experiment would strengthen the argument that NMDA inhibition is part of the 5HTR2C miRNA cluster and IGF2 up-regulation response to ketamine.

Ketamine is a NMDA receptor antagonist, and administration of antidepressant doses of ketamine rapidly increase AMPA receptor activity (Zarate et al. 2007; Maeng et al. 2008; El Iskandrani et al. 2015). It is thought that part of ketamine’s antidepressant effect depends on increasing AMPA receptor activity in the brain (Tizabi et al. 2012; Akinfiresoye and Tizabi 2013; Andreasen et al. 2013; Du et al. 2006). In fact, ketamine’s antidepressant effects in animal models are dependent on AMPA receptor activity, which can be studied using the AMPA receptor blocker NBQX (Li et al. 2010; Machado-Vieira
et al. 2009; Maeng et al. 2008; Autry et al. 2011; Koike, Iijima, and Chaki 2011). In our studies, the up-regulation of the 5HTR2C miRNA cluster was dependent on AMPA receptor signaling because NBQX attenuated the ketamine-mediated up-regulation of 5HTR2C cluster miRNAs (Figure 3B). Our study did not however, determine which signaling molecules downstream of AMPA receptors results in the up-regulation of the 5HTR2C miRNA cluster. Future experiments should determine what pathways downstream of AMPA receptors could potentially contribute to ketamine-mediated up-regulation of the 5HTR2C miRNA cluster. Increased AMPA receptor activity can increase expression of genes with calcium responsive elements, so it would be important to determine if this mechanism contributes to the regulation of 5HTR2C expression as well (Alt et al. 2006). Also, it was determined the inhibition of GSK3 with L803-mts was sufficient to up-regulate IGF2 (Figure 6F). It was not determined, however, what signaling molecules downstream of GSK3 resulted in up-regulation of IGF2. Future experiments should address this as well. It is possible that GSK3 inhibition regulates IGF2 expression by an epigenetic mechanism since GSK3 is known to participate in many epigenetic pathways which are involved in gene expression regulation. Of particular interest are GSK3’s roles in inhibiting histone deacetylase (HDAC) activity (Beurel et al. 2015). Inhibition of HDACs can promote gene expression and antidepressant responses (Fuchikami et al. 2016).

A ketamine metabolite called (R)-hydroxynorketamine was recently discovered to have robust antidepressant effects in the forced swim test, as soon as 1 hr after treatment and for as long as 3 days after treatment. (R)-hydroxynorketamine also lacked the detrimental side effects of ketamine. Further, (R)-hydroxynorketamine was found to
mediate its antidepressant effects independently of NMDA receptors. However (R)-hydroxynorketamine’s antidepressant effects did involve activation of AMPA receptors, which was determined using a pretreatment of NBQX which blocked (R)-hydroxynorketamine’s antidepressant effects measured 1 and 24 hr after administration in the forced swim test. The contribution of AMPA receptor signaling to (R)-hydroxynorketamine’s antidepressant effects was thought to occur in the hippocampus, but not the prefrontal cortex (Zabos et al. 2016). A future experiment that should be performed could determine if treatment of mice with an antidepressant dose of (R)-hydroxynorketamine up-regulates the 5HTR2C miRNA cluster or IGF2 in hippocampus. It might be that (R)-hydroxynorketamine results in increased inhibition of GSK3 as ketamine and other NMDA antagonists do, but by a mechanism further downstream of NMDA receptors. Also, the findings of antidepressant effects of (R)-hydroxynorketamine have only been found in mice thus far, and not human patients, so clinical trials will have to confirm the antidepressant effects in patients. If (R)-hydroxynorketamine is shown to have antidepressant effects in patients, without side-effects, and is determined to be more efficacious than ketamine, future pre-clinical studies should determine the molecular mechanism of this effect. For instance, perhaps (R)-hydroxynorketamine is more effective at inhibiting GSK3 than NMDA inhibition by ketamine or other NMDA inhibitors.

