NMDA Receptor Independent, Astrocytic Antidepressant Actions of Ketamine

BY

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B.S., Aquinas College, 2013

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Chicago, 2018

Chicago, Illinois

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Dedication

This dissertation is dedicated to my late brother Lennon Smith and my late sister Andrea Plesnicar. I would not have started a career in science without their support throughout of my life.

Acknowledgements

I would initially like to thank my committee members, Drs. Rasenick, Rao, Brodie, Weinberg, and Pradhan for their guidance throughout the years.

I would also like to thank the lab technicians, fellow graduate students and post-docs throughout the years, for their support and technical guidance, especially Sia Koutsouris, Andrew Czysz, Harinder Singh, Jeffrey Schappi, Bob Donati, Nick Senese, and Jiang-Zhou Yu.

Finally, I'd like to thank my family, Jerry Wray, Susan Wray, Peggy Wray, Joanie Giuliani, and Will Giuliani for their continued support throughout my time as a graduate student.

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List of Abbreviations

ANOVA	Analysis of variance
AC	Adenylyl cyclase
ADP	Adenine nucleotide diphosphate
ATCC	American Type Culture Collection
ATP	Adenine nucleotide triphosphate
β_2	Beta-2 adrenergic receptor
βγ	G protein betagamma subunit
BDNF	Brain derived neurotrophic factor
C6	C6 glioma cell line
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
CNS	Central nervous system
CREB	cAMP response element binding protein
Da	Dalton
DAG	Diacylglycerol
DAT	Dopamine reuptake transporter
DBS	Deep brain stimulation
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	Dithiothreitol
ECT	Electroconvulsive therapy
EDTA	Ethylenediaminetetracetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Epac	Exchange protein directly activated by cAMP
FACS	Fluorescence-activated cell sorting
FADS	Fatty acid desaturase
FRAP	Fluorescence recovery after photobleaching
FRET	Förster/fluorescent resonant energy transfer
G418	Geneticin
Gα	G protein alpha subunit
$G\alpha_s$	G protein alpha stimulatory subunit
$G\alpha_i$	G protein alpha inhibitory subunit
$G\alpha_q$	G protein alpha subunit activating PLC
GAP	GTPase activating protein
Gβγ	G protein betagamma subunit
GC-MS	Gas Chromatography -Mass Spectrometry
GDP	Guanine nucleotide diphosphate
GEF	Guanine exchange factors
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein

GPCR	G protein coupled receptor
GTP	Guanine nucleotide triphosphate
Gγ	G protein gamma subunit
GWAS	Genome-wide association study
HEK293	Human embryonic kidney 293 cell line
HeLa	HeLa cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
5-HTTLPR	Serotonin transporter-linked polymorphic region
IBMX	3-isobutyl-1-methylxanthine
kDa	Kilodalton
M	Molar
mM	Milimolar
mAb	Monoclonal antibody
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MAP	Microtubule-associated protein
MβCD	Methyl-β-cyclodextrin
MDD	Major depressive disorder
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
NET	Norepinephrine reuptake transporter

NGF	Nerve growth factor
nM	Nanomolar
PBS	Phosphate buffered saline
PC12	Pheochromocytoma 12 cell line
PDE	Phosphodiesterase
PET	Positron emission tomography
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PPM	Parts per million
PTM	post-translational modifications
PKA	cAMP dependent protein kinase
PET	Positron emission tomography
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROI	Region of interest
SCD1	Stearoyl-CoA desaturase-1
SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
siRNA	Small interfering RNA

SLC	Sodium-dependent large solute carrier
SNRI	Selective norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TrkB	Tropomyosin receptor kinase B
TMS	Transcranial magnetic stimulation
TX-100	Triton X-100 detergent
TX-114	Triton X-114 detergent
μM	Micromolar

Summary

Depression is a devastating mental illness that robs many of ability to experience the tremendous joy of being alive. It is diagnosed by its symptoms, the most prevalent being a depressed mood. While we now have a basic understanding of the disease, all of the treatments available fall short in some category. In fact, some patients do not respond to any current medical treatment. The anesthetic ketamine, when administered at sub-anesthetic doses, exerts powerful and rapid antidepressant effects. These effects are often seen even when behavioral therapy or other antidepressants have failed. There has been a push in the research community to understand how ketamine exerts its antidepressant effects, in order to develop safer alternatives and understand the biological underpinnings of depression. This thesis identifies and characterizes a novel antidepressant effect of ketamine. Data presented here may clarify some controversies surrounding the literature on ketamine and, more importantly, lead to the development of novel, rapid-acting antidepressants

1. Depression

1.1.1 Introduction to depression

Major depressive disorder (MDD) or depression is a debilitating mental illness that has been documented since antiquity. Aristotle described the disorder in depth in his *Problema* XXX.1. Today, depression is intensely researched and still discussed. While progress has been slow, and no clear understanding exists, we are beginning to understand some basic underlying neurobiology of MDD.

Depression is a common mood disorder that effects approximately 20% of people in the United States. As the leading cause of disability worldwide, it results in approximately 50 million years lived with disability (WHO 2017). In 2000 the economic cost of depression was estimated at $100 billion (Lepine 2011). Despite decades of rigorous research, no clear etiology or pathophysiology of MDD exists. Depression is typically characterized by symptoms, most prominent being a depressed mood and a diminished lack of pleasure in nearly all activities. These symptoms are often accompanied by sleep and weight disturbances, either insomnia or hypersomnia and increased or decreased weight gain. Patients will also experience fatigue, excessive feelings of guilt or worthlessness, diminished ability to think clearly (indecisiveness), and recurrent thoughts of death or

suicidal ideation. Suicide is the most disturbing negative result from depression and is currently the eighth foremost cause of death in the United States. Among adolescents, suicide is the second leading cause of death (Thapar 2012).

Like many diseases of the brain, depression is defined by its symptoms even though disturbances in sleep and weight can exhibit themselves in seemingly opposing manifestations. If such symptoms do have a biological underpinning, it is doubtful they are similar. Yet if two people exhibited such opposing symptoms and were diagnosed as depressed they would be given a similar treatment. Clearly, our understanding of depression is rudimentary. This is also highlighted by the statistic that only 66% of patients respond to therapy, conventional antidepressants, or a combination thereof. These non-responders will typically undergo multiple treatment paradigms only to discover that no treatment will offer them relief. Furthermore, conventional antidepressants exert their effect on their proposed targets within hours, yet clinical effects are not seen until weeks to months of chronic administration.

The dissociative anesthetic ketamine has been adopted by the psychiatric community as a novel rapid and powerful antidepressant requiring only one intravenous infusion that will have antidepressant effects in approximately 66% of patients who do not respond to conventional antidepressant treatment. Unfortunately, this still leaves a population that does not respond to pharmacological treatment. Furthermore, ketamine's use as

an antidepressant is still controversial and the exact antidepressant mechanism is unclear. This thesis investigates a novel mechanism of ketamine's antidepressant action.

1.2 Depression therapies

Depression is commonly treated with psychotherapy, antidepressants or both. Therapy and antidepressants have similar effectiveness in relieving depressive symptoms, although a combination of the two is more potent (Weitz 2015). The American Psychiatric Association recommends therapy for mild to moderate depression and pharmacological antidepressant treatment for moderate to severe symptoms (Weitz 2015). Intriguingly, symptom clusters are not an effective method to determine if one treatment will outperform the other (DeRubeis 2009). A recent imaging study has shown that patients with a hypoactive insula will be more likely to respond to psychotherapy and not antidepressants while patients with a hyperactive insula will respond to antidepressants and not psychotherapy (McGrath 2013). Meta-analyses have concluded that while both have similar effectiveness in relieving symptoms, therapy reduces relapse of depressive symptoms after discontinuation (DeRubeis 2009). Thus, it can be inferred that therapy produces lasting effects that antidepressants do not.

1.2.1 Antidepressants

Serendipity played a significant role in the discovery of compounds with antidepressant properties. Hoffman-La Roche Ltd USA developed isoniazid, the first antitubercular compound, which drastically reduced the death rate of tuberculosis. Given the success

of isoniazid chemists developed analogues in an attempt to increase effectiveness of treatment (Pletscher 1991). One compound, iproniazid, a monoalkyl derivative of its parent compound, produced unexpected side effects (Fox 1953). Despite patients suffering from tuberculosis when treated with iproniazid they experienced psychostimulation, euphoria, improved appetite and enhanced sleep (Pletscher 1991). A clinical trial with iproniazid with patients suffering from depression showed marked improvements in depressive symptoms in 70% of patients (Loomer 1958). Thus, iproniazid was the first successful compound to be used to treat depression as an off-label antitubercular compound. It was classified as a monoamine oxidase inhibitor (MAOI). MAO breakdown monoamines and catecholamines by oxidative deamination in the presynaptic terminal (Hillhouse 2016). There are two isoforms MAO_A and MAO_B; MAO_A primarily deaminates norepinephrine, epinephrine, serotonin, and melatonin, where MAO_B deaminates phenethylamine and benzylamine. Both enzymes deaminate dopamine. Inhibiting these enzymes increases neurotransmitter concentrations available for release. Later, it was discovered that iproniazid was a non-selective irreversible MAOI, inhibition of MAO_A was thought to be primarily responsible for the antidepressant effects of iproniazid. The compound was not without safety concerns as side effects included increased heart rate, sweating, and hypertension. These side effects were amplified if patients consumed foods containing high amounts of tyramine, such as cheese and chocolate, dubbed as the “cheese effect”. Ultimately, these side effects led to the withdrawal of iproniazid from the market. Soon other compounds

would be developed to selectively and reversibly target MAO_A (Lopez-Munoz 2007). A variety of MAOIs are still on the market, and these side effects are still of concern.

1.2.2 Tricyclic antidepressants

Much like MAOIs another class of compounds with antidepressant properties was discovered by chance. In an attempt to produce stronger antipsychotics Hafliger and Schinder synthesized analogues of the weak phenothiazine antipsychotic, promethazine. One compound first identified as G22355, now known as imipramine did show not antipsychotic effects but did show significant improvement depressive symptoms. Imipramine was approved by the FDA in 1959, establishing the class of compounds named tricyclic antidepressants (TCAs). Given imipramine's mechanism of action was unknown at the time its classification was based on its structure of three benzene rings (Hillhouse 2016). Later its diverse pharmacological profile was elucidated, and its primary mechanism of antidepressant action is thought to be inhibition of presynaptic serotonin and norepinephrine reuptake transporters (Cusack 1994). Much like MAOIs, although by a different mechanism, imipramine increases concentrations of serotonin and norepinephrine in the synaptic cleft (Hillhouse 2016).

1.2.3 Selective serotonin reuptake inhibitors

With mounting evidence suggesting serotonin played a significant role in both depression and the antidepressant action of many compounds, Eli lily began developing compounds that would selectively inhibit serotonin reuptake (Wong 1974). LY110140, now known as fluoxetine was synthesized, characterized as a selective serotonin reuptake inhibitor (SSRI), and eventually approved for the treatment of depression by the FDA in 1987. SSRIs are significantly more selective for inhibiting serotonin transporters than other reuptake transporters, such as norepinephrine transporters, and show nominal binding for other postsynaptic proteins (Owens 1997). Again, similar to TCAs and MAOIs, SSRIs increase the amount of serotonin in the synaptic cleft available to bind post synaptic receptors (Hillhouse 2016). It is noteworthy that, despite their widespread use, SSRIs are no more effective at ameliorating depression than the earlier-drugs. However, they are safer and less toxic.

1.2.4 Atypical Antidepressants

Bupropion was approved in 1989 as the first atypical antidepressant and exhibits a much different binding profile compared to conventional antidepressants. The drug's highest affinity is for the dopamine transporter and displays a lower affinity for the norepinephrine transporter. Unlike other antidepressants, it has no affinity for the serotonin transporter. Despite its unique binding profile bupropion shows similar

effectiveness in treating depression (Hillhouse 2016). Tianeptine, a drug that is thought to increase 5HT uptake is offered in the EU. How this contributes to antidepressant response is unknown (Samuels 2017). A recent report suggest that it may act through opioid receptors (Samuels 2017).

1.2.5 SNRIs

Given that some patients respond to TCAs, but not SSRIs, serotonin and norepinephrine reuptake inhibitors (SNRIs) were introduced in 1993. These compounds act similarly to TCAs but are more selective for their namesake transporters as they have no affinity for dopamine, serotonin, adrenergic, or histamine receptors. Thus, their side-effect profile is decreased compared to TCAs (Vaishnavi 2004).

1.2.6 Brain stimulation

Electroconvulsive therapy (ECT) was first proposed for schizophrenia by the Hungarian psychiatrist Ladislaw Meduna in 1934. He observed that post-mortem brains of patients suffering from schizophrenia showed decreased numbers of glia, which contrasted with glial hyperplasia seen in patients suffering from epilepsy. This astute observation led him to the hypothesis that schizophrenia may be treated by inducing seizures, which would increase the number of glial cells (Pinna 2016). In 1938 Bini and Cerletti performed the first ECT on a psychotic patient who went on to recover completely within a week. Notably, it has been adopted, modified, and proven to an effective treatment for depression. Moreover, ECT is more effective than conventional

antidepressants outperforming both TCAs and MAOIs (Pinna 2016). However, side effects such as memory loss and negative public perception blunt its widespread use. It now is typically reserved for patients experiencing extreme depressive symptoms (Pinna 2016). Transcranial magnetic stimulation (TMS) exploits the principle of induction to non-invasively stimulate the brain. Given the potent antidepressant effects of ECT, TMS has been posited to potentially be an effective treatment for MDD. Repetitive TMS (rTMS) delivers repetitive stimulation to the brain and has been approved for the treatment for MDD (Liu 2017). However, approximately only 25% patients respond to rTMS (O'Reardon 2007).

1.2.7 Antidepressants - concerns and controversies

A focus on monoamines has guided research and drug development, and the vast majority of marketed antidepressants in the United States act on monoaminergic uptake, catabolism or receptors. However, the rationale for this focus is weakened by the disconnect between the quick-acting effect antidepressants on monoaminergic targets (typically within a few hours of administration) and the therapeutically relevant response, which requires weeks to months, depending on the patient. Furthermore, only approximately 66% of patients respond to conventional or atypical antidepressants (Duman 2016). Many mechanisms have been proposed explaining the delayed onset of the therapeutic effects, although no clear mechanism has emerged. Increased

availability of monoamines after antidepressant treatment increases many downstream signaling pathways. Significant progress has been made parsing out which pathways are involved in the antidepressant response, although progress has not lead to development of novel, more effective antidepressants.

1.3 Methods to study depression

1.3.1 Animal Models of Depression

Given the ethical and practical challenges of studying human depression, various animal models have been developed to study depression. However, depression, like other disorders of the brain has proven difficult to model. Symptoms such as suicidal ideation, delusions, and guilt are impossible to replicate in animals. These symptoms may be unique to human depression and other human psychiatric disorders (Nestler 2010). Moreover, given the dearth of knowledge of the physiology and neurochemistry underlying or driving depressive symptoms in humans, animal models are difficult to validate. Consequently, a specific animal model is unlikely to reflect depression or any psychiatric disorder in its entirety. However, animal models have proven useful to understand aspects of the depression, and findings derived from animals have been replicated humans. Unfortunately, no advancement from an animal model has led to the successful development of a pharmacological antidepressant intervention.

Given the above considerations animal models of depression (like other diseases) need to be validated. The scientific community has long posited three types methods of validation, being construct, predicative and face validity. When developing or constructing an animal model of a disease it should be constructed by recreating an aspect of the disease found in humans. This may be accomplished by genetic, epigenetic, or biochemical means. When knowledge of these drivers is lacking, as is the case with depression, environmental factors may be employed. If a model has face validity it will reflect biochemical, physiological, or anatomical characteristics of the human disease. Again, a perfect recapitulation may not be possible. Predictive validity should allow an effective treatment to be transferable to the human disease. Unfortunately, predicative validity has not been effectively modeled in animal models of depression. This may be because many models have used conventional antidepressants to obtain a degree of validity. Although, upon further reflection, this approach seems more like an experimental tautology than effective method in creating models.

The rhesus monkey may provide the best animal model to understand MDD pathology in humans. These animals exhibit similar brain structure, social behavior and depressive symptoms as humans (Kalin 2003). However due to ethical concerns and costs they are not widely studied (Kalin 2003).

Given the lack of knowledge of genetic or physiological causes of depression models, have largely focused on exposing rodents to either acute or chronic stress in order to achieve validity. Acute exposure to stressors include the forced swim test, tail suspension test which were initially developed to screen for novel antidepressant compounds. Conventional antidepressants will increase the time an animal responds to these tests after a single treatment. Unfortunately, these models do not reflect human depression or the response to conventional antidepressants by any means. Furthermore, these tests are often passed as a measurement of depression and antidepressant action. Chronic exposure to stress offers a more valid model to study human depression. The chronic unpredictable stress paradigm involves exposing rodents to a random series of stressors which ultimately results in animals exhibiting symptoms of anhedonia (Willner 2005). Social defeat, another form of chronic stress comprises of introducing a rodent to a larger, more aggressive rodent over a period of time. Ultimately, the subordinate mouse displays depressive-like phenotypes including social withdrawal and anhedonia (Krishnan 2007). Rodents exposed to such chronic stress paradigms respond to chronic but not acute conventional antidepressant treatment (Krishnan 2007). Clearly, exposure to chronic stress paradigms are more valid than acute stress models. As knowledge of depression increases, researchers will be able to create more valid models, hopefully with a degree of predictive validity that has not been accomplished yet.

1.3.2 Cultured Cells

Given that no clear mechanism of action exists for any antidepressant compound, once a biochemical pathway is implicated in depression in humans or in animal models of depression, cultured cells offer a suitable, simplistic model. Moreover, antidepressant's influence of basic intracellular biochemical signaling is still being investigated. Given the numerous cell types in the human brain, cultured cells also provide a window into specific cell types. One example of this stems from research from our group. We have observed the effects of MDD and antidepressants in animal models of depression and humans. Since these effects have been seen in more complex systems, we employ C6 glioma cells to study the effect of antidepressants (Toki 1999) (Donati 2008). Other groups often culture hippocampal neurons to study the effects of antidepressants, as the hippocampus has repeatedly been implicated in MDD and the antidepressant response (Price 2010).

1.4 Etiology of Depression

1.4.1 Monoamine hypothesis of depression

The monoamine hypothesis of depression initially put forth by Schildkraut, was the first attempt to attribute depressive symptoms to biological underpinnings. Original evidence for the hypothesis largely came from observations after pharmacological interventions. Reserpine, a compound used to treat hypertensive vascular disease,

induced depression in select patients (Muller 1955). This effect was reproduced in animals and it was discovered that reserpine inhibits vesicular monoamine transporters, reducing monoamines available for release in vesicles, effectively causing a depletion in monoamines in the synapse (Hirschfield 2000). As the mechanism of action for compounds with antidepressant properties was found to increase monoamines through different mechanisms, a clear picture was beginning to emerge in the scientific landscape. Consequently, the monoamine hypothesis of depression, which states that patients with depression have decreased concentrations of serotonin, dopamine, and/or norepinephrine was born (Bunney 1965). Today this simplistic view of depression has been largely discarded, even though it has influenced the development of many successful antidepressants on the market.

1.4.2 Stress, neuroanatomy, and depression

While the exact cause of depression is unclear, a maladaptive response to chronic stress has repeatedly been implicated (Pittenger & Duman 2008). These responses are often manifested through a variety of cognitive, behavioral, biochemical and anatomical processes. Many of these maladaptive responses to stress have been recapitulated and studied in animals exposed to chronic stress.

While no distinct region has been determined to be the seat of depression, many brain regions, including the hippocampus, prefrontal cortex (PFC), and nucleus accumbens, have been implicated to various degrees using imaging studies and examining post-mortem tissue in depressed suicide victims. The diversity of brain regions implicated in depression may, at least in part, explain the variability in symptoms. Exposing rodents to chronic stress have also corroborated these neuroanatomical studies (Price 2010).

One highly repeatable and robust finding in humans implicates the hippocampus, in both depression and the antidepressant response. The hippocampus is important for the formation of long-term memories. Specifically, the hippocampus will shrink during depression from 8 up to 19% in volume (Price 2010). The precise mechanism for decreased hippocampal volume remains controversial, as some have reported reductions in neuronal cell bodies and dendrites, yet other have shown decreases in neuronal and glial cells (Sheline 2003). Chronic stress in rodents causes glial cell death and reduction in dendritic arborization in the hippocampus (Price 2010). These cellular changes are also reflected in impaired hippocampal long-term potentiation (LTP) and enhanced long-term depression (LTD) (Kim 2002). Interestingly, patients who have received pharmacological treatment show less reduction in hippocampal volume when compared patients who have not undergone treatment (Oakes 2017). Furthermore, a disparity of hippocampal volume is also present in patients who have recovered from

depression and those who have not. Specifically, those patients who have recovered display hippocampal volumes similar to control subjects (Oakes 2017).

Decreases in PFC volume have also been observed in depressed patients. These reductions have been attributed to decreased dendritic spine density, dendritic retraction, and glial cell atrophy (Drevets 2001) (Cook 2004) (Duman 2012). Specifically, pyramidal neurons in the medial prefrontal cortex (mPFC), but not the orbital PFC are reduced (Radley 2004) (Liston 2006). Furthermore, imaging studies in humans have shown hypoactivity in the mPFC in depressed patients (Liu 2017). This is also reflected as impaired attention in rodents exposed to chronic stress, as the mPFC plays a central role in attention (Liston 2006). A consistent and robust finding is the dramatic reduction of and proliferation of glial cells in the mPFC in humans and animal models of depression (Banasr 2007). This may contribute to reduced activity in the mPFC. Decreases in levels of BDNF in the PFC is also seen after chronic stress which is reversed upon successful antidepressant treatment (Sun 2013).

