

**Novel Treatment Strategies for Major Depressive Disorder:
Investigating Ketamine's Antidepressant Effects and the Role of the c-Jun N-
terminal Kinase Pathway**

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Abstract

Current first-line treatments for depression, namely monoamine-based drugs such as SSRIs, take weeks to show any clinical effects, and they are only effective in 60-70% of patients. There is therefore an urgent need to develop more rapid and efficacious treatments. Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist often used as a dissociative anesthetic, has been found to have rapid (within hours) antidepressant effects, even in historically treatment-resistant patients. Nevertheless, ketamine has its own limitations, such as unwanted side effects and abuse potential. The overarching goal of this thesis was to gain a better understanding of the antidepressant mechanisms of ketamine. Interestingly, we found that ketamine did not impact the typical stress hormone, corticosterone, nor did it modulate brain-region specific monoamine changes that were induced by acute (restraint) or systemic (lipopolysaccharide; LPS) stressors. However, ketamine did have anti-inflammatory actions, reducing interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α), and further still, repeated ketamine treatment promoted adult neurogenesis within the hippocampus. Notably, repeated ketamine also had an antidepressant-like behavioral effect that was still detectable 8 days after the final ketamine injection. In terms of potential mechanistic factors, ketamine increased active levels of the signaling factor c-Jun N-terminal kinase (JNK) within the cortex, and inhibition of JNK itself increased corticosterone levels. Intriguingly, JNK inhibition also modulated some stress-induced behavioral and monoaminergic changes, implying a diverse role for the protein. Overall, the data support a role for ketamine in neuroplasticity and immune function, and set the stage for future investigations into the pathways (i.e. JNK) associated with its antidepressant effects.

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Dedication

To my family, all of you. Only you know how long this road has really been, and your support has been absolutely paramount. Thank you.

Table of Contents

Abstract	ii
Acknowledgements	iii
Dedication	iv
Table of Contents	v
List of Figures	viii
List of Abbreviations	xi
Preface	xiii
Chapter 1. General Introduction	1
1.1 Depression Background	1
1.1.1 Risk: Genetic and Environmental.....	2
1.2 Pathophysiology	4
1.2.1 Monoamines.....	5
1.2.2 Neurogenesis and Neuroplasticity.....	7
1.2.3 Inflammation.....	9
1.2.4 HPA axis.....	11
1.2.5 Intracellular Pathways.....	13
1.2.5.1 Glucocorticoid Receptor.....	13
1.2.5.2 c-Jun N-Terminal Kinase.....	15
1.3 Treatment	18
1.3.1 Current Treatments.....	18
1.3.2 Ketamine.....	19
1.3.2.1 Background.....	19
1.3.2.2 Mechanisms.....	21
1.4 Research Objectives	25
Chapter 2. Ketamine modulates pro-inflammatory cytokines, but not stressor-induced neurochemical changes	27
2.1 Abstract	27
2.2 Introduction	27
2.3 Methods	29
2.3.1 Animals.....	29
2.3.2 Experiment 1.1: Ketamine treatment prior to an acute restraint stress.....	30
2.3.2.1 Open field series.....	31
2.3.2.2 Elevated plus maze.....	32
2.3.2.3 Corticosterone analysis.....	32
2.3.2.4 Monoamine detection.....	33
2.3.3 Experiment 1.2: Ketamine treatment prior to LPS.....	34
2.3.3.1 Sickness behavior.....	34
2.3.3.2 Corticosterone analysis.....	34
2.3.3.3 Monoamine detection.....	35
2.3.3.4 Plasma cytokine analysis.....	35
2.3.4 Statistical analysis.....	36