There is variability in the susceptibility of people to depression, which may depend on epigenetic mechanisms (Nestler 2013). This is true in mice as well. Some mice and mouse breeds tend to be more resilient in the face of stressors like chronic restraint stress, chronic unpredictable stress, isolation stress, defeat stress, and learned helplessness (Cabib et al. 2012; Radley et al. 2011). In our studies it was determined that approximately 76%
of wild-type C57BL/6 mice exposed to the learned helplessness test acquired learned-helplessness depression-like behavior (Figure 4A). In our studies it was found that miR1264-3p and miR448-3p were significantly up-regulated in the hippocampus of non-depressed mice as compared to depressed mice (Figure 5A). Similarly IGF2 expression was increased in the non-depressed group as compared to the depressed one (Figure 8B). In order to further characterize the relationship between miR448-3p, miR1264-3p and IGF2 expression and resilience endophenotypes, models of depression-like behavior that generate resilient and susceptible mice could be tested to determine if stress in parental animals determines miR448-3p, miR1264-3p and IGF2 expression levels and resilience in the offspring. Perhaps the offspring of resilient mice, that may have had increased GSK3 inhibition along with increased expression of miR448-3p, 1264-3p and IGF2, are also resilient and have increased expression of miR448-3p, 1264-3p and IGF2.

A single subanesthetic dose of ketamine administered 24 hr before the trial portion of the learned helplessness test had antidepressant effects (Beurel et al. 2011). The peak expression times of both the 5HTR2C miRNA cluster (Figure 2A) and IGF2 (Figure 6A) was also 24 hr, suggesting that these responses may contribute to the antidepressant effect of ketamine in the learned helplessness test. The antidepressant effects of ketamine in animal models however, is thought to be short-lived (Bechtholt-Gompf et al. 2011; Popik et al. 2008). Similarly the expression levels of both the 5HTR2C miRNA cluster (Figure 2A) and IGF2 (Figure 6A) returned to basal expression levels after only 48 hr, thus suggesting that there could be a correlation in the times of antidepressant responses of ketamine and the up-regulated expression times of the 5HTR2C miRNA cluster and IGF2. The short-lived antidepressant response of ketamine is also present in humans, which is
why multiple treatments are often used in order to maintain an effective antidepressant response (aan het Rot et al. 2010; Diamond et al. 2014; Rasmussen et al. 2013; Messer et al. 2010; Murrough et al. 2013; Liebrenz et al. 2009; Segmiller et al. 2013). Similarly in rodents both single and multiple treatments of ketamine are used to improve measures of depression-like behaviors (Yilmaz et al. 2002; Parise et al. 2013; Garcia et al. 2008; Gideons, Kavalali, and Monteggia 2014). One potential future experiment would be to treat mice with multiple administrations of an antidepressant dose of ketamine in a way that mimics a therapeutic treatment in the clinic in order to maintain antidepressant effects. It would be interesting to determine if chronic ketamine treatment for mice maintains up-regulation of the 5HTR2C miRNA cluster and IGF2 in hippocampus for as long as the treatment endures and mice remain sustained without depression-like behavior. Another concern regarding the timing of 5HTR2C miRNA cluster and IGF2 expression up-regulation after ketamine administration is that in patients ketamine elicits antidepressant effects in times shorter than 24 hr. There has also been a recent report of the prophylactic or depression-like behavior preventative capability of ketamine treatment against stress-induced depressive-like behaviors in mice (Brachman et al. 2016). In these experiments ketamine was administered in advance of exposure to stressors that trigger depression-like behavior. A future experiment could determine if the 5HTR2C miRNA cluster or IGF2 are required for the prophylactic effects of ketamine to prevent depression-like behavior induced by a stressor.

Previously it was found that a subanesthetic dose of ketamine had antidepressant effects in the learned helplessness paradigm (Beurel et al. 2011). In our study it was determined that blockade of either miR448-3p (Figure 5B) or IGF2 (Figure 7D) blocked
the antidepressant effects of ketamine in the learned helplessness test. It would be interesting to determine if treatment of mice with exogenous miR448-3p, which was the most robustly up-regulated miRNA by ketamine (Figure 1A), protects mice from depression-like behavior. Overexpression of IGF2 in mouse hippocampus protected mice from depression-like behavior as measured by the sucrose preference test and the forced swimming test (Luo et al. 2015). Overexpression of miR448-3p could also be achieved in mouse hippocampus to determine if it is sufficient to induce antidepressant effects. However, experimental manipulations that would overexpress miR448-3p or IGF2 in brain in order to test for protection from depression-like behavior are not as clinically useful as other methods of introducing potentially antidepressant molecules to the brain and hippocampus. For example a potential method of improving CNS delivery, while simultaneously avoiding peripheral effects, is by administering drugs intranasally (Illum 2000; Pires et al. 2009; Mistry et al. 2009; Dhuria et al. 2010). For example, intranasal administration of ketamine can be used to treat depression in patients (Andrade et al. 2015). Therefore future experiments should be done in which intranasal administration of both miR448-3p and IGF2 together are used, since our studies determined that they are both required for ketamine’s antidepressant effect, to determine if the synergy of these molecules is sufficient to have antidepressant effects in the learned helplessness measures of depression-like behavior.