While the PFC and the hippocampus both shrink in size and suffer from hypoactivity, the amygdala's activity and volume increase in patients with depression. This finding has been replicated in several types of imaging studies (Drevets 2003). Furthermore, evidence suggests that increased activity correlates with the intensity of negative symptoms of depression (Abercrombie 1998). These changes are also reflected in

enhanced synaptic plasticity and function in rats after exposure to chronic stress (Vyas 2004). BDNF is also increased in the amygdala after stress (Baxter 2002). Interestingly, these changes remain well after stressful stimuli is removed from the environment (Vyas 2004).

The nucleus accumbens (NAc) has also been repeatedly implicated in depression. With a role in reward processing, deficits in NAc functioning are thought to underlie symptoms of anhedonia (Nestler 2006). Rodents exposed to chronic stress exhibit changes in spine morphology, with an increase in spines and decreased postsynaptic densities on medium spiny neurons (Christoffel 2011). Interestingly, unlike the hippocampus and PFC BDNF is upregulated in the NAc in humans suffering from depression and rodents exposed to chronic stress (Krishnan 2007).

1.4.3 The glutamate system's contribution to depression

Recently, the glutamatergic system has become an attractive area of research in MDD as both a contributing factor and potential drug target. However, given it is a complex system comprised of neurons, glial, and many receptor subtypes progress has been slow. However, with essential roles in LTP, mood processing, learning, and cognition studies have consistently implicated the glutamatergic system in MDD.

Glutamate is the principle excitatory neurotransmitter and is released throughout the brain. It binds many receptors including the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainite, and N-methyl-D-aspartate (NMDA) receptors. Glutamate also binds the metabotropic glutamate receptors (mGluRs). All three ionotropic receptors are permeable to Na^+ ions. NMDA receptors are unique in that they also have a high permeability to Ca^{2+}. Both AMPA receptors and NMDA receptors play a fundamental role in synaptic plasticity (Morris 1986). When AMPA receptors conduct depolarizing current along the plasma membrane and the NMDA receptor is bound by glutamate, and the co-agonist glycine, the NMDA receptor will lose a Mg^{2+} blockade of its ion pore allowing the rapid influx of Ca^{2+}. This will initiate a variety of signaling cascades involved in LTP (Cole 1989). NMDA receptor signaling can also encourage cell survival or death pathways, depending on the location of the receptor on the synapse and the duration of activation (Hardingham 2002) (Hardingham 2010). Taken together it is clear that the NMDA receptor alone is crucial to the proper functioning of the synapse and is subject to extremely complex regulation and influence over signaling pathways (Fig. 1).

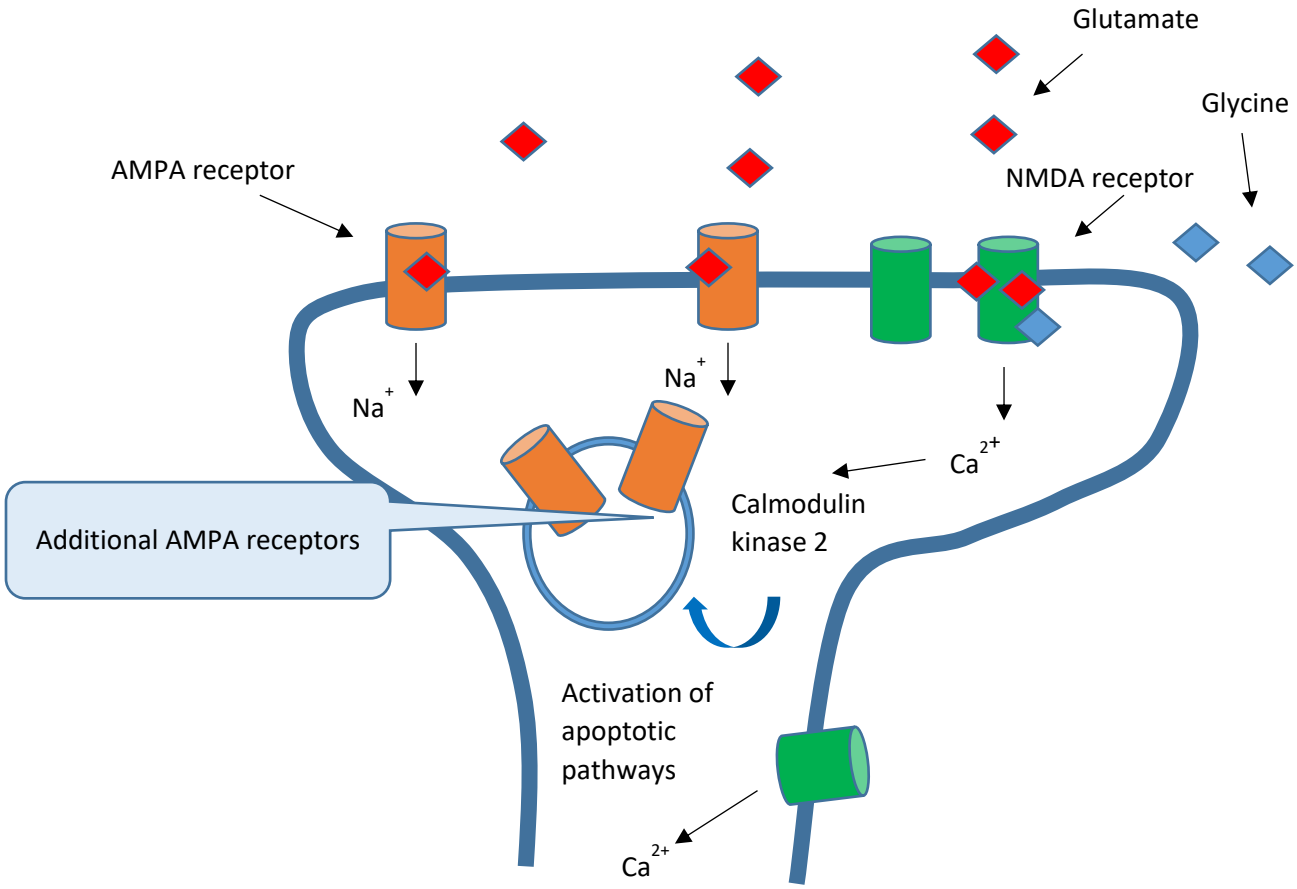


Figure 1. NMDA receptor signaling. NMDA receptors allow influx of calcium when the synapse is depolarized, and the receptor is bound by glutamate and glycine. NMDA receptors exhibit complex signaling mechanisms that can either induce insertion of AMPA receptors if activated on the synapse or apoptotic pathways if activated perisynaptically

Many divergent lines of evidence have reported the glutamatergic system in the pathophysiology MDD. High levels of glutamate have been reported in MDD patients in post-mortem and neuroimaging studies (Sanacora 2008) (Smoller 2016). Elevated amounts of glutamate have also been detected in the cerebrospinal fluid of patients with depression (Sanacora 2008). Many reports have also detailed alterations in NMDA receptor subunits in post-mortem tissue of suicide victims (Murrough 2017). Moreover, exposing mice to chronic stress has a variety effects on the glutamate system. Specifically, chronic stress reduces synaptic NMDA receptors and AMPA receptors, reduces glutamate synapse diameter, synapse density, and dendritic length (Popoli 2011). Clearance of glutamate by glial cells is also reduced after chronic stress leading to activation of extrasynaptic NMDA receptors and activation of apoptotic signaling pathways (Manji 2003).

1.4.4 Genetic contribution to depression

Even though stress may precipitate depression, a genetic contribution to the disease is well documented. Twin studies have revealed that the heritability of depression is approximately 40%, with some estimates as a high as 70%. This agrees with observations of depression amassing in families (Smoller 2016). While the genetic contribution to depression has been evident, sorting out predictive genes or single nucleotide polymorphisms that directly contribute to the development of the disease have been elusive (Smoller 2016).

Initially, genetic investigations into depression were limited to candidate genes. Most of these were chosen due to their relationship with antidepressant targets or preclinical studies in animals (Smoller 2016). Small, yet significant findings were reported in serotonergic genes and others relating to neurotransmission. However, none of these findings have been replicated in large genome-wide association studies (GWAS) (Wray 2012). Even more discouraging, many GWAS often employing thousands of controls and patients with depression have resulted in no significant findings. The first significant finding of a GWAS, was of a SNP in the SLC6A15 gene, which is involved in the transport of neutral amino acids (Kohli 2011). Unfortunately, this finding has not been supported by larger GWAS or meta-analyses (Hibar 2015). Even the GWAS with the most subjects to date, employing 9240 samples and 9519 controls did not reveal a single significant SNP or gene associated with depression (Dunn 2015). Recently, a GWAS study found two significant genes associated with depression - SIRT1 and LHPP.

Unlike previous GWAS, these compelling data were derived from a population of Han Chinese women. The authors credit reducing heterogeneity in their sample population to their success (Cai 2015). Further research is needed to determine the impact of these genes on the pathogenesis of depression. Clearly, more work is needed to fully grasp the complexity of the impact of genes on the etiology of depression.

1.4.5 BDNF

Brain-derived neurotrophic factor (BDNF) is a well characterized growth factor that is involved in many processes in the nervous system including synaptogenesis, synapse maturation, long-term potentiation, memory formation, and neuroplasticity (Park 2013) (Zheng 2012). A member of the neurotrophin family including neurotrophin-3, neurotrophin-4, and nerve growth factor, BDNF is expressed throughout the brain. Transcriptional regulation of BDNF is accomplished through nine promoters, each expressing specific transcripts that are translated into the same protein. The specific regulation and functional importance of such diverse transcriptional regulation is poorly understood. However, it is well established that neuronal activity increases the transcriptional activity of BDNF (Begni 2017). The neurotrophic factor is synthesized in astrocytes and in the soma of neurons, and ultimately released at pre- and postsynaptic terminals via activity dependent mechanisms (Lessmann 2009). Once released BDNF binds to the tropomycin receptor kinase B (TrkB) and p75 neurotrophin factor, albeit at a lower affinity (Sopper 1991) (Meeker 2015). Specifically, BDNF-TrkB signaling has been implicated in the antidepressant response (Bjorkholm 2016). BDNF binding of TrkB can lead to protein kinase C (PKC), mitogen-activated protein (MAP) kinase, and AKT-mTOR activation.

Chronic antidepressant treatment has been linked to upregulation of BDNF and to a variety of its downstream effects. The first line of evidence implicating BDNF in the delayed antidepressant response showed upregulation of BDNF and TrkB mRNA in the

hippocampus and cortex of rodents with a similar timeframe to the antidepressant response (Nibuya 1995) (Nibuya 1996). Further investigation revealed direct infusion of BDNF into the dentate gyrus (DG) or CA3, but not the CA1 region of the hippocampus mediated antidepressant effects in rodents (Shirayama 2002). BDNF inducible knockout adult mice, where BDNF was selectively knocked out in either the CA1 or DG showed that BDNF originating in the DG but not CA1 region was essential for the antidepressant response (Adachi 2008). Human studies further corroborate the role of BDNF in the pathology of depression, as hippocampal tissue from patients suffering from depression show decreased BDNF expression (Chen 2001).

The BDNF gene has a well characterized single nucleotide polymorphism (SNP) rendering the valine to be exchanged for a methionine at codon 66 commonly referred to as the val66met SNP. This polymorphism reduces the transport of BDNF to distal dendrites and inhibits its activity dependent release (Egan 2003) (Chen 2005). While this polymorphism is specific to humans, it has been replicated in rodents and extensively studied in the context of depression (Baj 2013). Mice homozygous for the met/met allele do not respond to the behavioral antidepressant effects of fluoxetine (Chen 2006). Patients with the val66met SNP show reduced response to antidepressant treatment (Bjorkholm 2016). Taken together, these studies show that BDNF plays a significant role in the antidepressant response.

1.4.6 Astrocytes and depression

Glial cells are the most abundant cell type in the brain and astrocytes are the largest, most diverse population of glia in the human brain (Rowitch 2010). Astrocytes are classified based on their morphology and the expression of specific biomarkers such as the intermediate filament glial fibrillary acidic protein (GFAP) and S100B. The population density of astrocytes is clustered in areas of neuronal cell bodies. Moreover, astrocytes extend processes dynamically ensheathing synapses, classically defined as the tripartite synapse (Heller 2015). Furthermore, astrocytes synthesize and release specific gliotransmitters that facilitate bidirectional crosstalk between astrocytes and neurons (Parpura 2012). Many of these gliotransmitters are released in a similar timeframe as synaptic communication dubbed “fast-acting” gliotransmitters. Intriguingly, astrocytes also release peptides, growth factors, cytokines, and metabolic substances defined as “slow-acting” gliotransmitters. These are released on a timescale of minutes to days, and influence metabolism, energy supply, inflammation, and development (Petrelli 2016). Thus, astrocytic influence over neuronal circuitry is becoming an ever increasingly recognized and studied level of information processing (Araque 2014).

Accumulating evidence indicates astrocytes may contribute to the pathophysiology of depression (Koyama 2015). Several post-mortem histological investigations from depressed-suicides using antibodies for the astrocyte specific markers S100B and GFAP

consistently report reduced number and density of astrocytes in brain regions including the prefrontal cortex, anterior cingulate cortex, and hippocampus (Coyle 2000) (Cotter 2001) (Rajkowska 2007). After exposure to chronic mild stress or chronic social defeat rodents show decreased somatic volume and number of astrocytes in the frontal cortex and hippocampus (Banasr 2010) (Gong 2012). Recent studies in rodents exposed to chronic stress have correlated reduction in hippocampal volume and volume of astrocytic cell bodies suggesting, astrocytes may play a central role in decreased hippocampal volume (Sanacora 2013). Moreover, treating animals with the SSRIs fluoxetine and paroxetine prevents stress induced loss of hippocampal astrocytes (Czeh 2006) (Sillaber 2008). Furthermore, ECT also upregulates GFAP protein in the hippocampus (Kragh 1993). These studies implicate astrocytes both in the pathology of depression and the antidepressant response.

1.5 G protein signaling

1.5.1 GPCRs

Increased serotonin and norepinephrine availability via transporter inhibition causes increased signaling through a plethora of receptors, most notable being G-protein-coupled receptors (GPCRs) (Table 1). With approximately 800 human genes encoding GPCRs, they are one of the largest family of proteins in the human genome (Oldham 2008). More importantly, GPCRs offer a prime target for pharmaceuticals as it is possible to design highly specific, high affinity agonists or antagonists for a single receptor.

Consequently, approximately 30-50% of drugs on the market primarily target GPCRs (Hopkins 2002).

Receptor	G-protein	expression
Serotonin receptors		
$5HT_{1A}$	$G\alpha_{i/o}$	Cortex, amygdala, hippocampus, dorsal raphe
$5HT_{1B}$	$G\alpha_{i/o}$	Basal ganglia, substantia nigra
$5HT_{1D}$	$G\alpha_{i/o}$	Hippocampus, Nacc, striatum, substantia nigra
$5HT_{1E}$	$G\alpha_{i/o}$	Cortex, hippocampus, dorsal raphe
$5HT_{1F}$	$G\alpha_{i/o}$	Cortex, hippocampus, dorsal raphe
$5HT_{2A}$	$G\alpha_{q}$	Cortex, basal ganglia
$5HT_{2B}$	$G\alpha_{q}$	Cortes, cerebellum
$5HT_{2C}$	$G\alpha_{q}$	Hippocampus, substantia nigra, basal ganglia
$5HT_{3}$	Ion channel	Hippocampus, cortex
$5HT_{4}$	$G\alpha_{s}$	Hippocampus, Nacc, striatum
$5HT_{5}$	$G\alpha_{s}$	Hippocampus, cortex, cerebellum
$5HT_{6}$	$G\alpha_{s}$	Hippocampus, cortex, striatum
$5HT_{7}$	$G\alpha_{s}$	Cortex, hypothalamus, thalamus
Norepinephrine Receptors		
α_{1A}	$G\alpha_{q}$	Hippocampus, cortex
α_{1B}	$G\alpha_{q}$	cortex
α_{1D}	$G\alpha_{q}$	
α_{2A}	$G\alpha_{i/o}$	cortex, midbrain
α_{2B}	$G\alpha_{i/o}$	diencephalon
α_{2C}	$G\alpha_{i/o}$	Hippocampus, cortex, cerebellum, basal ganglia
β_{1}	$G\alpha_{s}$	cortex, cerebellum
β_{2}	$G\alpha_{s}$	Hippocampus, cortex, piriform cortex

Table 1. GPCRs and G-protein

As antidepressants increase availability of monoamines in the synapse a variety GPCR signaling pathways will be activated throughout the brain. Here receptors for serotonin and norepinephrine, along with their cognate G-proteins, are listed.

GPCRs transduce myriad extracellular stimuli such as odorants, light, hormones, neurotransmitters and then activate intracellular signaling cascades. All receptors are composed of seven transmembrane-spanning alpha-helices, an intracellular carboxyl terminus, an extracellular amino terminus and three helical loops on either side of the plasma membrane. A highly conserved DRY/ERY motif is present on the third intracellular loop that binds a G-protein and is responsible for coupling of GPCR to a G-protein.

1.5.2 G-proteins

GPCRs activate intracellular signaling cascades by coupling with and ultimately activating GTP binding proteins (G-proteins). Hetereotrimeric G-proteins are composed of α, β, and γ subunits. Despite coupling to all GPCRs there are only 21 Gα subunits, 6 Gβ subunits and 12 Gγ subunits expressed in humans. Intracellular signaling specificity relies on the unique composition, which heterotrimeric G-protein the GPCR associates with or "couples". Upon ligand activation of the GPCR the receptor will undergo a conformation change that will cause the Gα subunit to exchange a guanosine diphosphate (GDP) for a guanosine triphosphate (GTP), which "activates" the hetereotrimeric protein. Thus, GPCRs act as guanine exchange factors (GEFs) when acting upon their cognate Gα subunit. G proteins primarily act through the Gα subunit, which once activated, will bind to and modulate a variety of downstream targets defined as effectors. However, Gβγ, which essentially function as one protein, can also

modulate a variety of proteins and effectors. To effectively turn off the intracellular signaling cascade all Gα subunits contain a GTPase domain, which will cleave a phosphate from the GTP rendering it a GDP to inactivate itself. The classic dogma of Gα signaling envisions the Gα subunit dissociating from the Gβγ subunits and binding to its cognate effector. This remains elusive to definitively test and the precise mechanism is unknown as some reports indicate a dissociation and others a conformational change (Oldham 2008).

The Gα subunit is composed of a conserved protein structure consisting of a helical and a GTPase domain. The GTPase domain is conserved even among monomeric G-proteins. Not only does this domain hydrolyze GTP it also provides binding surface for GPCRs, effectors, and the Gβγ dimer. This domain contains three switch regions named switches I, II and III. Six α-helix bundles comprise the helical domain, which sequesters bound nucleotides inside the protein (Fig. 2). The helical domain is unique to the Gα subunit (Oldham 2008).

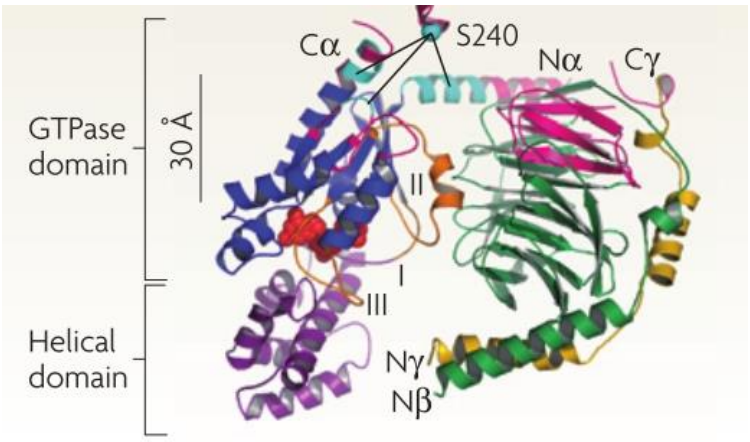


Figure 2. Crystal structure of heterotrimeric G-protein

Crystal structure reveals switch regions I, II, and III in Gα subunit. Furthermore, association with Gβγ dimer at GTPase domain of Gα is shown. Adopted from (Oldham 2008).

Heterotrimeric G-proteins are targeted and tethered to the plasma membrane by numerous fatty acid post-translational modifications. The Gα subunit also has a polybasic positively charged amino-terminus which aids in its association to the negatively charged plasma membrane. These covalent lipid modifications are sometimes reversible and influence GPCR signaling (Escriba 2007). Interestingly, the Gα subunits undergo a diverse range of acylation modifications, which targets the protein to unique plasma membrane domains and influences protein-protein interactions (Fig. 3) (Oldham 2008). The $G\alpha_i$ family is the only Gα member that exclusively undergoes myristoylation (14 carbon saturated fatty acid), which is an irreversible modification (Escriba 2007). Most Gα subunits with the exception of $G\alpha_t$ and $G\alpha_{gust}$, undergo a palmitoylation (16 carbon saturated fatty acid). This modification is reversible and the Gα subunit will undergo rapid palmitate turnover after GPCR activation. Moreover, depalmitoylation mediates subcellular redistribution of the Gα subunit from the plasma membrane to the cytosol (Wedegaertner 1996) (Yu 2002). Furthermore, acylation of the Gα subunit can target the hetereotrimeric g-protein to specific plasma membrane microdomains (Allen 2007). The Gγ subunits can be farnesylated or geranylgernylated at their carboxyl terminals.

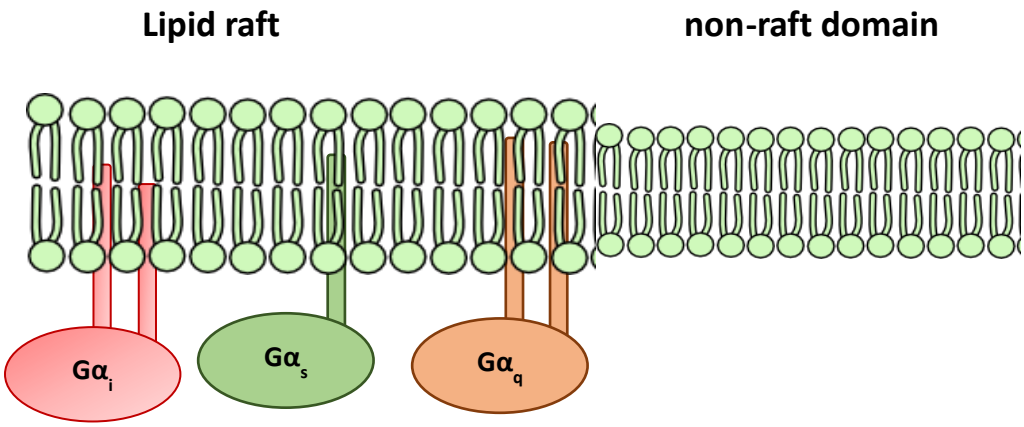


Figure 3. Lipid modification of Gα subunit

The Gα subunit undergoes a variety of acylation post-translation modifications. $G\alpha_s$ is singly palmitoylated. $G\alpha_i$ is palmitoylated and myristoylated. $G\alpha_q$ is doubly palmitoylated.

Heterotrimers are classically defined by the function of their Gα subunit. The $G\alpha_q$ subunit, often referred to as $G\alpha_q$ activates phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol trisphosphate (IP_3). The $G\alpha_{12/13}$ subunit primarily modulates actin dynamics (Milligan 2006). The stimulatory subunit ($G\alpha_s$) and inhibitory subunit ($G\alpha_i$) will bind to adenylyl cyclase after GTP induced activation and either stimulate or inhibit the ability of adenylyl cyclase to produce cyclic adenosine mono-phosphate (cAMP). Once a single Gα subunit is activated by a GPCR, it can activate numerous effectors, thereby amplifying the extracellular signal. Whereas these pathways are each Gα's primary mode of action, many will deviate from their simplistic categorization and modulate other signaling pathways (Table 2) (Milligan 2006).

	Effect
$G\alpha_s$	activate adenylyl cyclase to increase cAMP
	activate GTPase of tubulin
	activate potassium channels
	activate c-Src kinase
$G\alpha_{i/o}$	inhibit adenylyl cyclase to decrease cAMP
	activate ERK/MAP kinase
	decrease opening probability of calcium channels
	activate potassium channels
$G\alpha_q$	activate phospholipase C
	activate p63-RhoGEF
	activate potassium channels
$G\alpha_{12/13}$	activate phospholipase D
	activate iNOS
	activate p115RhoGEF
	activate PDZ-RhoGEF

Table 2. Functions of G-proteins

The Gα subunits initiate many cellular signaling pathways besides their canonical effector. Table 2 lists a few of the known biochemical events. Table adopted from *(Milligan 2006).*

1.6 Depression, antidepressants, $G\alpha_s$, and cAMP

1.6.1 Translocation of $G\alpha_s$

A well-established biochemical phenomenon involved in GPCR signaling and depression is the translocation of $G\alpha_s$ from lipid raft domains to non-raft domains of the plasma membrane. Once localized in non-raft domains it can more effectively bind and activate adenylyl cyclase.

$G\alpha_s$ plasma membrane localization has been proposed to be a biomarker of depression and successful antidepressant response, given in the depressed state $G\alpha_s$ is localized in lipid rafts, and in the non-depressed state $G\alpha_s$ localized in non-raft domains (Donati 2008). Non-raft $G\alpha_s$ localization may modulate numerous GPCRs that are coupled to $G\alpha_s$ (Table 1). The first line of evidence of this effect reported increased production of cAMP from membranes prepared from the cortex and hypothalamus of rats treated with tricyclic antidepressant for 15-22 days or treated with ECT (Menkes 1983). This effect was ablated by the addition of colchicine initially implicating microtubules in the antidepressant increase in cAMP (Menkes 1983).

While an exact mechanism detailing the biochemical events that drive $G\alpha_s$ from lipid rafts to non-rafts is unknown, recent evidence from our group suggests that it involves a disruption of the $G\alpha_s$ and tubulin complex. It is well documented that $G\alpha_s$ binds tubulin with an affinity of ~130 nm and tubulin is enriched in lipid rafts (Allen 2007) (Schappi

2014). TCAs and SSRIs reduce association of tubulin and $G\alpha_{s,}$ specifically in lipid rafts (Singh 2018). Interestingly, increasing the acetylation of α-tubulin at lysine 40 with histone deacetylase (HDAC) 6 specific inhibitors also reduces the association tubulin and $G\alpha_s$, which results in $G\alpha_s$ translocating out of lipid rafts. Indeed, HDAC 6 inhibitors such as tubastatin show antidepressant effects in humans and animal models of depression (Singh 2018). Furthermore, the successful translocation is dependent on the isoform of adenylyl cyclase. When HEK 293 cells are treated with antidepressants $G\alpha_s$ does not translocate from lipid rafts. However, when adenylyl cyclase 6 (which is not endogenously expressed in HEK 293 cells) is expressed in HEK 293 cells, they respond to antidepressant treatment (Schappi *in prep*). Other groups have also suggested that adenylyl cyclase isoforms act as cellular scaffolds for specific G-proteins (Sadana 2009).

After a 3-day treatment in neural derived cells and 3-week treatment in rats, all classes of antidepressants mediate the translocation of $G\alpha_s$ from lipid raft domains to non-raft domains in a dose-dependent manner (Toki 1999) (Csysz 2015). Indeed, disruption of lipid rafts by caveolin depletion or methyl-β-cyclodextrin (MβCD) increases intracellular cAMP (Allen 2009). Other psychotropic drugs such as stimulants, anxiolytics, and antipsychotics do not influence the plasma membrane distribution of $G\alpha_s$ (Csysz 2015). Furthermore, this translocation is specific to the $G\alpha_s$ subunit as all other classes of Gα do not modulate their plasma membrane distribution (Toki 1999) (Donati 2005) (Schappi *in*

prep). It is currently unknown if rapid acting antidepressants, such as ketamine, influence the plasma membrane distribution of $G\alpha_s$ and consequently cAMP.

1.6.2 cAMP and depression

Studies from post-mortem tissue from depressed suicides have shown that $G\alpha_s$ is localized in inhibitory lipid rafts in both the cortex and cerebellum in the depressed state (Donati 2008). Adenylyl cyclase activity is also reduced in membranes prepared from the frontal cortex of suicide completers despite total $G\alpha_s$ and $G\alpha_i$ expression not differing from controls (Cowburn 1994). This same effect is also seen in the periphery as platelets from depressed patients show reduced capability to produce cAMP, yet levels of $G\alpha_s$ remain unchanged (Hines 2005) (Mooney 2013). Increasing intracellular cAMP by selectively inhibiting the cAMP specific phosphodiesterase type 4 (PDE4) with the high affinity PDE4 inhibitor rolipram, produces antidepressant effects in rodents when administered chronically but not acutely (Itoh 2004) (Zhang 2002). Moreover, decreased PDE4 activity achieved by inhibiting the PDE4 phosphorylating (and regulating protein) Cdk5 in the ventral striatum increases intracellular cAMP and promotes antidepressant-like effects (Plattner 2015). Isoforms PDE4A and PDE4B are upregulated upon chronic antidepressant treatment, also suggesting that cAMP is upregulated after antidepressant administration (Takahashi 1999). Furthermore, recent positron emission tomography (PET) studies have shown that cAMP is globally decreased in patients suffering from depression and upon successful remission of symptoms after

antidepressant treatment with SSRIs, cAMP increases to levels seen in control patients (Fujita 2016).

1.6.3 PKA, CREB, and Depression

As cAMP increases it may activate many intracellular targets. Protein kinase A may be the most thoroughly characterized, and also is a well-documented player in depression and in the antidepressant response (Dwivedi 2008). PKA is an enzyme that exists as a tetramer in its inactive state with the two catalytic units suppressed by two regulatory units. As intracellular cAMP levels increase, cAMP will bind to the two regulatory units which will allow the catalytic units to disassociate. The two catalytic units are then free to phosphorylate serine or threonine residues on downstream targets in the cytosol or in the nucleus (Scott 1990). Once translocated to the nucleus the catalytic subunits will phosphorylate cAMP-response element binding protein (CREB) and induce transcription of genes with a cAMP-response element (CRE) consensus sequence in their promoter. One target that is critical to the antidepressant response is BDNF, as described in detail above (Nibuya 1995) (Dwivedi 2008). Furthermore, independently of transcriptional regulation, PKA can modulate synaptic plasticity, neurotransmitter release, receptor desensitization, and cell survival. The role of these effects is not as well characterized in the context of depression (Lara 2003) (Riccio 1999).

CREB has been consistently and robustly implicated in depression and the antidepressant response. Indeed, CREB is upregulated after chronic antidepressant in rodents, yet upregulation of CREB has varied effects on animal behavior (Nibuya 1996) (Malberg 2005). For example, CREB overexpression in the nucleus accumbens results in a pro-depressive phenotype in rodents (Pliakas 2001). Likewise, increased CREB expression in the baso-lateral amygdala also produced pro-depressive behavior (Wallace 2004). Similarly, inhibition of CREB in the nucleus accumbens by transgenic expression of a dominant negative mutant of CREB produces antidepressant effects in rodent models of depression (Newton 2002).

However, many studies have shown increased CREB expression to be necessary for clinical antidepressant effects which are reviewed in (Dwivedi 2008). Overexpression of CREB in the hippocampus induces antidepressant behavioral effects in animal models of depression (Chen 2001). A report from depressed patients have shown reduced CREB expression in the temporal cortex (Dowlatshahi 1998). Postmortem tissue from depressed suicide completers has shown significantly decreased CREB expression both in the prefrontal cortex and hippocampus (Dwivedi 2003). Both CREB and phosphorylated CREB were found to be significantly reduced in the orbitofrontal cortex of depressed patients (Yamada 2003). In the periphery, the $G\alpha_s$ coupled agonist isoproterenol stimulated phosphorylated CREB was reduced in fibroblasts isolated from depressed patients (Manier 2000) (Akin 2005). Moreover, phosphorylation of CREB was

increased in T-lymphocytes in depressed patients who responded to antidepressant treatment, but not in antidepressant non-responders (Koch 2002). These peripheral effects may be the result of $G\alpha_s$ localized in inhibitory lipid rafts.

1.6.4 Lipid rafts

The original Singer-Nicolson model of the plasma membrane postulated the lipid bilayer as a fluid and homogenous two-dimensional plane where proteins could diffuse unimpeded (Singer 1972). This model has been extensively reimagined and updated as we continue to learn more about the complexity and heterogeneity of the lipid and protein content of the plasma membrane. It is now known that the inner and outer leaflet of the plasma membrane are composed of hundreds of different lipids and proteins resulting in an extremely diverse two-dimensional plane (Ingolfsson 2014).

Furthermore, discrete membrane compartmentalization occurs though a variety of intermolecular forces among lipids, membrane proteins, and cytoskeleton proteins. Specifically, these membrane compartments are defined as lipid rafts, which are small (25-100nm diameter) temporally transient domains of the membrane dependent on cholesterol, rich in saturated fatty acids, sphingolipids, and cytoskeletal components that organize proteins and modulate signaling events (Nicolau 2006) (Allen 2007). Lipid rafts exist in two forms. One mediated by the scaffolding protein caveolin which causes

the membrane to invaginate named, caveolae (Latin for little caves). The other being planar lipid rafts which are composed of cholesterol, sphingolipids, saturated fatty acids, and cytoskeletal proteins but contain flotillin in lieu of caveolin, and therefore do not invaginate. Caveolin and subsequently caveolae are highly expressed in hippocampal neurons, oligodendrocytes, dorsal root ganglia, and astrocytes (Trushina 2006). The majority of neurons lack caveolin and therefore do not have caveolae, nevertheless neurons do express flotillin which is a component of planar lipid rafts (Lang 1998). Protein localization in lipid rafts has been shown to alter a variety of events involved in signaling including ligand binding and both inhibition and enhancement of GPCR mediated second messenger production (Fig. 4) (Allen 2007).

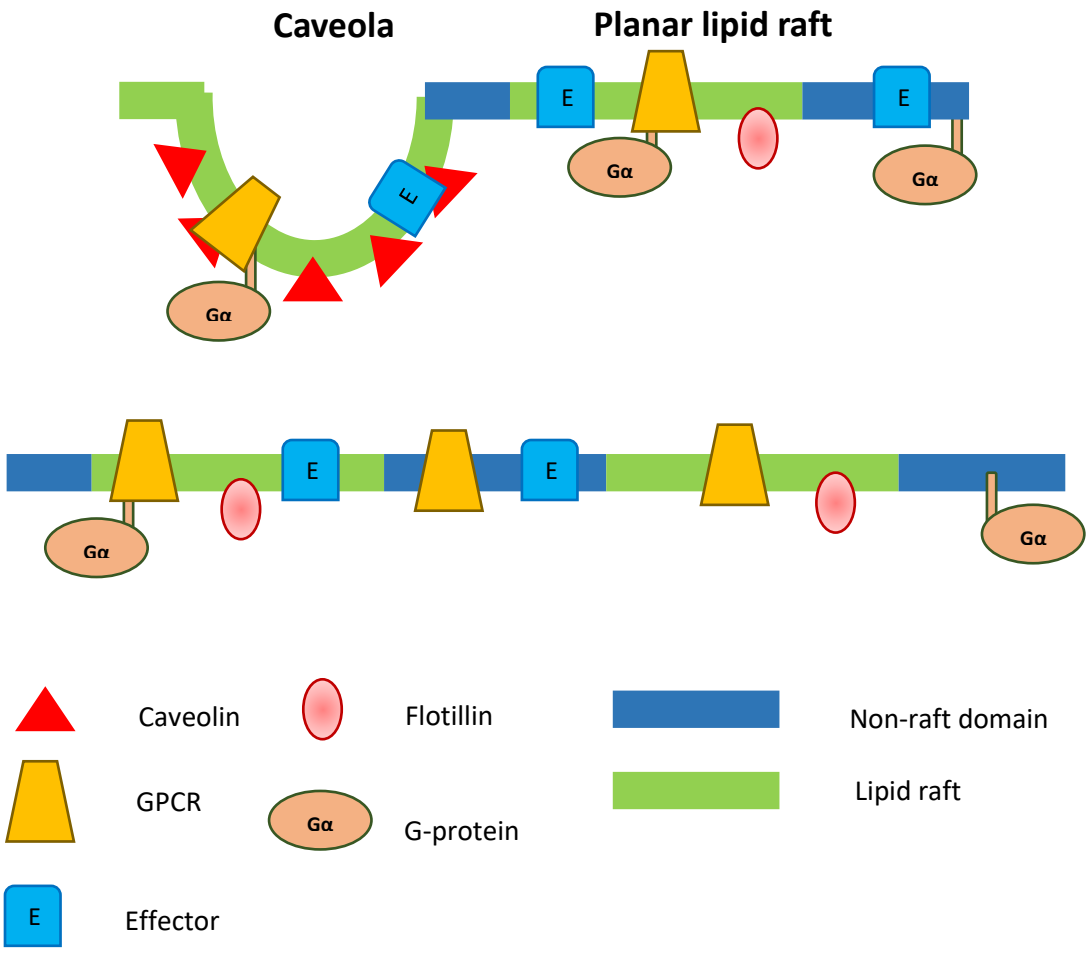


Figure 4. Lipid rafts act as signaling organizers.

Caveolin are required for invagination of caveola. Planar lipid rafts lack caveolin but contain flotillin. Both types of lipid rafts organize GPCRS, G-proteins and effectors.

1.6.5 Antidepressants and lipid rafts

Our group and others have shown that a variety of antidepressant compounds accumulate in lipid rafts. Eisensamer first reported that TCAs and SSRIs accumulate in lipid raft fractions when added directly to sucrose density gradient isolated lipid rafts (Eisensamer 2005). Our group treated cultured C6 glioma cells over three days and isolated lipid rafts, which revealed accumulation of SSRIs and to a reduced degree, TCA antidepressants (Erb 2016.) Other psychotropic compounds with similar lipophilicity did not accumulate (Erb 2016). More importantly the antidepressant escitalopram accumulated in a dose and treatment duration manner and its inactive isomer (R)-citalopram did not accumulate in lipid rafts, indicating the stereospecificity of this effect (Erb 2016). Where proteins are often thought as a general target for stereospecific molecules lipid species are also stereospecific, and lipid-protein interactions also allow unique binding targets giving rise to the possibility that escitalopram binds to a specific protein, lipid, or protein-lipid binding partners present in lipid rafts (Hurley 2001). It is currently unknown if rapid acting antidepressants such as ketamine also accumulate in lipid rafts.

1.6.6 Methods to study lipid rafts

Given lipid rafts submicroscopic nature, they cannot be visualized with traditional microscopic techniques. However, many complementary methods exist to study their role in organization and signaling. The majority of the techniques employ specific

detergents, which can either solubilize lipid raft domains or non-raft domains. Specifically, the non-ionic detergent Triton X-100 (TX100) will solubilize non-raft domains and buoyant lipid rafts will float on top of a sucrose density gradient after centrifugation. Other detergent such as octylglucoside, Brij 96, Brij 98, and tween 20 are also used. Given that cholesterol is necessary to organize lipid rafts, reagents that chelate cholesterol, such as methyl-β-cyclodextrin (MβCD), may be used to grossly disrupt lipid rafts to determine the cellular relevance of membrane protein lipid raft localization (Simons 2000). Model membranes composed of simplistic membrane fatty acids labeled with heavy atoms has allowed visualization of lipid raft like domains using electron microscopy (Edidin 2003). Recently, a stimulated emission depletion (STED) study in cultured cells have shown transient organization of membrane proteins in 100nm clusters, which were dependent on cholesterol and not perturbed by protein disruption, strengthening the case for the existence of lipid rafts (Saka 2014). Unfortunately, direct visualization of lipid rafts *in vivo* has yet to be performed and will require technical advances.

1.6.7 Lipid raft controversy

While generally accepted as an integral mediator of cell signaling and plasma membrane organization among cell biologists, lipid rafts are somewhat controversial among some (Munro 2003). This controversy has largely been a result of the technical challenges associated with studying lipid rafts and often conflicting data are obtained as a result of small changes in experimental paradigms (Munro 2003). One well characterized

example of detergent dependent protein localization is the epidermal growth fact receptor (EGFR). If Brij 98 is used to extract membranes the EGFR is localized in lipid raft fractions but if TX100, Brij 96, or octylglucoside is employed the EGFR appears to be localized in non-raft fractions (Pike 2005). These conflicting results highlight the technical challenges associated with using different experimental methods to study lipid raft protein localization. Ultimately, superior techniques will need to be developed to study and even visualize lipid rafts *in intro* and *in vivo.*

1.7 Methods to study $G\alpha_s$ lipid raft localization

1.7.1 Biochemical methods

As stated before $G\alpha_{s\ is}$ localized in lipid rafts in the depressed states and translocated to non-raft regions after effective antidepressant treatment. One method to study the translocation is by isolating lipid rafts by TX100 and centrifugation to float lipid rafts on top of sucrose density gradients. The lipid rafts can then be isolated, sucrose washed off, and samples immunoblotted for proteins of interest. This can be performed in a variety of tissues including brain, liver, and blood, or in any type of cultured cells. Obtaining enough material from cultured cells requires hundreds of millions of cells. Specific regions from rat brains must be pooled from at least 2 animals, for our protein of interest ($G\alpha_{s)}$ (Toki 1999). The requirement for such large amount of material often causes lipid raft isolation to be slow and laborious. We also employ either a TX100 and sucrose density gradient or sucrose density gradient with a detergent. If a detergent is

not used, harsh mechanical force is required to break apart lipid rafts from the plasma membrane.

1.7.2 Microscopy based techniques

Recently a higher throughput technique was developed to study the antidepressant mediated translocation from lipid rafts to non-raft regions. Fluorescent recovery after photobleaching (FRAP) provides a method to study the lateral mobility of proteins in the two-dimensional space that is the plasma membrane. Briefly, a dye, fluorophore, or fluorescently tagged protein will be localized or expressed solely in the plasma membrane, baseline fluorescence measured and then bleached with an intense laser. The intensity of fluorescence is then measured over time as it returns toward baseline.

As the speed of diffusion of the molecule of interest increases it will increase the rate which fluorescence intensity approaches baseline and vice versa. To determine if $G\alpha_s$ exodus from lipid rafts would alter the FRAP of $G\alpha_s$, a functional $G\alpha_s$-GFP was created and expressed in C6 glioma cells. Given that lipid rafts are considered stiff compartments of the plasma membrane due to enriched cholesterol and saturated fatty acid content, it was hypothesized that after antidepressant mediated translocation from lipid rafts $G\alpha_s$-GFP mobility would increase consequently increasing $G\alpha_s$-GFP FRAP. Surprisingly, the mobility and FRAP of $G\alpha_s$-GFP was actually decreased. Cholesterol chelation with MβCD leading to gross lipid raft disruption has the same effect upon $G\alpha_s$-GFP FRAP as antidepressants. These findings further support the notion that the

decreased $G\alpha_s$-GFP mobility can be used as a proxy of $G\alpha_s$-GFP plasma membrane localization. It is now thought that as $G\alpha_s$-GFP translocates from lipid rafts it increases its association with the much larger 12 transmembrane spanning protein adenylyl cyclase, which decreases mobility of $G\alpha_s$-GFP. This remains to be confirmed by knocking down adenylyl cyclase expression and performing FRAP (Csysz 2015). The $G\alpha_s$-GFP FRAP assay is currently being developed as a medium-high throughput assay to identify potential novel antidepressant compounds. Ideally, thousands of compounds will be screened over a few days. Currently, the assay allows a few compounds to be screened for potential antidepressant action over the course of an afternoon, which is a drastic increase compared to isolating lipid rafts via biochemical methods.

1.8 Ketamine

Ketamine has recently received wide recognition as a novel, rapid and potent antidepressant, especially where traditional treatment with behavioral therapy and conventional antidepressants have failed. This finding has galvanized researchers in efforts to understand its mechanism of action and develop alternative rapid acting antidepressants (Sanacora 2015).