It will be important to determine the downstream targets of ketamine-mediated up-regulation of the 5HTR2C miRNA cluster and IGF2. There are currently many software resources available to make predictions about potential mRNA targets of miRNAs. However, there are often many predicted targets for each miRNA, leaving the experimenter
unsure of which targets to pursue. Also, since miRNAs do not typically lead to mRNA level changes or degradation, rather they block translation of mRNA, validating a miRNA target is usually done at the protein level (Oulas et al. 2015). One future experiment could modulate miR448-3p expression in vivo, by the introduction of miR448-3p or an miR448-3p inhibitor, then follow this treatment with RNA-Seq analysis of hippocampal tissue. This experiment will not likely identify the target of miR448-3p that is important for ketamine’s antidepressant effects, however changes in gene expression patterns may indicate which pathways are modulated by miR448-3p. This could provide a more specific description of the pathways modulated by miR448-3p that are important for ketamine’s antidepressant effects. A similar approach could also be taken to determine the specific pathways modulated by IGF2 that are important for ketamine’s antidepressant effects. One study did find that miR448-3p reduced protein expression of Kruppel-like factor 5 (KLF5) in adipocytes, however it is not clear how KLF5 repression could contribute to ketamine’s antidepressant effects (Kinoshita et al. 2010). IGF2, however, is much more well-studied and may contribute to ketamine’s antidepressant effects by binding IGF1 receptors which induces AKT signaling which has been determined to play an important role in antidepressant effects in brain (Beaulieu 2012; Werner and LeRoith 2014).

It will also be important to determine in future experiments if 5HTR2C cluster miRNAs or if IGF2 levels in the serum correlate with mouse resilience or with ketamine responsiveness. This would allow for the ability to measure 5HTR2C cluster miRNAs and IGF2 throughout the course of an ongoing experiment. Also, since diagnosis of patients susceptible to depression sometimes relies on self-reporting or on psychiatric examinations such as the Hamilton Depression Rating Scale, which are not always accurate, it would be
helpful to determine if 5HTR2C cluster miRNAs and IGF2 levels in human serum could predict depression susceptibility or responsiveness to ketamine or other antidepressants treatments in patients with depression (Bagby et al. 2004). Biomarkers of this nature are highly needed in the clinic since patients require different treatments for depression, and it can take much time to identify the best course of treatment for each individual (Young et al. 2016). Similarly it would be important to find if genetic aberrations, such as single-nucleotide polymorphisms (SNPs), or if epigenetic variations in DNA methylation at the 5HTR2C miRNA cluster or IGF2 genetic loci predict susceptibility to depression or ketamine responsiveness (Alhajji and Nemeroff 2015). Analysis of postmortem tissue from depressed patients or from patients treated with ketamine will also be particularly valuable in order to determine if 5HTR2C cluster miRNAs or IGF2 levels change under these conditions. Ultimately it will be crucial to determine if treatments that increase 5HTR2C cluster miRNAs or IGF2 levels in human patients proves to mediate antidepressant effects.

This work described two novel molecular outcomes of administration of an antidepressant dose of ketamine. Regulation of the 5HTR2C miRNA cluster has not been reported in any study to date to be modulated by any psychiatric drug. Thus, the findings that the 5HTR2C miRNA cluster contributes to the antidepressant effects of ketamine and to mouse resilience should stimulate further interest in this area of study. IGF2 has been described previously to contribute to antidepressant responses, but no study has investigated the importance of IGF2 expression for ketamine’s antidepressant effects. Therefore, our studies offer new and exciting contributions to the field of psychiatry which will hopefully benefit depressed patients in the future.
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