1.8.1 A brief history of ketamine

Ketamine's inception came after the chemist Calvin Stevens at Parke Davis Company desired to synthesize a phencyclidine (PCP) analog, to mimic PCP's safe yet potent

anesthetic qualities but with decreased duration and delirium. Ketamine was first identified as CI-581 having a similar structure as PCP but a marked decrease in anesthetic potency compared to its parent compound (Fig.5). Having both a ketone and an amine in its structure, it was renamed ketamine (Fig.5). It's first clinical trial as an anesthetic was performed by Domino and Corssen, two professors from the University of Michigan, who reported the drug to be a safe and effective anesthetic appropriate for clinical use (Domino 1965). The researchers later reported that ketamine was capable of producing a delirium or hallucinogenic state similarly to PCP, albeit not nearly as pronounced (Corssen 1966). After Domino's wife heard stories of the delirium or disconnected state ketamine was capable of producing, she coined the term "dissociative anesthetic", and the name stuck. Ketamine was approved by the FDA in 1970, marketed under the tradename Ketalar as a dissociative anesthetic, and is still in clinical use today.

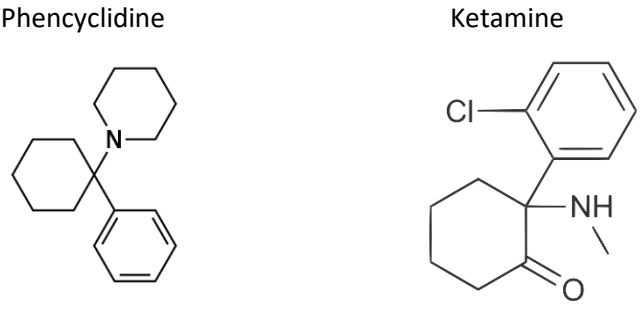


Figure 5. Chemical structure of PCP and ketamine

PCP (left) produced powerful anesthetic effects but also intense hallucinations. Ketamine (right) was initially synthesized to mimic PCPs anesthetic properties.

1.8.2 Ketamine pharmacology and metabolism.

Ketamine is classically defined as a non-competitive NMDA receptor antagonist and binds inside the open ion channel to occlude the flow of ions (MacDonald 1987). The NMDA receptor must be open to allow ketamine to penetrate and bind inside the ion pore (MacDonald 1987). Similar to its parent molecule PCP, ketamine can remain bound inside the ion channel after the pore closes (Huetnner 1988). Thus, the molecule displays "trapping" characteristics. Ketamine dissociates from the open channel faster than PCP; thus, it has lower trapping properties than PCP (Johnson 2006). Furthermore, ketamine is not selective for specific NMDA receptor subunit composition (Chen 2009). Ketamine is typically sold as a racemic solution and the isomers display different affinities. The (R) isomer displays a K_i of 1.4 μM and the (S) isomer displays an increased K_i of 0.3 μM (Ebert 1997).

Ketamine permeates the blood brain barrier and shows high brain permeability (Zanos 2016). Ketamine's bioavailability varies depending on the method of administration. Intravenous administration of ketamine yields 100-93% bioavailability. Intramuscular injection of 0.5 mg/kg ketamine displays a bioavailability of 93% and the peak concentration of 240 ng/mL (Grant 1981). Given orally, ketamine has a markedly decreased bioavailability, at the same dose of 17%, and peak concentration of 45 ng/mL (Grant 1981). The metabolic half-life of ketamine is approximately 3 hours and is not considerably different between the isomers (White 1985). Ketamine is converted into a wide range of metabolites via demethylation and hydroxylation. Interestingly, (2R,6R)-

hydroxynorketamine (HNK) shows antidepressant activity and may play a role in the antidepressant action of ketamine. It is currently unknown if (2R,6R)-HNK exerts influence on $G\alpha_s$ plasma membrane microdomain localization.

1.8.3 Ketamine as a recreational drug

The delirium, as it was first described, produced by ketamine has made it a popular recreational drug, especially in the electronic dance music scene. Ketamine is often sold as a powder under various names including Special K, Vitamin K, Cat Valium, Super Acid or Purple (Tyler 2017). Users typically experience a sense of euphoria at low doses, and hallucinations with out-of-body experiences (or a dissociative state) at higher doses. The out-of-body experience is regularly referred to as the K-hole (Muetzefeldt 2008). Consequences of chronic long-term recreational use of ketamine include memory problems, ulcerative cystitis, and a decreased sense of well-being (Morgan 2010) (Shahani 2007). Chronic high dose recreational use has been associated with changes in white and gray brain matter (Roberts 2014) (Liao 2011). Although death from ketamine overdose is extremely rare, it remains a concern (Bokor 2014). Given its potential for abuse, in 1999 it was listed as a Schedule III non-narcotic substance under the Controlled Substance Act (Tyler 2017).

1.8.4 Ketamine as an anesthetic

Despite ketamine's capability of inducing delirium and euphoria, it remains one of the safest anesthetics available in the clinic today, as it has a large therapeutic window and does not cause respiratory depression. Even at sub-anesthetic doses of 0.2-0.8 mg/kg

ketamine can deliver analgesia and a state of restfulness, and general anesthesia is achieved at doses of 0.5-1.0 mg/kg. (Allen 2005) (Domino 1984). A variety of mechanisms mediating ketamine's anesthetic properties have been proposed over the years. Indeed, the small hydrophobic molecule passes the blood brain barrier, binds to many protein targets and elicits numerous effects at various concentrations thought to contribute to its anesthetic properties (Table 3). However, it is largely accepted that ketamine elicits its anesthetic properties primarily by non-competitively antagonizing the NMDA receptor. Not only does it act as an open channel blocker by binding inside the receptor to prevent the flow of ions through the receptor it also allosterically binds to the outside surface of the NMDA receptor to reduce channel opening probability (Orser 1997). Furthermore, ketamine has a slow-off rate or a high trapping rate, that is even after the receptor closes as glutamate becomes unbound, ketamine remains bound inside the pore of the receptor causing continual antagonism. This high trapping rate is thought to be a key transducer of its anesthetic effects as NMDA antagonists with low trapping such as memantine do not produce considerable anesthetic effects (Sleigh 2014). Further strengthening the theory that NMDA antagonism mediates ketamine's anesthetic effects is the phenomena of (S)-ketamine acting as an approximate fourfold more potent anesthetic compared to (R)-Ketamine, and (S)-ketamine having an approximate fourfold increased affinity for the NMDA receptor compared to the (R) isomer.

1.8.5 Ketamine as an antidepressant

As mentioned above ketamine has galvanized researchers' attempts to identify novel antidepressant targets in order to develop new antidepressants that act as rapidly and potently as ketamine. The first preclinical data came 25 years before the first clinical trial and actually reported the tricyclic imipramine to be a significantly superior antidepressant (Sofia 1975). The first clinical trial to test ketamine's antidepressant potential was conducted at Yale with 7 patients. A subanesthetic dose of 0.40mg/kg was administered over the duration of 40 minutes and then depressive symptoms were evaluated using the Beck Depression Inventor and the Hamilton Depression Rating Scale. Researchers reported an appreciable improvement from depressive symptoms within three hours that lasted up to three days (Berman 2000). Interestingly, the initial report was largely ignored in the scientific community as researchers and clinicians alike didn't think such rapid acting antidepressant activity was feasible. Additional clinical trials have shown antidepressant effects of ketamine lasting up to two weeks after one treatment (Murrough 2013).

Target	K_i (nM)	concentration	effect	reference
NMDA receptor	1,190		Antagonist in rat cortex	PDSP K_i database
	659		-	(Roth 2013)
	661		-	PDSP K_i database
		5-50 μM	NMDA receptor blockade which decreases phosphorylation of eEF2, which rapidly increases BDNF translation	(Autry 2011)
K-opioid receptor	> 10,000		-	PDSP K_i database
	25,000	EC_{50} 29 μM	agonist	(Nemeth 2010)
	4,200.0		-	(Hustveit 1995)
μ-opioid receptor	2,500.0		-	(Hustveit 1995)
δ-opioid receptor	1,000.0		-	(Hustveit 1995)
B_2AR	> 10,000		-	PDSP K_i database
D1	> 10,000		-	PDSP K_i database
D2	1000.0	EC_{50} of 0.9 ± 0.4 μM	Agonist	(Kapur 2002)
$5ht_{2A}$	1500.0		-	(Kapur 2002)
DAT		66.8 μM	Inhibition of dopamine transport	(Nishimura 1999)
NET		62.9 μM	Inhibition of norepinephrine transporter	(Nishimura 1999)
SERT		162 μM	Inhibition of serotonin transporter	(Nishimura 1999)
HCN1		EC_{50} 16 μM	Selectively inhibits currents from Hyperpolarization-activated cyclic nucleotide–gated channels (HCN)1 subunit-containing channels	(Chen 2009)
Muscarinic	1,800.0		-	(Hustveit 1995)
unidentified		25 μM	Inhibition of ATP-Evoked Exocytotic Release of BDNF from Vesicles in Cultured Rat Astrocytes	(Stenovec 2016)
		10 – 30 μM	Potentiates 5-HT3 receptor-mediated currents in rabbit nodose ganglion neurones	(Peters 1991)
		15mg/kg	Increase in BDNF in rat hippocampus	(Garcia 2008)
		30mg/kg	Upregulation and activation of AMPA receptors	(Zanos 2016)
		30mg/kg	Phosphorylation of TrkB downstream targets including mTOR	(Li 2010)
		7mg/kg	ketamine accelerates differentiation of adult hippocampal neural progenitors into functionally mature neurons	(Ma 2017)
BK channels		100 μM	Inhibition of inward currents in cultured microglial cells	(Hayashi 2011)

L-type VDCC		10 μM	Inhibition of L-type voltage dependent calcium channels	(Baum 1991)

Table 3. Ketamine's promiscuous nature. Ketamine binds many targets at various concentrations throughout the brain. Some targets may be responsible for its anesthetic properties while others may elicit antidepressant effects. Other effects are mediated by unknown targets*. (Ki determinations were provided by the National Institute of Mental Health's Psychoactive Drug Screening Program (PDSP)).*

Ketamine's mechanism of antidepressant action is still highly controversial and many of its proposed mechanisms for antidepressant activity are detailed in (Table 3). Furthermore, the antidepressant mechanism may involve targets other than NMDA receptor antagonism, given that other NMDA receptor antagonist have failed clinical trials, and do not provide quick-acting, robust, and persistent antidepressant effects in animals and humans (Newport 2015) (Zanos 2016). Memantine, which acts as an antagonist and displays a similar NMDA receptor trapping profile did not outperform placebo in the remission of symptoms of major depressive disorder or bipolar disorder (Zarate 2006b) (Smith 2013) (Anand 2012). For all three studies, memantine was administered orally at a daily dose of approximately 20 mg. Similarly to ketamine and memantine, lanicemine binds within the ion pore of the NMDA receptor albeit with lower trapping than ketamine and memantine (Sanacora 2014). Interestingly, lanicemine significantly reduces depressive symptoms at 80 and 110 minutes after one intravenous infusion but fails to maintain a reduction in depressive symptoms 1 or 3 days after administration (Zarate 2013). However, when lanicemine is administered daily, depressive symptoms are significantly reduced after three weeks of treatment (Sanacora 2014). Traxoprodil an NMDA antagonist that binds to a site outside of the ion pore on the GluN2B subunit was developed by Pfizer as a potential rapid acting antidepressant. In the lone clinical study investigating the potential antidepressant effects patients received one intravenous infusion and antidepressant activity was determined 2, 5, 8, 12, and 15 days after infusion. Strangely, significant reduction of depressive symptoms was only recorded at 5 days after infusion (Preskorn 2014).

Further raising the possibility of an NMDA independent target of ketamine is the curious phenomenon of the anesthetic and antidepressant efficacies of ketamine isomers. If ketamine's antidepressant action was mediated by NMDA antagonism the (S) isomer should have greater antidepressant activity much like what is seen in ketamine's anesthetic qualities as discussed above. However, the (R) isomer of ketamine produces more potent and longer lasting antidepressant effects, yet the (S) isomer has a fourfold higher affinity for the NMDA receptor (Zhang 2014). These different efficacies also suggest that ketamine may be exerting its antidepressant effects independently of NMDA receptor antagonism.

1.8.6 Ketamine's clinical controversy

A number of prominent psychiatrists have issued statements of caution and concern over the use of ketamine as an antidepressant. First and foremost, ketamine is a known drug of abuse, and those suffering from depression have an increased risk to develop drug habits and ultimately addiction (Sanacora 2015). The precise mechanisms causing euphoria and hallucinations are unknown. Studies have also shown that ketamine binds to μ-opioid receptors and upregulates ERK1/2 phosphorylation (Kekesi 2011) (Gupta 2011). Ketamine's anti-nociception properties are blocked by μ and λ antagonists, but not by κ antagonists. These studies indicate that ketamine acts as an agonist on μ-opioid

agonists, which are known to be highly addictive (Sanacora 2015). Furthermore, choice preference can be enhanced by the drug in rodents (Suzuki 1999). Conversely, intracranial self-stimulation is not enhanced by ketamine (Hillhouse 2014). Clearly, further studies are needed to determine ketamine's abuse potential before it is readily available to the population.

1.8.7 BDNF is a key mediator of ketamine's antidepressant action

As discussed above BDNF is involved in the pathology of depression and the antidepressant action of conventional antidepressants. Ketamine is no exception. Despite the controversy of ketamine's antidepressant mechanism of action, the increase of BDNF is reliably reproduced and consistently shown to be necessary for its robust and rapid antidepressant effects (Björkholm 2016). The first attempt to explain ketamine's antidepressant mechanism of action was published 8 years after the first clinical trial and detailed an increase in BDNF in the hippocampus of mice treated with an antidepressant dose of ketamine (Garcia 2008). Shortly thereafter, Duman and colleagues reported TrkB activation via phosphorylation after ketamine treatment, essentially showing that BDNF was acting on its cognate receptor (Li 2010). It was then proposed that by blocking NMDA receptors calcium influx was decreased which relieved inhibition of translation by reducing phosphorylation of eukaryotic elongation factor 2 (eEF2) to ultimately increase translation of synaptic related proteins including BDNF (Autry 2011). Unfortunately, many NMDA antagonists have a similar effect *in vivo* and *in vitro* but fail to produce significant antidepressant effects in humans (Newport 2015).

Interestingly, antidepressant behavioral effects of ketamine are ablated in forebrain specific conditional TrkB knockout mice. Injecting a BDNF neutralizing antibody in the forebrain of mice also inhibits ketamine's behavioral effects (Lepack 2014). Ketamine mediated synaptogenesis was ablated in rodents with the val/met BDNF SNP. Clinical trials have shown that patients with depression and the val/val BDNF gene show increased antidepressant response to ketamine compared to those with who carried the val/met allele (Laje 2012). Furthermore, ketamine responders produce a significant increase in BDNF in blood serum compared to non-responders (Haile 2014). Taken together, it appears BDNF plays a central role in mediating ketamine's antidepressant effects even if the exact mechanism of increased synthesis and release is unclear.

1.9 Specific Aims

Given the controversy surrounding ketamine's antidepressant mechanism of action this thesis investigates the effects of ketamine on the plasma membrane localization of $G\alpha_s$. We posited that ketamine would have an effect similar to classical antidepressants but with a short treatment duration. Furthermore, since classical antidepressants require a 2-week treatment in rats or 3-day treatment in cultured cells we sought to determine the role of altered gene expression in the role of $G\alpha_s$ redistribution.

1.9.1 Aim 1: Determine the effect of ketamine on $G\alpha_s$ plasma membrane distribution

If ketamine does influence $G\alpha_s$ the effect should occur more rapidly compared to classical antidepressants that is on a timescale reflecting its robust antidepressant effects.

1.9.1.1 Aim 1.1: Evaluate the role of NMDA receptor antagonism in $G\alpha_s$ plasma membrane distribution

Since ketamine is canonically defined as a NMDA receptor antagonist and C6 cells express NMDA receptors, the translocation may be dependent NMDA antagonism.

1.9.2 Aim 2: Determine the functional consequences of altered $G\alpha_s$ localization.

If ketamine does alter $G\alpha_s$ plasma membrane localization it should increase intracellular cAMP and initiate biochemical involved in the antidepressant response.

1.9.3 Aim 3: Evaluate chronic antidepressant treatment and ketamine treatment effects on gene expression

SSRIs, TCAs require extended treatment to elicit the translocation of $G\alpha_s$. Given the duration of treatment required to observe the effect, altered gene expression may play a role in $G\alpha_s$ redistribution. Ketamine induced $G\alpha_s$ redistribution may share a similar molecular mechanism, albeit with different kinetics.

Chapter II

2. Methods

2.1 Drug Treatments.

C6 glioma cells were cultured in DMEM (Corning, Manassa, VA), 4.5 g of glucose and 10% newborn calf serum at 37 °C in humidified 5% CO_2 atmosphere to a confluence of ~80% before drug treatments. Treatment with 1, 3 or 10 µM ketamine was for 15 minutes or 24 hours. Treatment with AP-V, memantine, or MK-801 was for 15 minutes. If signaling events were being investigated C6 glioma cells were serum starved for approximately two hours before experimental challenge. After 15-minute ketamine treatment cells were rinsed twice with pre-warmed phosphate buffered saline (PBS) to remove debris and wash away unbound drugs.

2.2 TX100 Lipid Raft Isolation.

As published earlier (Zhang 2010) Cells were washed and harvested in ice-cold 1X PBS. Briefly, C6 cells were extracted in 1 mL of ice-cold lysis buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 1mM DTT; 1% Triton X-100; Protease inhibitor cocktail). Following 30 min incubation on ice, the lysates were homogenized and gently mixed with 1 mL ice-cold 80% sucrose in TME (10mM Tris HCl; 1mM $MgCl_2$; 1mM EDTA; pH 7.5; 1mM DTT; protease inhibitors) and loaded in the bottom of a centrifuge tube. Samples were overlaid by syringe and fine needle with 1 mL each of 30% sucrose, 15% sucrose, and

finally 5% sucrose. Sucrose gradients were centrifuged at 200,000*g* in an SW55-Ti rotor in a Beckman Ultra centrifuge at 4^{o} C overnight for 16-18 hours. Lipid rafts exist between 5% and 15% sucrose layers and appear as opaque white clusters. Raft fractions were collected, and sucrose removed via sequential mixing in Wash buffer (10mM HEPES pH 7.4, 150mM NaCl, 1mM DTT) and centrifugation at 40000*g* at 4 C for 20 min. (~4-5X) until a pellet emerges. Lipid raft pellets were reconstituted in 50uL of TME buffer. Protein content was determined by absorbance at 280 nm on a nanodrop UV-Vis spectrophotometer.

2.3 Percoll plasma membrane isolation.

As previously published (Singh 2018), following 15-minute ketamine treatment, C6 cells were immediately placed on ice and scraped in detergent-free Tricine buffer (250Mm sucrose, 1Mm EDTA, 20Mm Tricine, pH 7.4). The cellular material was homogenized and centrifuged at low speed (1,500*g* for 5 minutes at 4°C) to precipitate nuclear material and unlysed cells. The resulting supernatant was collected, mixed with 30% Percoll in Tricine buffer and subjected to ultracentrifugation for 45 minutes (Beckmann SwTi-55 rotor, 77,000x*g*, at 4°C) to collect plasma membrane fraction (PM). The plasma membrane appears as a fluffy opaque band near the top of the centrifuge tube. The band is collected and washed in Wash buffer via sequential mixing in Wash buffer (10mM HEPES pH 7.4, 150mM NaCl, 1mM DTT) and centrifugation at 40000*g* at 4 C for 30 minutes (~4-5X) until a pellet emerges. Protein was resuspended in appropriate buffer.

2.4 Detergent free sucrose density gradient lipid raft isolation.

As previously published (Singh 2018), following experimental challenge, C6 cells were immediately placed on ice and scraped in detergent-free Tricine buffer (250Mm sucrose, 1Mm EDTA, 20Mm Tricine, pH 7.4). The cellular material was homogenized and centrifuged at low speed (1,500*g* for 5 minutes at 4°C) to precipitate nuclear material and unlysed cells. The resulting supernatant was collected, mixed with 30% Percoll in Tricine buffer and subjected to ultracentrifugation for 25 minutes (Beckmann MLS50 rotor, 77,000xg, at 4°C) to collect plasma membrane fraction (PM). The plasma membrane appears as a fluffy opaque band near the top of the centrifuge tube. PMs were collected and sonicated (3x30sec bursts). The sonicated material was mixed with 60% sucrose (to a final concentration of 40%), overlaid with a 35-5% step sucrose gradient and subjected to overnight ultracentrifugation (Beckman MLS50 rotor, 87,400xg at 4°C). Fractions were collected every 400uL from the top sucrose layer and proteins were precipitated using 0.25 volume trichloroacetic acid (TCA) deoxycholic acid in double distilled water to precipitate proteins. TCA was allowed to evaporate overnight. Protein was then resolubilized in loading buffer and fractions were loaded by equal volume into gel.

2.5 Fluorescence recovery after photobleaching (FRAP).

As previously described (Czysz 2015) C6 cells were transfected with GFP-Gαs and cells expressing the fluorescent construct were selected with G418. Cells were plated on glass microscopy dishes and treated with 1, 3, and 10uM ketamine for 15 minutes or 24

hours. For imaging, drug was washed out for one hour prior and media was replaced with low serum (2.5% NCS) phenol red-free DMEM to limit fluorescent background. Temperature was maintained at 37°C using a PeCon temperature-controlled stage during imaging. Imaging utilized a Zeiss LSM 710 confocal microscope at 512 x 512 resolution with an open pinhole to maximize signal but minimize photobleaching. One hundred fifty data points, approximately 300 ms apart (including 10 pre-bleach values) were measured for each cell. Zeiss Zen software was used to calculate FRAP recovery half-time utilizing a one-phase association fit, correcting for total photobleaching of the analyzed regions.

2.6 SDS-PAGE and Western Blotting.

As previously described (Zhang 2010) samples were assayed for protein via a Nanodrop 2000c spectrophotometer or BCA if buffer contained detergents and equal quantities were loaded onto Stain-Free acrylamide gel for SDS-PAGE (Bio-Rad, Hercules, CA, USA). Gels were transferred to Nitrocellulose membranes (Bio-Rad, Hercules, CA USA) for western blotting. The membranes were blocked with 5% nonfat dry milk diluted in TBST (10 mM Tris–HCl, 159 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 hour. Following the blocking, membranes were washed with TBST and then incubated with an anti-$G\alpha_s$ monoclonal antibody (NeuroMab clone N192/12, Davis, CA, USA, catalog #75-211), caveolin-1 (BD Biosci #610059), β-actin (SIGMA Clone AC-74), p-CREB (Cell Signaling #9198), CREB (Cell Signaling #9197), BDNF (Cell Signaling #3987), NR1 (Millipore 05-432) SCD1 (Abcam ab39969), FADS1 (Abcam ab59031), FADS2 (Abcam ab71289) overnight at

4°C. Membranes were washed with TBST and incubated with a secondary antibody HRP-linked anti-mouse antibody IgG or HRP-linked anti-rabbit antibody IgG cell signaling for 1 h at room temperature, washed, and developed using ECL Luminata Forte chemiluminescent reagent (Millipore, Billerica, MA, USA). Blots were imaged using Chemidoc computerized densitometer (Bio-Rad, Hercules, CA, USA) and quantified by ImageLab 3.0 software (Bio-Rad, Hercules, CA, USA). In all experiments, the original gels are visualized using BioRad stainfree technology to verify protein loading or β-actin.

2.7 Depletion of NR1 NMDA subunit.

Expression of NR1 was inhibited with a SMARTpool: ON-TARGET plus Grin1 (24408) siRNA (Catalog # L-080174-02-0005) and scrambled control siRNA (Dharmacon, Inc, Pittsburg, PA). Briefly, C6 cells at 40% confluence were transfected with 50 µM siRNA using DharmaFECT-1. The reduced expression level of the NR1 subunit was determined by Western blot analysis. Various concentrations of siRNA were tested until the optimum concentration of 50 µM siRNA determined.

2.8 Primary astrocyte culture.

P3 Wistar rats were sacrificed and brains removed and placed into a HBSS. Olfactory bulb and cerebellum were removed, and the remaining cortex was cut into small pieces, 2.5% trypsin was mixed with the tissue and incubated at 37^{o} C for 30 minutes. Tissue was then centrifuged at 300*g* and supernatant aspirated. The pellet was resuspended in astrocyte plating medium (DMEM, high glucose + 10% heat-inactivated fetal bovine

serum + 1% Penicillin/Streptomycin) and seeded at $10x10^6$ cells per T-75 flask. Medium was changed every 2 days until cells reached confluency. To obtain an enriched astrocyte culture, microglia were removed by shaking flasks for 180 rpm for 30 minutes and discarding medium. Oligodendrocyte precursor cells were removed by shaking flasks at 240 rpm for 6 hours. Remaining astrocytes were seeded into flasks at $5x10^5$ density. Astrocyte medium was changed every 2-3 days and astrocytes allowed to mature for 14 days before experimentation.

2.9 Viral Infection and cAMP Quantification.

C6 glioma cells were grown on glass bottom microscope dishes and infected with ($1.09x10^9$ VG/mL) cADDIS BacMam virus encoding the green "up" cAMP sensor (Montana Molecular, Bozeman, MT, USA) and grown for 24-26 hours before live imaging under a 40x objective on a Zeiss 880. Cells were serum starved with 1% serum for 2-3 hours before drug treatments. Images were taken every 30 seconds. Average responses from 4-10 cells were selected from the visual field and fluorescence was normalized to baseline fluorescence for each experiment.

2.10 Isoproterenol cAMP dose response.

C6 cells were plated in 96-well black-sided clear-bottom plates (Costar 3603, Corning Inc.) 24 hours before measurement at a density of 48,000 cells per well. At time of plating each well was infected with 20 μl of baculovirus expressing Green cADDis Upward cAMP sensor (Montana Molecular) and supplemented with sodium butyrate at

a final concentration of 2 mM. Final volume of each well was brought up to a volume of 140 μl with culture media (DMEM supplemented with 10% newborn calf serum). 24 hours after plating, media was replaced with fresh culture media, with or without Ketamine at a final concentration of 10 μM. 15 minutes later culture media was replaced with 200 μl DPBS.

GFP signal intensity was determined on a Biotek Synergy H4 plate reader (Biotek Instruments Inc.) using in-built monochromator with excitation set to 488 nM and emission set to 525 nM. Wells were read from the bottom with detector set to 90% sensitivity. Each condition was measured in duplicate wells for each experiment.

Blank subtracted baseline measurements were obtained for each well before isoproterenol challenge. Wells containing only 200 μl DPBS were used for blank subtraction. Following baseline measurement either isoproterenol or vehicle (ddH2O) was added to each well and GFP signal intensity was measured. In dose response experiments F_0 was defined as the mean signal intensity of the vehicle + vehicle condition. Isoproterenol EC_{50} and maximal efficacy were calculated by fitting the data to a standard agonist concentration versus response curve (Hill slope = 1). For time course experiments, signal intensity was measured with a 39 s read interval for the duration of the experiment.

2.11 Immunoprecipitation.

As previously described (Singh 2018), plasma membrane or lipid rafts were isolated using either percoll or TX100, respectively and an aliquot was saved as starting material.

50 µL protein G dynabeads (Thermofisher Catalog # 10003D) were transferred and washed with PBS. Beads were blocked for one hour in 5% milk and washed with PBS. 5µg of α-tubulin (Sigma catalog T9206) antibody was added with PBS and conjugated to Dynabeads for 2 hours at room temperature and then washed with PBS. 20µg of plasma membrane protein or 5µg of lipid raft protein was added and incubated overnight at 4^{o}C with IP buffer (10mM Tris, 150mM NaCl, 60mM β-octyl glucoside, pH 7.4, protease inhibitors). After overnight immunoprecipitation the supernatant was removed, and beads were washed PBS and 0.1% tween. Proteins were boiled in biorad 2x Laemmli Sample Buffer (catalog #1610737) and loaded onto 15% gel for western blot analysis.

2.12 RNA isolation and RNAseq.

RNA was isolated with the RNeasy Mini Kit (Qiagen Inc.). Total RNA concentration was measured using a nanodrop. 200 ng was used to create sequencing libraries of with the TrueSeq RNA Sample Prep Kit v2-Set B (RS-122–2002, Illumina Inc, San Diego, CA). 3 individual libraries were normalized. Pooled libraries were then clustered on cBot (Illumina) using the TruSeq SR Cluster Kit v3—cBot—HS(GD-401–3001, Illumina Inc, San Diego, CA) sequencing was then performed as 50 bp, single reads and 7 bases index read on an Illumina HiSeq2000 instrument using the TruSeq SBS Kit HS- v3 (50-cycle) (FC-401–3002, Illumina Inc, San Diego, CA).

2.13 RNA expression level analysis.

Expression level estimation of Illumina 50 bp paired-end reads to the ray reference genome [University of California, Santa Cruz (UCSC) Genome Browser version mm9] was performed using TopHat software, which employs Bowtie as an internal read mapper). TopHat aligns reads to a reference genome. Read mapping (TopHat) was performed on default settings and -G option, which supplies TopHat with gene model annotation of known transcripts (Illumina iGenome UCSC mm9.gtf annotation file). After read mapping, transcripts were built employing Cufflinks software. Expression level estimation was documented as fragments per kilobase of transcript sequence per million mapped fragments.

2.14 Statistical Analysis.

Western blot bands were quantified using Biorad image lab software. Control values were set to one and compared to treatment values. The graphs are represented with either fold change or percent change with p values. Data are represented from at least three biological replicate experiments. Statistical significant differences ($p < 0.05$) were determined by unpaired t-test or two-way ANOVA, or if variances were unequal a two-way Kruskal–Wallis test was performed followed by Dunn's post hoc test for multiple comparisons. Unpaired t-test for from control vs treatment conditions were performed followed by Welch's correction. In cAMP dose response experiments F_0 was defined as the mean signal intensity of the vehicle + vehicle condition. Isoproterenol EC_{50} and

maximal efficacy were calculated by fitting the data to a standard agonist concentration versus response curve (Hill slope = 1). For time course experiments, signal intensity was measured with a 30 second read interval for the duration of the experiment. Results are represented as mean ± S.E.M. All statistical analysis was performed with the Prism version 5.0 software package for statistical analysis (GraphPad Software Inc., San Diego, CA).

Chapter III

3.Ketamine Results

3.1 Brief ketamine treatment redistributes plasma membrane $G\alpha_s$ into non-raft regions of the plasma membrane of C6 glioma cells

$G\alpha_s$ localization in lipid rafts is decreased after 3-day treatment with 10 µM antidepressants in C6 cells and 3-week treatment in the frontal cortex of rat brains (Toki 1999) (Donati 2005). Moreover, after i.p. injection of 30 mg/Kg ketamine in rodents the concentration of ketamine plateaus at approximately 10 µM in the brain (Zanos 2016). To test if ketamine induced a similar distribution, C6 cells were treated for 15-minutes or 24 hours with 10 µM ketamine and lipid raft fractions were isolated using the detergent free sucrose density gradient method. The results show ketamine rapidly decreased $G\alpha_s$ from lipid raft fractions compared to cells treated with vehicle at 15-minute and 24-hour time points (Fig.6A.). Further experiments were restricted to 15-minute treatment, as this treatment duration provides a model for the quick acting effects of ketamine.

Antidepressant induced translocation of $G\alpha_s$ from lipid rafts has also been measured using FRAP as discussed above (Czysz 2015). To test if ketamine influenced the fluorescence recovery rate of $G\alpha_s$-GFP, C6 glioma cells stably transfected with moderate levels $G\alpha_s$-GFP were treated with 10 µM ketamine for 15-minutes or 24 hours. Indeed, ketamine treatment for both durations decreased the rate of diffusion of $G\alpha_s$-GFP

indicating increased interaction between $G\alpha_s$-GFP and its much larger cognate effector adenylyl cyclase (Fig. 6B) (Czysz 2015). Data presented here indicate ketamine mediates an antidepressant signature after only 15 minutes treatment analogous to 3-day treatment with conventional antidepressants. These data also corroborate the data derived from lipid-raft isolation and immunoblotting.

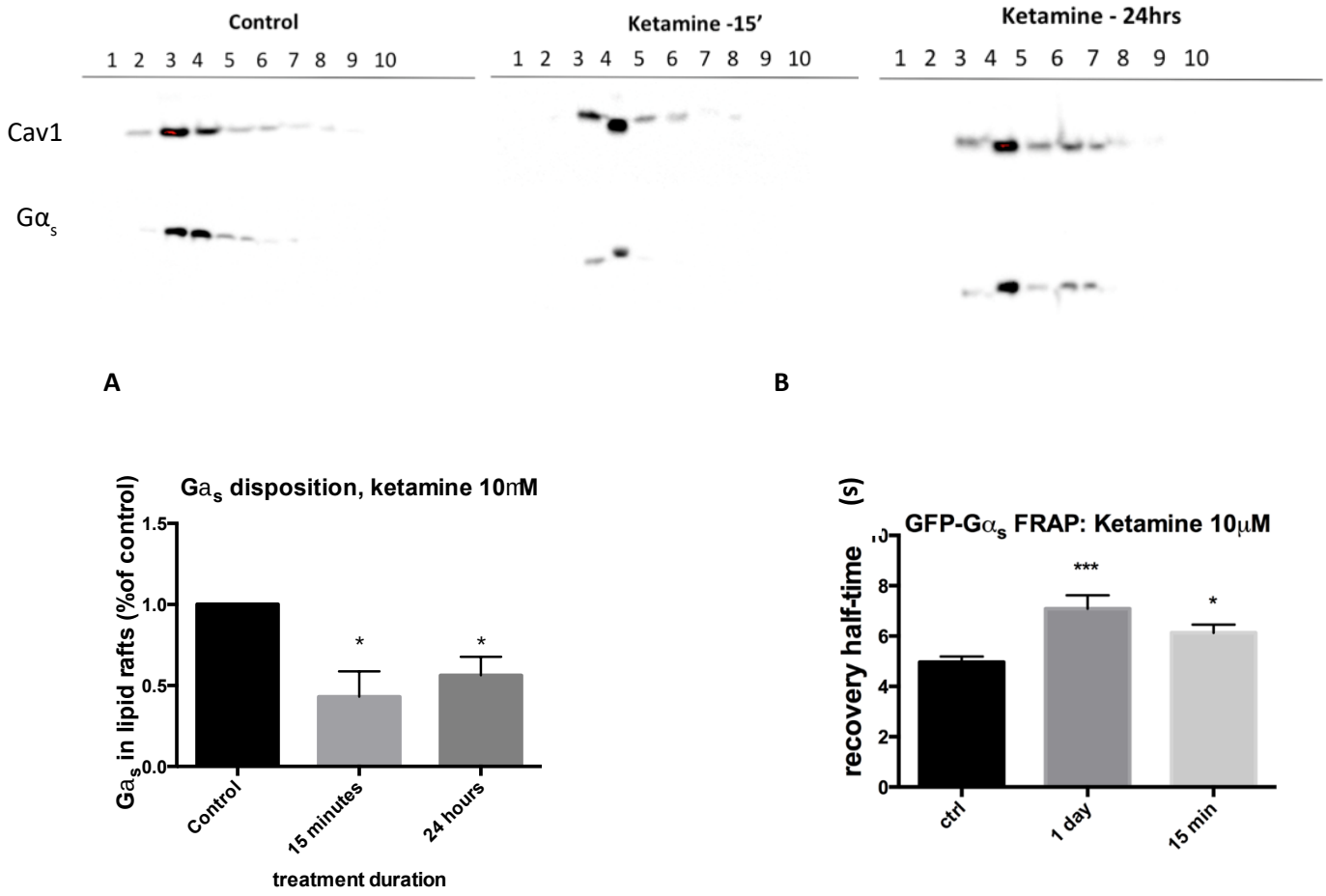


Figure 6. **Ketamine decreases $G\alpha_s$ lipid raft localization** (A) C6 cells were treated with 10 μM ketamine for 15 minutes or 24 hours and lipid raft fractions isolated and probed for $G\alpha_s$. Both groups show a statistical decrease of $G\alpha_s$ in lipid raft fractions indicating ketamine mediated $G\alpha_s$ translocation from lipid rafts into non-raft regions of the plasma membrane, blots were re-probed for caveolin-1 to confirm lipid raft fractions. (B) $G\alpha_s$ - GFP monoclonal C6 cells were treated for 15 minutes or 24 hours and $G\alpha_s$ lateral mobility analyzed by fluorescence recovery after photobleaching (FRAP), which revealed a statistical increase of recovery half-time consistent with augmented association of $G\alpha_s$ and adenylyl cyclase (n≤4) *$p < 0.05$; ***, $p < 0.001$

3.2 Ketamine translocates $G\alpha_s$ from lipid rafts in a dose-dependent manner at clinically relevant concentrations

Patients are commonly treated with one 0.5 mg/Kg infusion of ketamine over 40 minutes, which provide antidepressant effects within four hours and on average often last up to one week, with some patients experiencing remission for up to 3 months (Abdullah 2016). Ketamine serum levels after 10 minutes and 30 minutes increase to 1.3 µM then fall to 1.0 µM, after one ketamine sub-anesthetic infusion (Sos 2105). To determine if ketamine translocation of $G\alpha_s$ from lipid rafts occurs at clinically relevant concentrations, C6 cells were exposed to 1, 3, and 10 µM ketamine for 15-minutes and lipid raft fractions were isolated using the TX100 sucrose density gradient lipid raft isolation protocol in order to load all experimental conditions on one gel. There was a dose-dependent decrease of $G\alpha_s$ from lipid raft isolate at concentrations achieved in human subjects (Fig.7A.). Furthermore, these biochemical data are verified by a dose-dependent decrease in $G\alpha_s$ mobility as measured by FRAP after 15-minutes ketamine treatment (Fig.7B.). Data presented here indicate that ketamine may result in decreased levels of $G\alpha_s$ in lipid raft fractions in humans after one sub-anesthetic intravenous infusion of ketamine.

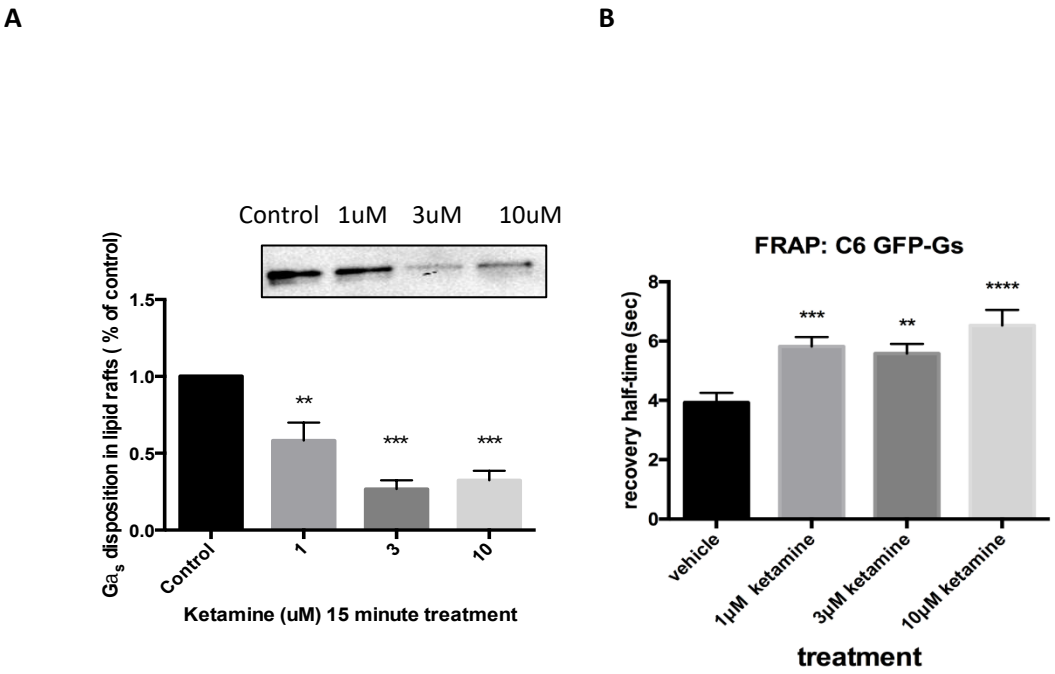


Figure 7. Ketamine decreases Gα$_s$ lipid raft localization in a dose dependent manner. Dose response of ketamine mediated Gα$_s$ translocation was analyzed by (A)TX100 lipid raft isolation and (B) FRAP, both methods reveal a dose-dependency of ketamine for Gα$_s$ translocation, which occurred at clinically relevant levels of drug (n≤4) **, $p < 0.01$; ***, $p < 0.001$; **** $p< 0.0001$

3.3 Ketamine-induced translocation of $G\alpha_s$ returns to baseline after 24 hours

The duration of ketamine's antidepressant effects is often highly variable lasting from 24 hours to up to 3 weeks, with an average remission of depressive symptoms for one week (Abdullah 2016) (Murrough 2013). We sought to determine the duration of decreased lipid raft isolate $G\alpha_s$ localization. C6 glioma cells were treated with 10 μM ketamine for 15-minutes, drug washed off with prewarmed PBS and cells collected at 15-minutes, 1, 6, 12, and 24 hours after drug treatment. Lipid rafts fractions were isolated via the TX100 sucrose density gradient lipid raft isolation protocol to load all experimental conditions on one gel and $G\alpha_s$ quantified by immunoblotting. Decreased levels $G\alpha_s$ from lipid rafts was significantly sustained for 12 hours after transient 15-minute treatment. $G\alpha_s$ lipid raft localization returned to baseline levels after 24-hours (Fig.8).

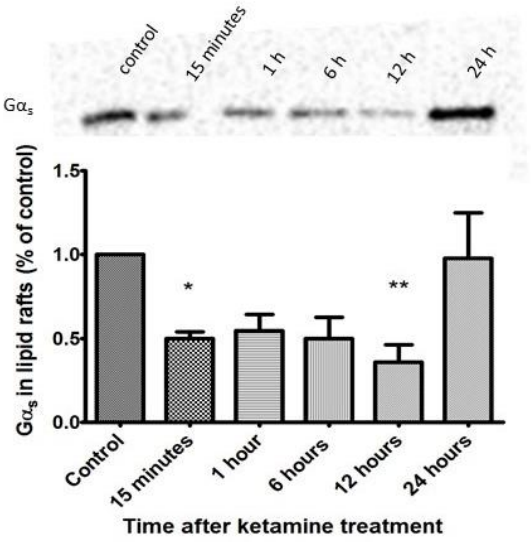


Figure 8. $G\alpha_s$ lipid raft localization returns after 24 hours C6 cells were treated with ketamine for 15 minutes and drug was washed off. Cells were collected at 15 minutes, 1, 6, 12 and 24 hours afterward, lipid rafts were isolated and probed for $G\alpha_s$ indicating the localization of $G\alpha_s$ in non-raft regions is maintained for 12 hours after 15-minute treatment with significant reductions occurring at 15 minutes and 12 hours (n=4). *$p <$ 0.05; **, $p < 0.01$. Ketamine effects were no longer evident by 24 hours past treatment.

3.4 Other NMDA antagonists do not affect $G\alpha_s$ localization

C6 cells provide an excellent, simplistic model to study conventional antidepressants effect on $G\alpha_s$ plasma membrane localization as they lack monoamine transporters, which are the canonical targets of a majority of FDA approved antidepressants (Erb 2016). Given C6 cells express NMDA receptors, the canonical target of ketamine's action, we questioned whether $G\alpha_s$ lipid raft exodus was mediated by NMDA antagonism. To test this, C6 cells were treated with 10 μM of the NMDA antagonists MK801, memantine or AP5 for 15 minutes and $G\alpha_s$ lipid raft localization was evaluated via TX100 sucrose density gradient lipid raft isolation. No other NMDA receptor antagonist had a significant effect on $G\alpha_s$ lipid raft localization suggesting that ketamine may be acting on a target other than the NMDA receptor (Fig. 9A). These data were further corroborated via FRAP (Fig. 9B). Interestingly, these data suggest NMDA antagonism may not be the sole target responsible for ketamine's antidepressant action (Newport 2015) (Zanos 2016).

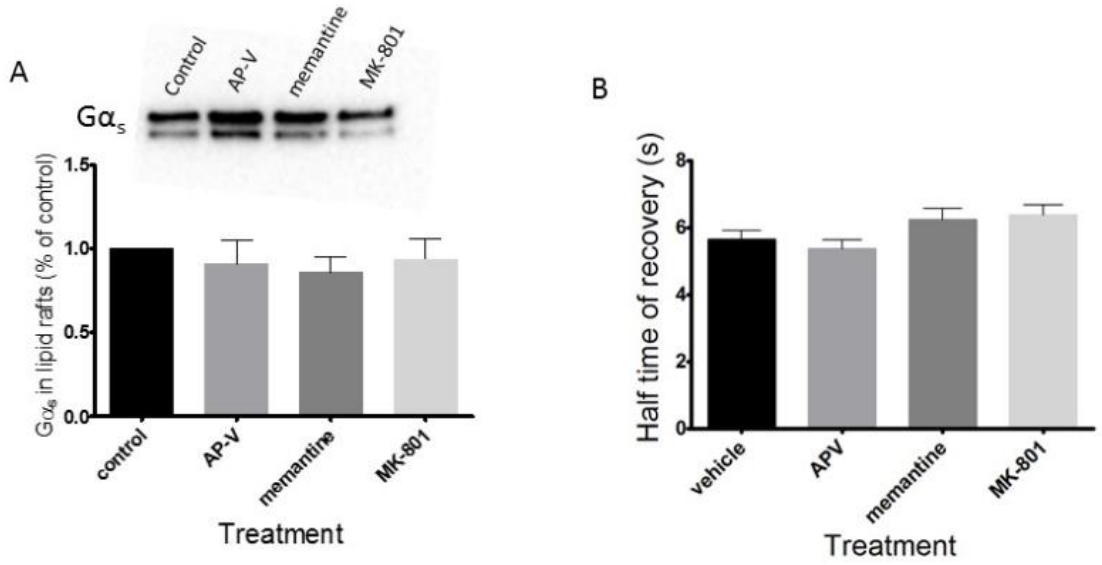


Figure 9. Other NMDA receptor antagonist do not mediate $G\alpha_s$ translocation from lipid rafts (A) C6 cells were treated with NMDAR antagonists AP-V, memantine, and MK-801 for 15 minutes and $G\alpha_s$ translocation was analyzed by (A) lipid raft isolation (n=3) and (B) FRAP (n≥48). Both methods reveal no effect upon $G\alpha_s$ lipid raft localization, indicating ketamine mediated $G\alpha_s$ translocation may result from an NMDAR independent target of ketamine.

3.5 μ-opioid receptor agonist and κ-opioid receptor antagonist do not affect $G\alpha_s$ localization

Ketamine has been shown to bind to opioid receptors. While the literature is conflicting, the prominent consensus is that ketamine acts both as an agonist on μ-opioid receptors (MOR) and antagonist on κ-opioid receptors (KOR). To test if either action played a role in the ketamine mediated translocation of $G\alpha_s$, C6 cells were treated with either the MOR agonist DAMGO or the KOR antagonist nor-BNI and exposed to FRAP. Indeed, C6 cells express both MOR and KOR (Talbot 2010) (Olianas 2012). Neither compound influenced the fluorescent recovery rate of $G\alpha_s$-GFP (Fig 10). These data suggest that ketamine does not act through opioid receptors in our model.

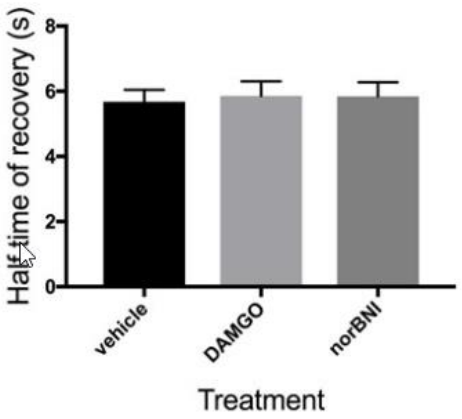


Figure 10. MOR agonism or KOR antagonism does not influence $G\alpha_s$ plasma membrane localization. $G\alpha_s$-GFP C6 cells were treated for 15 minutes with 10 µM DAMGO or 100 nM nor-BNI and $G\alpha_s$ -GFP lateral mobility analyzed by FRAP (n≥75), which indicated no change of recovery half-time suggesting MOR agonism or KOR antagonism does not mediate $G\alpha_s$, plasma membrane redistribution.

3.6 Ketamine treatment enhances isoproterenol stimulated cAMP accumulation in NR1 knock down C6 cells

As antidepressant treatment translocates $G\alpha_s$ from lipid rafts, $G\alpha_s$ increases association with adenylyl cyclase, in turn increasing intracellular cAMP. To test if ketamine mediates an increase in intracellular cAMP, we measured cAMP in live C6 cells using exchange factor directly activated by cAMP (EPAC)-based fluorescent biosensors developed by Montana Molecular and measured fluorescence intensity every 30 seconds. (Tewson 2015). The "upward" fluorescent sensor increases fluorescent intensity as intracellular cAMP concentration increases with a dynamic range between 10-100 µM cAMP. When 10 µM ketamine was added there is no significant increase in basal fluorescence over the duration of 20 minutes (Fig 11). However, when stimulated by a $G\alpha_s$-coupled agonist, ketamine-treated cells show both a quicker increase in and more robust fluorescence indicating a more facile coupling of $G\alpha_s$ and adenylyl cyclase (Fig.12.A). To determine if intracellular ketamine mediated accumulation of cAMP occurs independently of NMDA receptor antagonism the NR1 subunit of the NMDA receptor was knocked down as described in methods (Fig.12.B), which is necessary for the proper assembly and trafficking of the NMDA receptor. After NMDA receptor knock down, cAMP induced fluorescence was measured every 30 seconds for 15 minutes. The ketamine pretreated NR1 knock down group mimics ketamine pre-treated cells in that cAMP mediated fluorescence increased both more quickly and robustly compared to C6 cells that were treated with vehicle (Fig.12.A). These data further support the possibility of a NMDA receptor independent antidepressant target of ketamine.

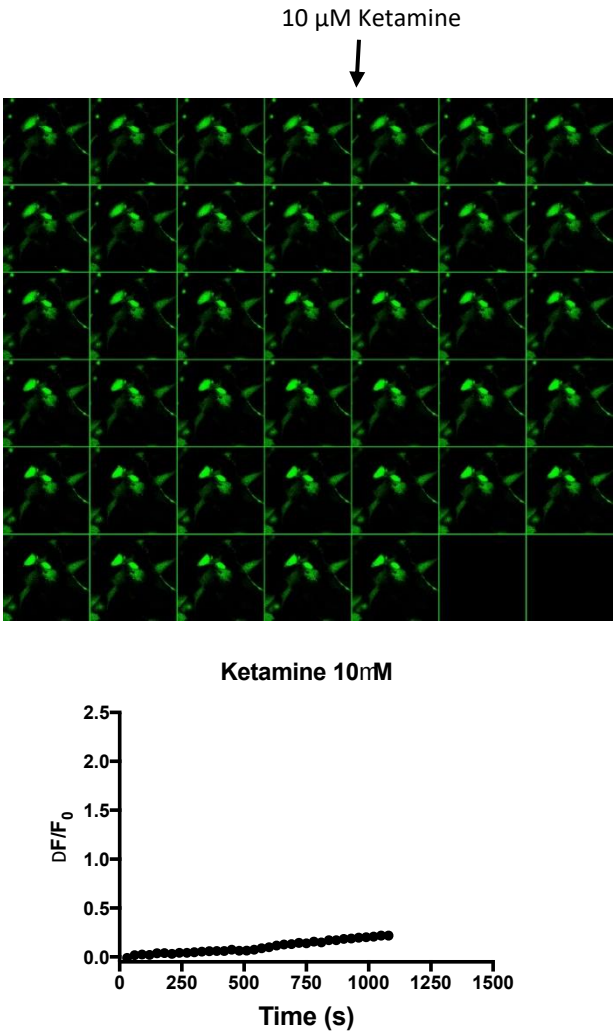
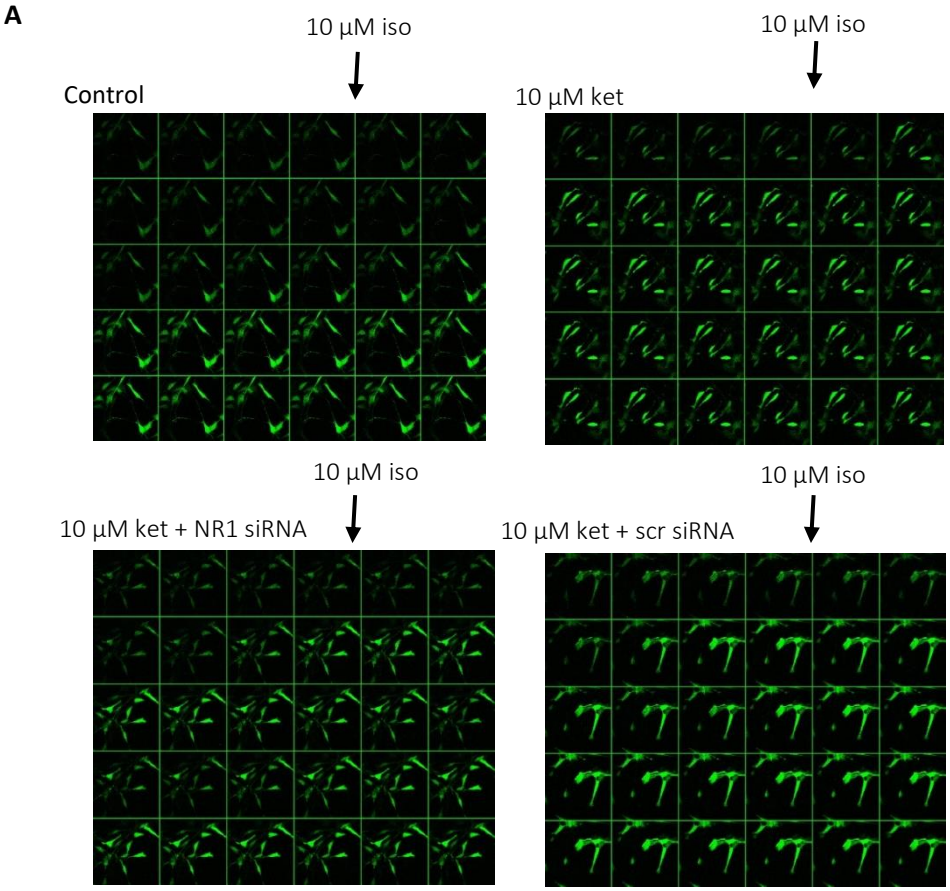


Figure 11. **Ketamine treatment alone does not increase cAMP** (A) C6 cells were infected with ($1.09x10^9$ VG/mL) cADDIS virus and imaged every 30 seconds and 10 µM ketamine was added after the fourth frame (n=2)


A
Control
10 µM iso
10 µM ket
10 µM iso
10 µM ket + NR1 siRNA
10 µM iso
10 µM ket + scr siRNA
10 µM iso

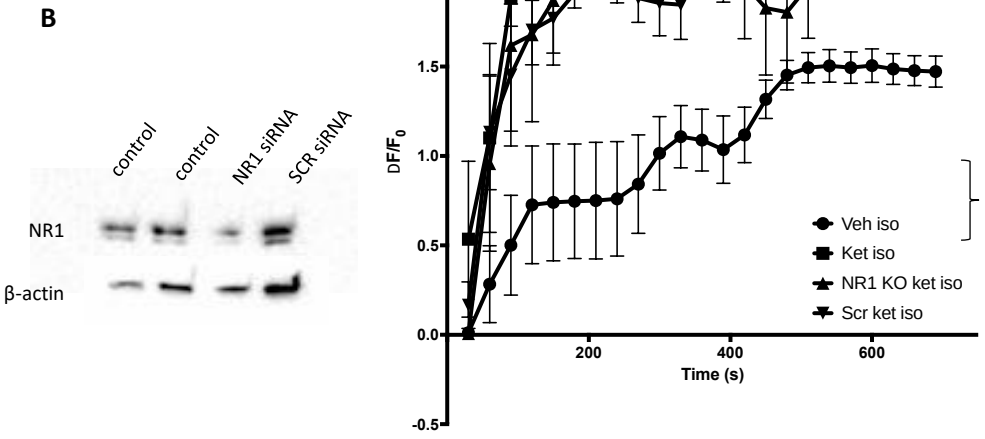

B
control
control
NR1 siRNA
SCR siRNA
NR1
β-actin
DF/F0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
200
400
600
Time (s)
Veh iso
Ket iso
NR1 KO ket iso
Scr ket iso

Figure 12. Ketamine treatment results in increased accumulation of cAMP, independent of NMDARs (A) Clockwise from top left; vehicle pretreatment with addition of isoproterenol; ketamine pretreatment with isoproterenol added after frame 4; NR1 siRNA pretreatment, ketamine pretreatment with addition of isoproterenol; scrambled siRNA pretreatment, ketamine pretreatment with the addition of isoproterenol. All ketamine pretreated groups showed a quicker and more robust statistical increase in fluorescence indicating relieved inhibition of lipid rafts upon $G\alpha_s$ and a more facile interaction of $G\alpha_s$ and adenylyl cyclase and NMDAR-independent effects of ketamine (B) Cells were exposed to vehicle, 50 nM NR1 siRNA or 50 nM scrambled siRNA for 24 hours. Western blotting confirmed knock down of the NR1 subunit after cAMP accumulation was determined (n=4). *$p < 0.05$; **** $p< 0.0001$

3.7 Ketamine increases efficacy of isoproterenol but not potency

To determine if ketamine influences $G\alpha_s$-coupled GPCR potency and efficacy, the isoproterenol mediated cAMP accumulation C6 cells was examined. C6 cells were seeded in a 96-well plate, treated with ketamine, and challenged with increasing doses of isoproterenol. Dose response reveals ketamine treatment significant increases percent stimulation (efficacy) over baseline (180 ± 10) compared to control (130 ± 11) while EC50 (potency) of ketamine (-9.0 ± 0.23) compared to (-8.2 ± 0.28) was unaffected (Fig.13). These results suggest that $G\alpha_s$ is coupling more effectively to adenylyl cyclase.

	Vehicle	Ketamine	Significance
log(EC_{50}) (M)	-8.2 ± 0.28	-9.0 ± 0.23	No
% stimulation over basal	130 ± 11	180 ± 10	*

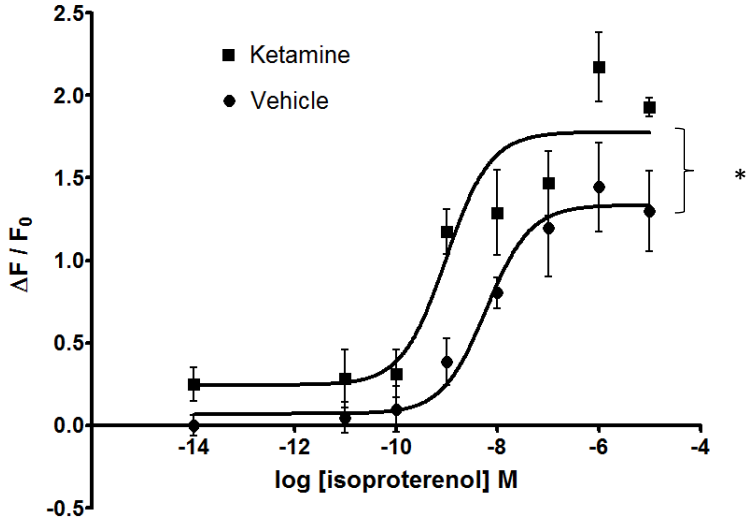


Figure 13. cAMP dose-response.C6 cells in a 96-well plate were infected with ($2.18x10^8$ VG/mL) cADDIS virus and treated with 10 µM ketamine or vehicle for 15 minutes. Vehicle or isoproterenol was added to each well and GFP signal intensity was measured. Data reveals statistical increase in efficacy but not potency. (n=3) *$p < 0.05$

3.8 Transient Ketamine treatment mediates sustained phosphorylation of CREB

When the intracellular concentration cAMP increases it binds to the two regulatory subunits of protein kinase A (PKA) which then release the active subunit of PKA, which is then free to phosphorylate numerous targets. One target associated with antidepressant activity is the phosphorylation of cAMP response element binding protein (CREB) at serine-133 (Ser-133). Phosphorylated CREB (pCREB) then translocates to the nucleus where it acts as a transcription activator on genes involved in growth and survival, neuroprotection, and synaptic plasticity (Lonze 2002). CREB is both up-regulated and phosphorylated at Ser-133 after chronic SSRI and TCA treatment in animals and cultured cells (Gass 2007) (Nibuya 1996). We sought to evaluate the effect of ketamine-mediated cAMP elevation on CREB phosphorylation. To test this, C6 cells were treated with 10 μM ketamine for 15-minutes, drug was washed off and cells harvested at 15 minutes, 1, 2, 6 and 24 hours after drug treatment. The data indicate a sustained increase of pCREB after a transient exposure to ketamine. Initial increase in pCREB is significant increase is shown at 15 minutes and again at two hours. Elevated pCREB is sustained for 24 hours after initial ketamine treatment although not at statistical significance (Fig.14). Total cellular CREB content remains constant. Increased pCREB is consistent with an increase of cAMP production. Interestingly, the addition of an agonist was not necessary to observe the effects of elevated levels of cAMP. This

may be due to factors present in serum, which may stimulate $G\alpha_s$ coupled receptors and canonical GPCR signal amplification.

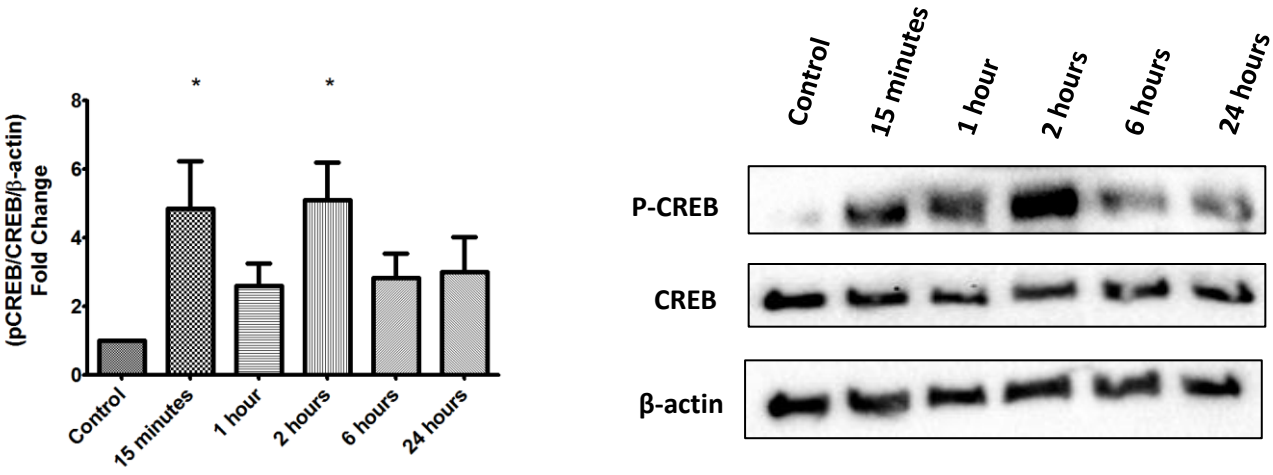


Figure 14. Ketamine increases phosphorylation of CREB. Western blot analysis of CREB. C6 cells were treated for 15 minutes with 10 µM ketamine and collected at the indicated time point and probed for (A) phosphorylation of CREB at Ser-133 which showed elevated levels for 24 hours and statistically significant increases at 15 minutes and 2 hours (n=5) *$p < 0.05$;

3.9 Transient Ketamine treatment increases in phosphorylation of Eukaryotic Elongation Factor 2 in C6 cells

Monteggia and colleagues have shown that NMDA receptor blockade by ketamine and MK801 results in decreased threonine-56 (Thr-56) phosphorylation of eukaryotic elongation factor 2 (eEF2) by attenuating the influx of calcium and the activity calmodulin dependent eEF2 kinase (eEF2k) (Autry 2011). Decreased p-eEF2 relieves inhibition of protein synthesis ultimately resulting in the rapid translation of target mRNA, including BDNF (Autry 2011). PKA also phosphorylates eEF2K to ultimately increase eEF2 Thr-56 phosphorylation (Browne 2002). Furthermore, C6 cells express NMDA receptors primarily composed of GluN1, GluN2C/D and GluN3 subunits, and given the presence of the GluN3 subunit; lack the ability to maintain a magnesium dependent blockade (Palygin 2011). Since the magnesium blockade must be removed for ketamine to bind NMDA receptors, NMDA receptors in C6 cells may be more sensitive to ketamine than neuronal NMDA receptors. Effective binding of ketamine may decrease the influx of calcium, which would cause decreased phosphorylation of eEF2. Given, the aforementioned and data presented here indicating ketamine mediated increased cAMP and PKA dependent phosphorylation, we sought to determine the effect of ketamine on p-eEF2 levels in our model. To evaluate p-eEF2, C6 cells were treated with 10 µM ketamine for 15 minutes, drug was washed off and cells harvested at 15 minutes, 1,2,6 and 24 hours after drug treatment. The data indicate a sustained increase in p-eEF2 on Thr-56 after transient treatment with ketamine. A statistically significant increase of p-eEF2 occurs 1 hour after drug washout (Fig.15).

Total eEF2 protein levels remain constant. These data indicate that eEF2k may be phosphorylated by PKA after ketamine treatment in C6 cells and are consistent with increased cAMP levels.

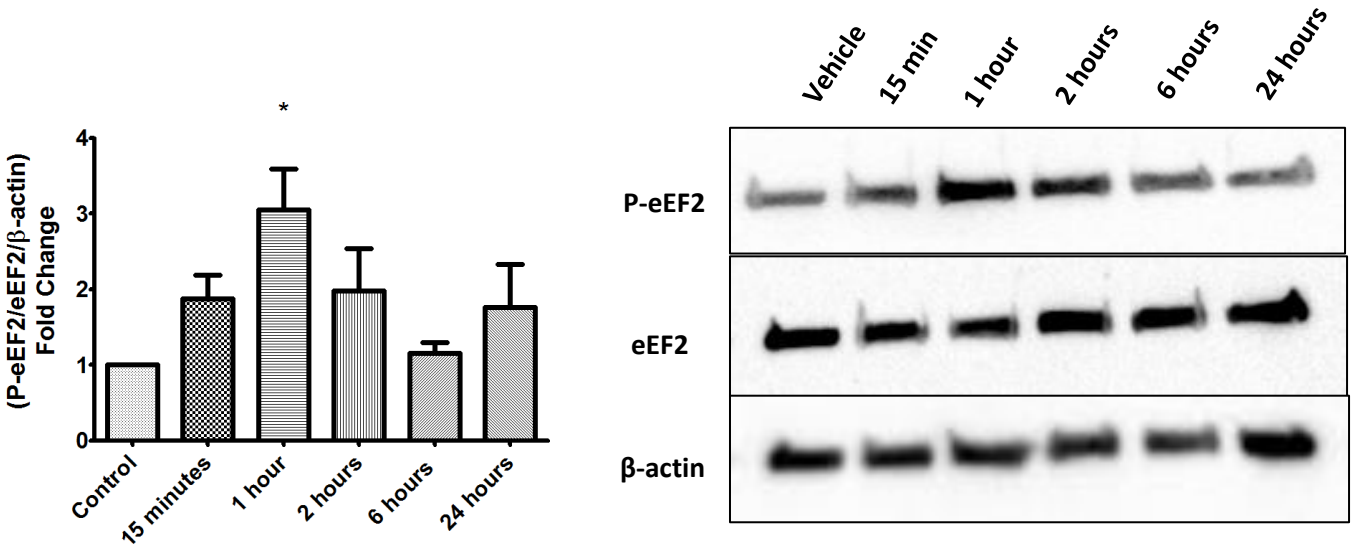


Figure 15. Ketamine increases phosphorylation of eEF2. C6 cells were treated with 10 µM ketamine for 15 minutes and collected at various timepoints and P-eEF2 levels at Thr-56 and total eEF2 levels were determined. Phosphorylation of eEF2 at thr-56 showed increased levels for 24 hours reaching statistical significance at 1-hour C6 cells (n=4), *, $p < 0.05$

3.10 Ketamine increases BDNF in a cAMP dependent manner in primary astrocytes

Numerous reports indicate that chronic treatment with conventional antidepressants increase BDNF mRNA and protein levels in the rodent brain and in cell cultures (Nibuya 1995) (Altar 1999) (Coppell 2003) (Calabrese 2007). Emerging evidence indicates acute ketamine treatment also results in increased levels of BDNF (Li 2010) (Autry 2011) (Zanos 2016). Furthermore, ketamine treatment does not elicit an antidepressant behavioral response in mice lacking forebrain BDNF, indicating the necessity of BDNF for the ketamine mediated antidepressant response in animal models of depression (Autrey 2011). Taken together, these studies indicate that BDNF is a necessary component of successful antidepressant action. Given that PKA phosphorylation of CREB has been shown to increase the transcription of BDNF, we sought to determine the effects of these cellular events. C6 cells were transiently treated with ketamine for 15 minutes and cells were collected after 1 hour and 24 hours. Only at 24 hours after ketamine treatment was there a significant increase in BDNF indicating the possibility of a cAMP/PKA/pCREB mediated transcriptional dependent increase (Fig. 16A).

Next, we sought to validate the physiologic relevance of ketamine mediated, cAMP-dependent increase of BDNF in primary rat astrocytes. Primary rat astrocytes were isolated and allowed to mature for 20 days before experimentation. Staining for GFAP confirmed presence of astrocytes (Fig. 16B). After maturation, primary astrocytes were transiently treated with vehicle, 10 µM ketamine, or 10 µM ketamine and 1 µM of the cell permeable cAMP competitive antagonist cAMPs-Rp and were collected after 24

hours. The data indicate a significant increase in BDNF after 24 hours, which was attenuated by cAMPs-Rp. These data suggest a ketamine-induced, cAMP-dependent, BDNF production in astrocytes.

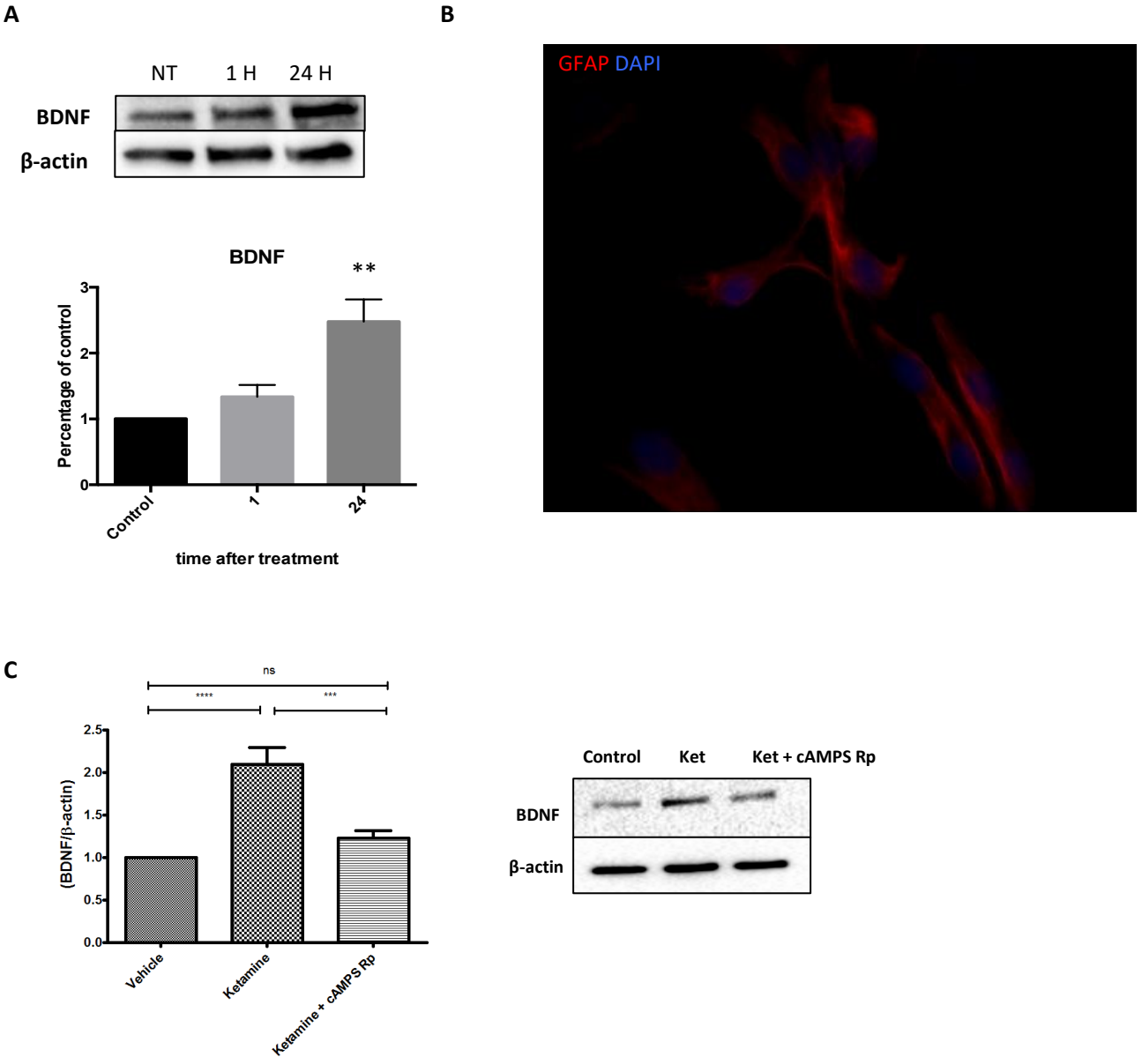


Figure 16. Ketamine increases of BDNF. (A) C6 cells were treated with ketamine for 15 minutes, drug washed out and cells collected 1 or 24 hours later. Only the ketamine treated group showed a statistical increase in BDNF after 24 hours. (n=5) Primary rat astrocytes were isolated (B) staining for GFAP confirmed primary astrocytes (C) ketamine or ketamine plus 1 µM cAMPS-Rp and cells collected 24 hours later, the ketamine treated group showed a statistical increase in BDNF which was abolished by cAMPS-Rp. (n=5), ** $p < 0.01$; ***, $p < 0.001$; **** $p< 0.0001$

3.11 Ketamine metabolite (2R,6R)-hydroxynorketamine (HNK) increases cAMP accumulation and decreases FRAP

A recent report indicates that the ketamine metabolite (2R,6R)-HNK produces rapid and robust antidepressant effects through increased expression of BDNF, GluA1, GluA2, and de-phosphorylation of eEF2 in synaptoneurosomes through an unknown mechanism independent of NMDA receptor antagonism (Zanos 2016). Suzuki *et al.* have suggested a possible role of NMDA receptor antagonism but at high non-physiological concentrations (Suzuki 2017) (Zanos 2017). We questioned if (2R,6R)-HNK decreases lateral mobility of $G\alpha_s$-GFP similarly to ketamine. A 15-minute transient treatment was sufficient to reduce $G\alpha_s$-GFP lateral mobility indicating increased association of $G\alpha_s$ and adenylyl cyclase (Fig 17A). To determine the functional consequences of augmented $G\alpha_s$ and adenylyl cyclase coupling, C6 cells were infected the EPAC-based fluorescent biosensor previously used, treated with 10 µM (2R,6R)-HNK for 15 minutes, drug washed off, stimulated with isoproterenol and fluorescence was measured. Indeed, (2R,6R)-HNK robustly increased cAMP mediated fluorescence (Fig.17B) indicating a possible role of cAMP in the antidepressant effects of (2R,6R)-HNK.

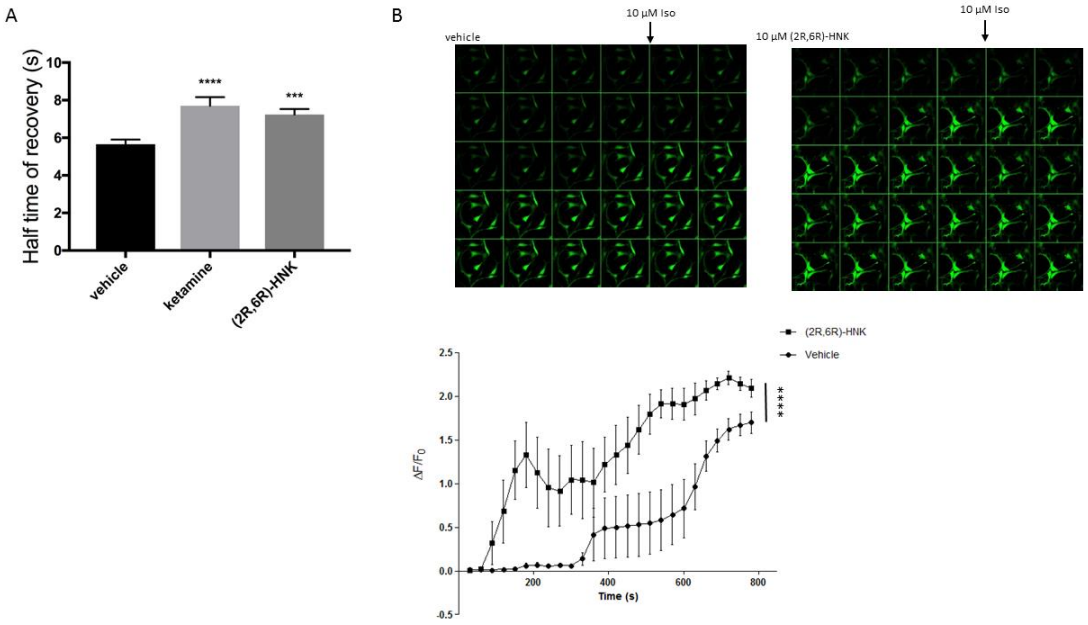


Figure 17. (2R,6R)-Hydroxynorketamine attenuates Gα_s lipid raft localization and cAMP accumulation similarly to ketamine. (A) Gα_s-GFP C6 cells were treated for 15 minutes with 10 µM (2R,6R)-HNK and Gα_s lateral mobility analyzed by FRAP (n≥75), which indicated a statistical increase of recovery half-time indicating augmented association of Gα_s and adenylyl cyclase (B) C6 cells were infected with ($1.09x10^9$ VG/mL) cADDIS virus and imaged for cAMP every 30 seconds and 10 µM drug was added after the fourth frame. (n=4), ***, $p < 0.001$; **** $p< 0.0001$

3.12 Ketamine does not disrupt $G\alpha_s$/tubulin complexes

A recent report from our group detailed the disruption $G\alpha_s$/tubulin complex after 3-day treatment of several antidepressants in C6 cells (Singh 2018). We sought to determine if ketamine acted through a similar mechanism but with a shorter treatment duration. C6 cells were treated 10 μM ketamine for 15-minutes and either plasma membranes were isolated with percoll or lipid rafts were isolated using the detergent free lipid raft isolation protocol. No changes were seen in $G\alpha_s$/tubulin association after ketamine treatment from either group (Fig.18). These data suggest ketamine mediated translocation of $G\alpha_s$ occurs by a different mechanism than classical antidepressants. Given that ketamine acts much more rapidly, it follows that it acts via a different mechanism.

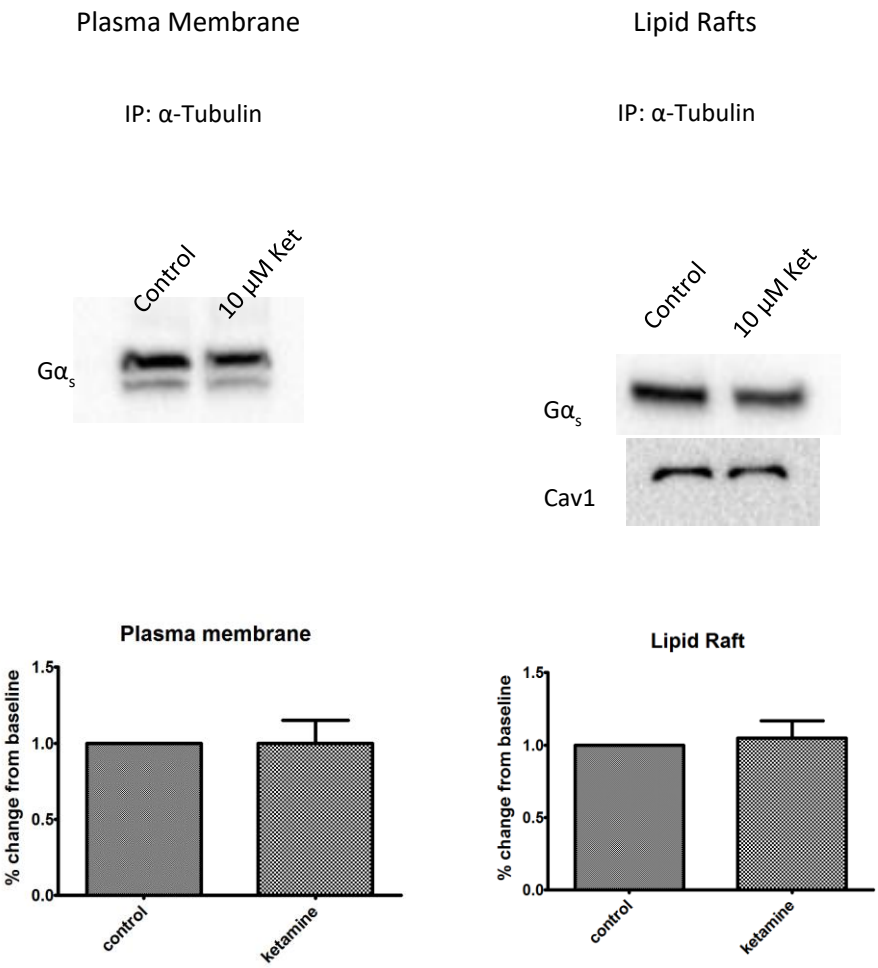


Figure 18. $G\alpha_s$/tubulin complexes remain after ketamine treatment. C6 cells were treated with 10 µM and treated with ketamine, plasma membrane or lipid raft fraction were collected, and immunoprecipitated with α-tubulin. Ketamine treatment did not disrupt $G\alpha_s$ and tubulin binding (n=6).

Chapter IV

4.1 RNAseq Results

4.1.1 Antidepressant treatment increases desaturase expression

Interestingly, stearoyl-CoA desaturase 1 (SCD1), stearoyl-CoA desaturase 2 (SCD2), fatty acid desaturase 1 (FADS1), and fatty acid desaturase 2 (FADS2) mRNA was significantly elevated in groups treated with either antidepressant. Furthermore, sterol regulatory element-binding transcription factor 1 (SREBF1), the transcription factor regulating desaturase expression, was also upregulated (Table 4). Desaturase enzymes are expressed in the endoplasmic reticulum and are involved with the synthesis of saturated fatty acid to polyunsaturated fatty acids (PUFAs). That is, they introduce double bonds onto long fatty acid carbon chains. SCD1 and SCD2 are the rate limiting enzymes in the production of monounsaturated fatty acids (MUFAs) (Fig. 17). FADS1 and FADS2 then can introduce further bonds onto MUFAs to synthesize PUFAs. (Fig. 18). Their specific function is detailed in Table 4 as each has a unique ΔX function, indicating that the desaturase will introduce a double bond at the carbon on the acyl chain of the fatty acid. For example, SCD1 will desaturate the 16-carbon chain saturated fatty acid palmitate at the Δ9 carbon to synthesize palmitoleic acid. However, all desaturase proteins have been shown to non-specifically desaturate fatty acids.

Strikingly, ketamine had no effect on desaturating enzyme mRNA or any other gene. This may be due a cell collection time that was not in line with increased levels of mRNA. This remains to be tested.

Gene	function	Escitalopram p value	Escitalopram Q value	Imipramine p value	Imipramine Q value	Ketamine P Value	Ketamine Q value
FADS1	Δ5 desaturase	2.68 E-7	.0037	1.24 E-8	3.47 E-5	<0.5	<0.5
FADS2	Δ6 desaturase	0.004	.058	1.92 E-6	0.001	<0.5	<0.5
SCD1	Δ9 desaturase	3.92 E-6	.009	2.24 E-11	1.56 E-7	<0.5	<0.5
SCD2	Δ9 desaturase	9.06 E-6	.016	4.87 E-09	1.7 E-5	<0.5	<0.5
INSIG1	SREBf regulatory protein	3.52 E-6	.009	3.42 E-7	.0004	<0.5	<0.5
SREBf1	Transcription factor regulating enzymes above	0.0003	0.24	7.55 E-5	0.03	<0.5	<0.5

Table 4. RNAseq reveals increased expression of desaturates. Mature primary astrocytes were cultured and treated with escitalopram or imipramine for 3 days, RNA isolated, and expression evaluated via RNAseq. All desaturating enzymes were significantly upregulated and most remained significantly upregulated after correction for false discover rate ($Q<0.05$).

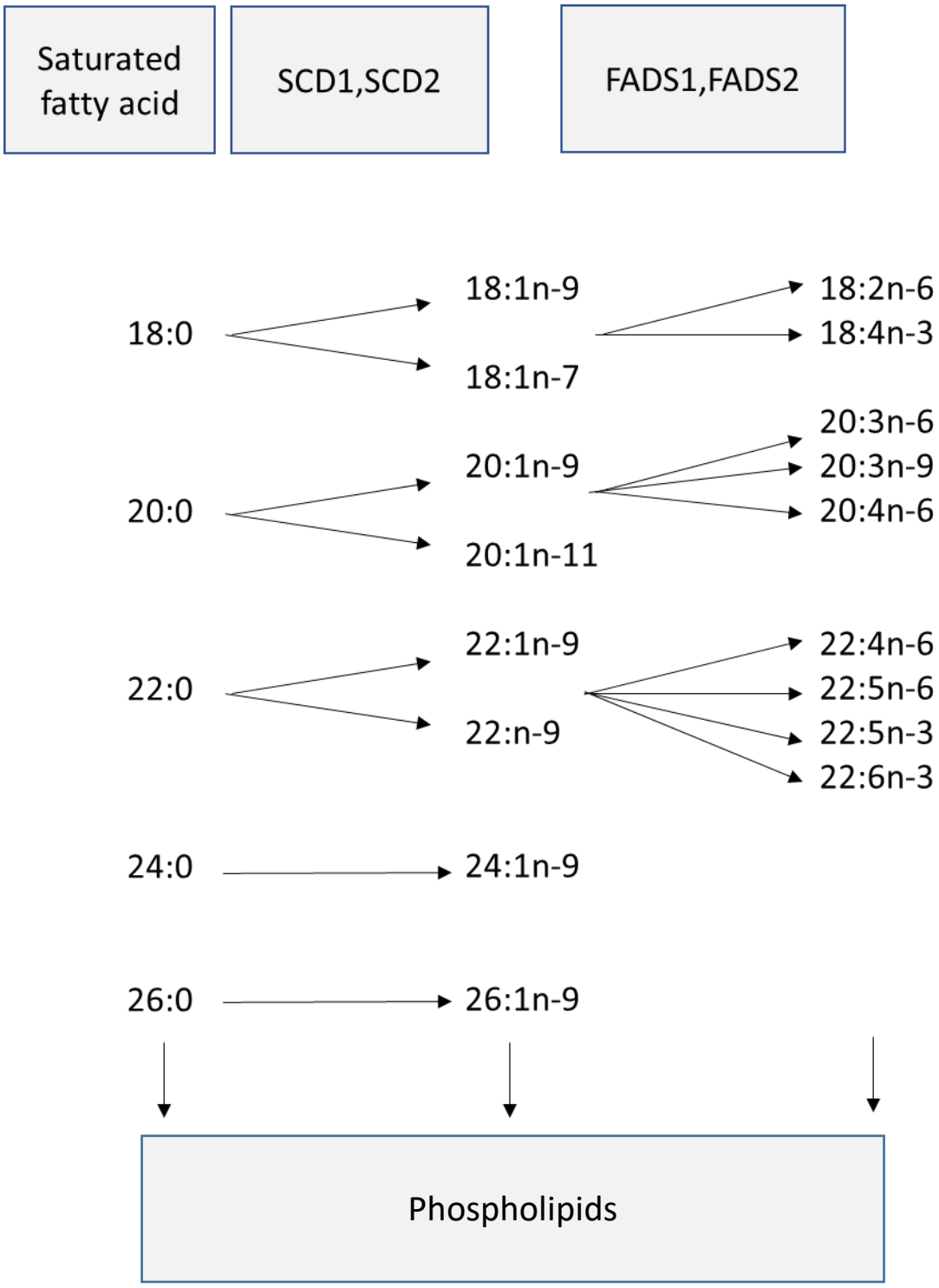


Figure 19. PUFA synthesis. SCD1, SCD2, FADS1, and FADS2 desaturating activity upon a variety of saturated fatty acids and MUFAs

4.1.2 Antidepressant treatment increases desaturase protein expression in C6 cells

To ensure elevated protein levels of enzymes identified by RNAseq are present in our current model used to study $G\alpha_s$ translocation, C6 cells were treated with antidepressants, collected at various timepoints and immunoblotted for desaturases. 10 µM escitalopram significantly elevated levels of SCD1, FADS1, and FADS2 at 2 and 3 days of treatment (Fig. 19). Similarly, 10 µM imipramine significantly increased levels of FADS2 at 2 and 3 days of treatment. SCD1 and FADS2 were significantly increased after 3 days of imipramine treatment (Fig. 19). Immunoblotting was restricted to FADS1, FADS2, and SCD1 as antibodies for SCD2 and SREBF1 were either unavailable or unreliable. Interestingly, treatment of C6 cells with PUFAs translocates $G\alpha_s$ from lipid rafts (Czysz *unpublished*). These results suggest that the upregulation of desaturating enzymes may be involved in the antidepressant mediated translocation of $G\alpha_s$ from lipid rafts to non-raft domains. Cells were also treated with 10 µM ketamine for 15 minutes or 24 hours, drug was washed out, cells collected after 24 hours initial treatment and immunoblotted for desaturating enzymes. No significant changes were detected.

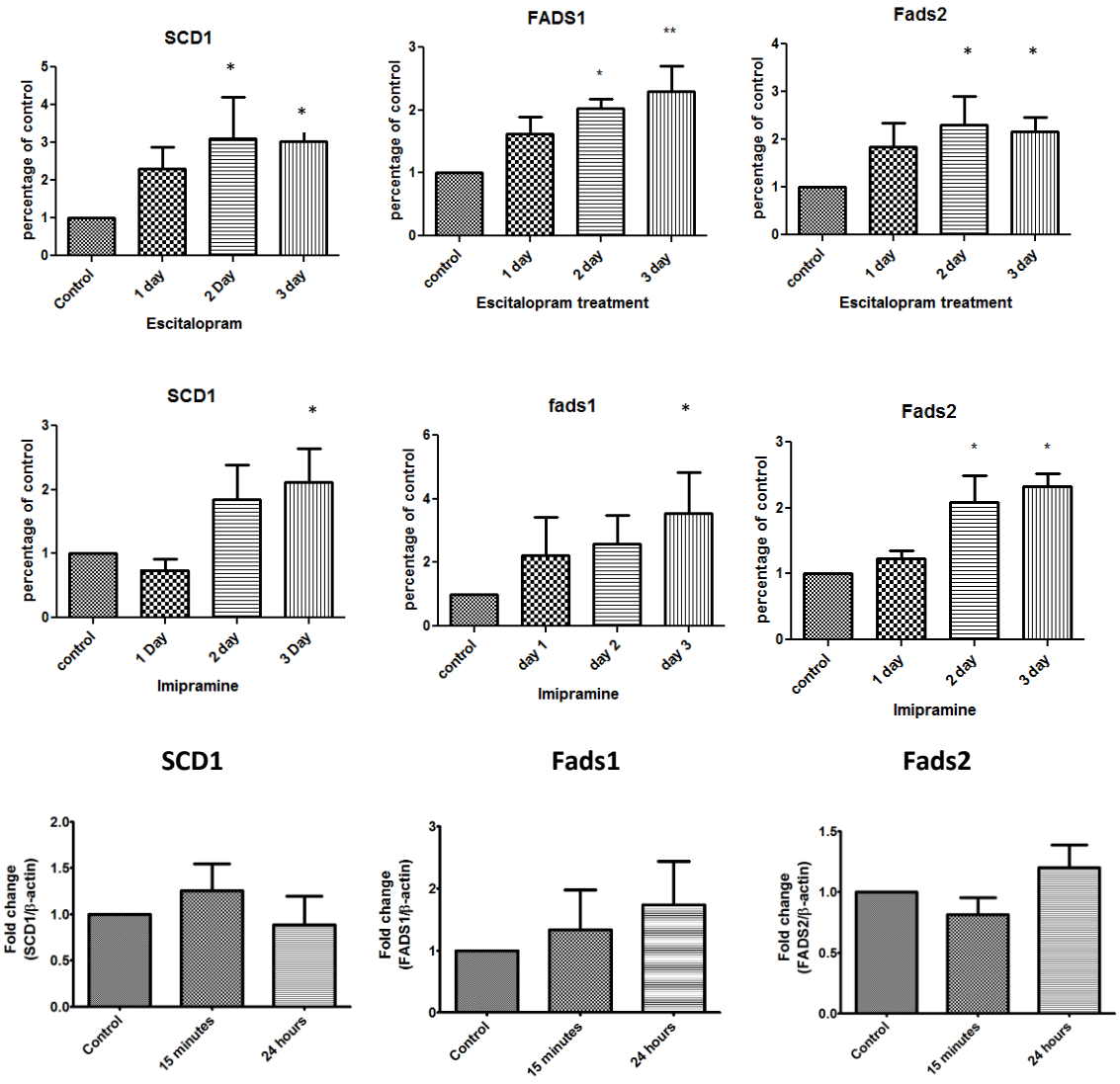


Figure 20. Desaturating enzymes are upregulated by antidepressant treatment in C6 cells. C6 cells were treated with 10 µM imipramine or escitalopram for 1, 2, or 3 days, cells collected, and protein levels evaluated by immunoblotting. Ketamine treatment was for 15 minutes or 24 hours, then drug was washed off and cells collected 24 hours after initial treatment. Imipramine and escitalopram treatment significantly increases desaturating enzymes by day 3 of treatment. (n≥5), *,$p < 0.05$, **, $p < 0.01$

Chapter V

5.1 Discussion

This thesis presents the novel finding that ketamine mediates translocation of $G\alpha_s$ from lipid raft microdomains to non-raft domains. The subsequent biochemical events may contribute to the antidepressant action of ketamine. The delayed increase in BDNF compared to eEF2-translational dependent BDNF production suggests that, whereas this effect may be contributing to the sustained antidepressant effects of ketamine, it is doubtful that it is contributing to the immediate antidepressant effects. However, elevated levels of astrocytic cAMP may mediate other unknown biochemical events lending to ketamine's rapid antidepressant activity. As discussed above, cAMP is globally reduced in brains of MDD patients (Fujita 2017). Nonetheless, ketamine has a metabolic half-life of approximately six hours and remission of depressive symptoms on average persist for two weeks, sometimes lasting up to three months, indicating the necessity of both rapid and sustained activation of cellular pathways essential for persistence of antidepressant effects (Abdullah 2016). Interestingly, other NMDA antagonists do not cause selective redistribution of $G\alpha_s$. Thus, these effects may occur independently of NMDA receptor antagonism. Given that intracellular cAMP accumulation was maintained after knocking down the expression of NMDA receptors, the functional consequences also appear to be independent of NMDA receptor antagonism. Thus, giving rise to the possibility of an unidentified target mediating the translocation of $G\alpha_s$. Furthermore, these data are in contrast to other reports, where other NMDA

antagonists elicit a similar biochemical antidepressant response to ketamine (Li 2010) (Autry 2011).

It may also be possible, yet unlikely given cAMP accumulation was maintained after knocking down the NMDA receptor, that ketamine elicits a unique NMDA receptor-biased signaling event that mediates $G\alpha_s$ translocation and subsequent cAMP accumulation. To test this alternate hypothesis, the NMDA receptor expression would need to be completely ablated by superior methods compared to siRNA presented in this study. This could be accomplished by techniques such as Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated genes (CRISPR/cas9). Furthermore, despite ketamine's previously discussed polyvalent nature (Table 3) none of its known actions are capable of increasing cAMP accumulation. Data presented here, and the aforementioned discussed studies additionally support the novelty of ketamine induced $G\alpha_s$ translocation mediating cAMP accumulation.

The ketamine metabolite (2R,6R)-HNK elicits a similar response as ketamine in both reduction of $G\alpha_s$-GFP plasma membrane mobility and increased cAMP accumulation. The study that first identified (2R,6R)-HNK as rapid acting antidepressant also demonstrated that it acted on a target independent of the NMDA receptor. It may be possible that ketamine and (2R,6R)-HNK may be acting through the same unknown target in our model to ultimately increase cAMP. This remains to be tested.

To the best of the author's knowledge, this is the first report to show cAMP dependent production of BDNF after an antidepressant dose of ketamine in astrocytes or cultured glial tumor cells. This effect may be specific to astrocytes or other glia, as reports on primary neurons and animals show sub-anesthetic ketamine mediates decreases of p-eEF2 and if intracellular levels of cAMP were being upregulated phosphorylation of eEF2 should also increase (Autry 2011) (Zanos 2016). Furthermore, studies examining the effect of ketamine on p-CREB are conflicting (Xue 2016) (Réus 2015) (Du 2017) (Autry 2011) (Zanos 2016). When primary neurons or synaptosomes are isolated from rodents treated with an antidepressant dose of ketamine, no increase in p-CREB is detected. However, when homogenate from the hippocampus or the cortex of rodents treated with ketamine a consistent increase in p-CREB is observed (Xue 2016) (Réus 2015). Considering that brain homogenate contains a variety of cells, including astrocytes, the increase in p-CREB and cAMP may not occur in neurons. One difference between astrocytes and neurons explaining this possibility is their lipid raft composition. Astrocytes are enriched in caveolae (containing caveolin) and neurons primarily have only planar lipid rafts, which contain flotillin. Interestingly, a small population of hippocampal neurons contain caveolae, a brain region repeatedly shown to be involved in the pathology of depression and the antidepressant response to therapy and antidepressants (Allen 2007). This possibility remains to be tested directly.

Considering that BDNF is an essential component of ketamine's and conventional antidepressant's effective antidepressant action, and astrocytes synthesize and release a wide variety of neurotrophic factors, including BDNF, one may speculate that a major loss of neurotrophic producing cells or a loss of astrocytic neurotrophic production would result in depression. Parallel to this concept one would expect increased astrocytic neurotrophic production to exert antidepressant effects. Indeed, a recent study has shown that both SSRIs and TCAs increase BDNF mRNA in primary astrocytes (Wang 2017). Furthermore, overexpressing BDNF in hippocampal astrocytes *in vivo* produces antidepressant-like behavioral effects in animal models of depression (Quesseveur 2013). Further research is needed to understand astrocytic-derived BDNF release as the mechanism remains unknown (Wang 2017). Considering that we have shown that ketamine increases astrocytic BDNF by a cAMP dependent mechanism, it may be possible that astrocyte cAMP plays a role in both the synthesis and release of ketamine mediated astrocytic BDNF, as cAMP is a secretagogue in astrocytes (Parpura 2009). Given that astrocytes release slow gliotransmitters, including BDNF, a key component of antidepressant action, targeting astrocytes to release BDNF over long periods of time could potentially have powerful and long-lasting antidepressant effects. Intriguingly, it is established that astrocytic cAMP induces slow gliotransmitter exocytosis, while Ca^{2+}governs fast gliotransmitter release (Vardjan 2015). Furthermore, astrocytes also synthesize and release a host of other neurotrophic factors that may have antidepressant qualities (Wang 2017).

Many *in vivo* studies do not show an increase in BDNF until 24 hours after ketamine treatment, which is well after the observation of behavioral effects (Zanos 2016) (Popp 2016) (Cui 2018). Thus, it can be inferred that there may be an additional unidentified biochemical event mediating ketamine's rapid antidepressant effects (Zanos 2016) (Popp 2016) (Cui 2018). Recently, a convincing pair of studies detailed the rapid antidepressant action of ketamine was caused by suppressed neuronal bursting in the lateral habenula. In this study employing a genetically bred animal model of depression, increased lateral habenula bursting was caused by increased clearance of K^+. By an unknown mechanism, astrocytic Kir4.1 expression was increased in the lateral habenula of the depressed rat, thus decreasing K^+ ions in the extracellular synaptic space. Reduction of K^+ caused a decrease in resting membrane potential in neurons, which caused T-type voltage sensitive calcium channels to become activated, initiating neuronal bursting. This activity was ablated by ketamine and T-type voltage sensitive calcium channel antagonists, which also rescued the depressed phenotype along a similar timeframe (Cui 2018) (Yang 2018). These studies also raise the possibility of targeting astrocytes or T-type voltage sensitive calcium channels for the treatment of MDD.

Astrocytes also play a key role in the formation of LTP. Astrocytic processes surround synapses and actively engage in bidirectional communication. During the initial stages of LTP induced by Pavlovian threat conditioning, synapses swell in size. To allow for

changes in synaptic morphology astrocytic processes must withdraw (Ostroff 2014). Thus, it can be inferred that dynamic actions of astrocytes are necessary for the initial stages of LTP for some forms of learning. Moreover, during many physiological conditions such as sensory stimulation and reproduction astrocytes exhibit extraordinary morphological plasticity (Zorec 2015). Interestingly, the precise mechanisms governing such remarkable plasticity are unknown, but are thought to involve cAMP (Zorec 2015). Increasing intracellular cAMP with a variety of compounds in astrocytes causes distinct and rapid morphological changes (Won 2000) (Gharami 2004) (Vardjan 2014). These changes are temporally in line with memory consolidation and involve cytoskeleton reorganization (Safavi-Abbasi 2001). Furthermore, after Hebbian learning paradigms, changes in astrocytic morphology have been reported (Ostroff 2014). Reduction of baseline astrocytic cAMP may impair astrocyte plasticity and contribute to the pathophysiology of MDD. Directly increasing astrocytic cAMP may prove to alleviate such symptoms.

Previous reports from our group show that disrupting microtubules causes liberation of $G\alpha_s$ from lipid rafts into non-raft regions (Csysz 2015) (Singh 2018). This effect is also observed as decreased association of $G\alpha_s$ and tubulin in lipid raft fractions (Singh 2018). Interesting, ketamine has no effect on $G\alpha_s$ and tubulin in plasma membrane or lipid raft isolate. Thus, it can be inferred that ketamine influences $G\alpha_s$ localization via a distinct mechanism. This is line with its rapid activity.

Given its small and lipophilic nature, ketamine crosses the BBB and also partitions into the plasma membrane. In fact, many anesthetics are known to partition into the plasma membrane and many have posited that this phenomenon may underlie their anesthetic activity (Jerabek 2010). Two general hypotheses exist; one being anesthetics fluidize the plasma membrane altering protein function and/or anesthetics bind specific proteins in the plasma membrane. The plasma membrane fluidization theory is attractive as anesthetic potency is strongly correlated with the potential of the compound to induce plasma membrane fluidization (Boren 2007). Interestingly, some anesthetics display preference for interacting with lipid raft over non-raft domains (Bandeiras 2013). Unfortunately, ketamine was not investigated in the latter study. However, ketamine has been shown to increase lateral pressure of model membranes but not model membrane thickness (Jerabek 2010). A similar force may be acting upon $G\alpha_s$ in cellular membranes. Furthermore, a recent report details an anesthetic altering plasma membrane localization in nanoscale domains of a transmembrane protein within minutes of treatment (Bademosi 2018). Thus, ketamine may be acting in a similar manner on $G\alpha_s$. This possibility remains to be tested.

While we have proposed that a disruption of $G\alpha_s$/tubulin complexes are mediated by antidepressant treatment, results presented here suggest desaturating activity may also play a role (Singh 2018). The upregulation of all desaturating enzymes may be increasing

the synthesis of PUFAs (Fig 19). Studies suggest that these lipids would be incorporated into the plasma membrane, which would increase fluidity (Czysz 2013). Moreover, many groups, including ours, have shown the movement of lipid raft proteins to non-raft domains after treatment with PUFAs (Huster 1998) (Diaz 2002) (Jaureguiberry 2014) Czysz unpublished). An alternative possibility may be that desaturating enzymes are introducing a double bond into the palmitate that targets $G\alpha_s$ to the plasma membrane. Specifically, SCD1 does introduce a double bond onto palmitate rendering it into palmitoleic acid (Fig. 20). If such a modification were to take place, $G\alpha_s$ would exhibit preference for non-raft domains of the plasma membrane, as MUFAs are not favored in cholesterol-rich lipid rafts.

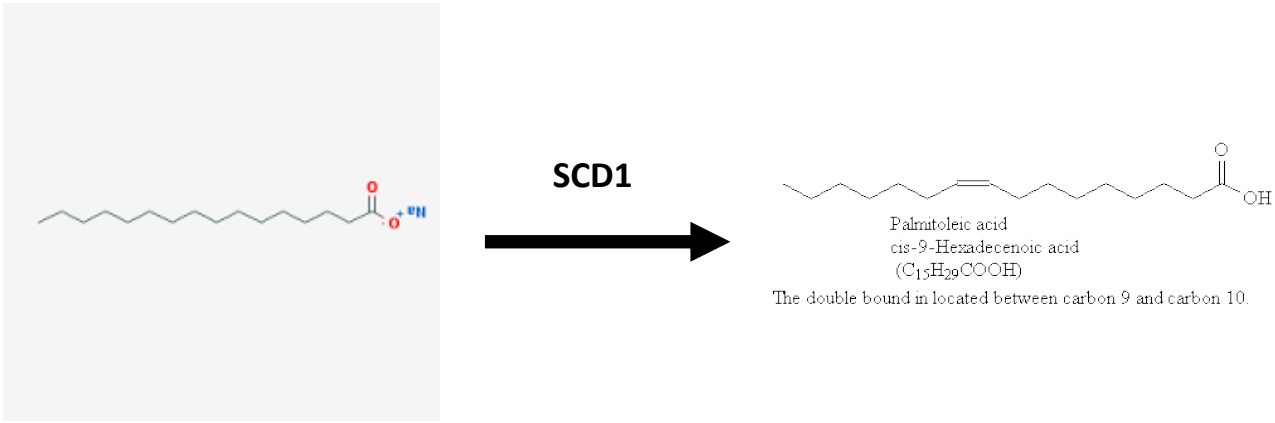


Figure 21. SCD1 desaturating palmitate. SCD1, a Δ9 desaturating enzyme could potentially render the palmitate (left) attached to $G\alpha_s$ to palmitoleic acid (right). Increased unsaturated fatty acids present in the plasma membrane would increase plasma membrane fluidity.

These results suggest a possible dynamic interplay between plasma membrane fluidity lipid rafts and $G\alpha_s$/tubulin complexes involved in the plasma membrane localization of $G\alpha_s$. It remains to be tested if plasma membrane lipid composition is altered after antidepressant treatment. Finally, if ketamine is also altering plasma membrane fluidity, albeit much more rapidly, these results point to a common mechanistic force shared between ketamine and classical antidepressants, mediating the translocation of $G\alpha_s$.

Future directions

Given that all of this experimental work was performed *in vitro* and mostly in C6 glioma cells, experiments are needed to replicate what we have shown *in vivo*. Furthermore, many questions remain could be answered using *in vitro* methods.

The pertinent questions are listed as follows:

- Does ketamine mediate translocation of $G\alpha_s$ specifically in astrocytes or in neurons as well? Specific to caveolae or planar lipid rafts?

- Does ketamine mediate cAMP accumulation specifically in astrocytes and not in neurons? Specific to caveolae or planar lipid rafts?

- Are there specific brain regions that respond to ketamine in regard to the translocation of $G\alpha_s$ from lipid raft domains to non-raft regions?

- Does the translocation of $G\alpha_s$ and subsequent increase in intracellular cAMP contribute to the antidepressant behavioral effects of ketamine?

- How does ketamine cause $G\alpha_s$ to translocate from lipid-raft domains to non-raft regions? Does it involve protein or lipid targets in the plasma membrane? Does it involve plasma membrane "fluidization"?

- Does the upregulation of desaturating enzymes change the lipid composition of the plasma membrane?

Conclusion

Our group has suggested that the translocation of $G\alpha_s$ from lipid rafts, is a cellular hallmark of antidepressant action and it may provide a biosignature to identify compounds with antidepressant potential. All classes of antidepressants display a similar biosignature where other psychotropic drugs such as stimulants and anxiolytics are without effect. The notion that ketamine and (2R,6R)-HNK displays this signature along a treatment duration relevant for the antidepressant effects of that compound, is

intriguing. Data presented in this thesis also implicates astrocytic cAMP in ketamine's potent antidepressant effects. While we have identified a few biochemical events, there may be many left unidentified. Furthermore, data presented here suggests that ketamine and other antidepressants share a common mechanism which alters plasma membrane fluidity. This remains to be tested. Finally, this thesis also suggests that screening for the classical antidepressant biosignature may identify novel rapid acting antidepressants.

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Data presented in this thesis was published in:

Wray, N. H., Schappi, J. M., Singh, H., Senese, N. B., & Rasenick, M. M. (2018). NMDAR-independent, cAMP-dependent antidepressant actions of ketamine. *Molecular Psychiatry*, *2*, 1. https://doi.org/10.1038/s41380-018-0083-8

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