2.4 Results	36
2.4.1 <i>Experiment 1.1: Ketamine treatment prior to an acute restraint stress</i>	36
2.4.1.1 <i>Open field series</i>	36
2.4.1.2 <i>Elevated plus maze</i>	38
2.4.1.3 <i>Corticosterone analysis</i>	38
2.4.1.4 <i>Monoamine detection</i>	38
2.4.2 <i>Experiment 1.2: Ketamine treatment prior to LPS</i>	39
2.4.2.1 <i>Sickness behavior</i>	39
2.4.2.2 <i>Corticosterone analysis</i>	40
2.4.2.3 <i>Monoamine detection</i>	40
2.4.2.4 <i>Plasma cytokine analysis</i>	41
2.5 Discussion	41
2.6 Conclusions	45
Chapter 3. Ketamine increases hippocampal neurogenesis, but does not influence stressor-induced neurochemical changes	58
3.1 Abstract	58
3.2 Introduction	58
3.3 Methods	61
3.3.1 <i>Animals</i>	61
3.3.2 <i>Experiment 2.1a: FST following a single ketamine treatment</i>	62
3.3.2.1 <i>FST procedure</i>	62
3.3.3 <i>Experiment 2.1b: FST following repeated ketamine treatment</i>	63
3.3.4 <i>Experiment 2.2: Repeated ketamine treatment and CUS</i>	63
3.3.4.1 <i>CUS procedure</i>	64
3.3.4.2 <i>DCX immunohistochemistry</i>	65
3.3.4.3 <i>Corticosterone analysis</i>	66
3.3.4.4 <i>Monoamine detection</i>	66
3.3.4.5 <i>Open field series</i>	67
3.3.4.6 <i>Elevated plus maze</i>	68
3.3.4.7 <i>Y-maze</i>	68
3.3.4.8 <i>Sucrose preference test</i>	69
3.3.5 <i>Statistical analysis</i>	70
3.4 Results	70
3.4.1 <i>Experiment 2.1a: FST following a single ketamine treatment</i>	70
3.4.2 <i>Experiment 2.1b: FST following repeated ketamine treatment</i>	70
3.4.3 <i>Experiment 2.2: Repeated ketamine treatment and CUS</i>	71
3.4.3.1 <i>DXC immunohistochemistry</i>	71
3.4.3.2 <i>Corticosterone analysis</i>	71
3.4.3.3 <i>Monoamine detection</i>	72
3.4.3.4 <i>Open field series</i>	72
3.4.3.5 <i>Elevated plus maze</i>	73
3.4.3.6 <i>Y-maze</i>	73
3.4.3.7 <i>Sucrose preference test</i>	74
3.5 Discussion	74
3.6 Conclusions	78

Chapter 4. Endogenous JNK affects stress relevant processes, and its phosphorylation state can be influenced by acute ketamine treatment	88
4.1 Abstract	88
4.2 Introduction	88
4.3 Methods	90
4.3.1 <i>Animals</i>	90
4.3.2 <i>Experiment 3.1a: Investigating the role of JNK in the behavioral and neurochemical response to acute stress</i>	91
4.3.2.1 <i>Treatments</i>	91
4.3.2.2 <i>Open field test</i>	92
4.3.2.3 <i>Elevated plus maze</i>	92
4.3.2.4 <i>Corticosterone analysis</i>	93
4.3.2.5 <i>Monoamine detection</i>	93
4.3.3 <i>Experiment 3.1b: Inhibition of p38 prior to acute stress</i>	94
4.3.3.1 <i>Treatments</i>	95
4.3.3.2 <i>Corticosterone analysis</i>	95
4.3.3.3 <i>Western blot analysis</i>	95
4.3.4 <i>Experiment 3.2: Ketamine’s effects on JNK in the context of stressor exposure</i>	97
4.3.4.1 <i>Treatments</i>	97
4.3.4.2 <i>Nestlet test</i>	97
4.3.4.3 <i>Western blot analysis</i>	98
4.3.5 <i>Statistical analysis</i>	99
4.4 Results	99
4.4.1 <i>Experiment 3.1a Investigating the role of JNK in the behavioral and neurochemical response to acute stress</i>	99
4.4.1.1 <i>Open field test</i>	99
4.4.1.2 <i>Elevated plus maze</i>	100
4.4.1.3 <i>Corticosterone analysis</i>	100
4.4.1.4 <i>Monoamine detection</i>	101
4.4.2 <i>Experiment 3.1b Inhibition of p38 prior to acute stress</i>	102
4.4.2.1 <i>Corticosterone analysis</i>	102
4.4.2.2 <i>Western blot analysis</i>	102
4.4.3 <i>Experiment 3.2 Ketamine’s effects on JNK in the context of stressor exposure</i>	103
4.4.3.1 <i>Nestlet test</i>	103
4.4.3.2 <i>Western blot analysis</i>	103
4.5 Discussion	104
4.6 Conclusions	110
Chapter 5. General Discussion	122
References	132
Appendix A. Supplementary figures	162

List of Figures

Chapter 1

- Figure 1.1: Simplified diagram of the proposed mechanisms underlying ketamine's antidepressant effects.....24

Chapter 2

- Figure 2.1: Timeline for Experiment 1.1.....47
- Figure 2.2: Effects of a single ketamine injection and restraint stress on open field performance.....48
- Figure 2.3: Effects of a single ketamine injection and restraint stress on novel exploration and social preference-avoidance.....49
- Figure 2.4: Effects of ketamine and restraint stress on elevated plus maze behavior..50
- Figure 2.5: Effect of a single ketamine injection (5 mg/kg) and restraint stress on plasma corticosterone levels.....51
- Figure 2.6: Effects of a single ketamine injection (5 mg/kg) and restraint stress on monoamine levels.....52
- Figure 2.7: Timeline for Experiment 1.2.....53
- Figure 2.8: Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on sickness rating.....54
- Figure 2.9: Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on plasma corticosterone.....55
- Figure 2.10: Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on monoamine levels.....56
- Figure 2.11: Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on plasma cytokines.....57

Chapter 3

- Figure 3.1: Effects of single and repeated ketamine treatment (10mg/kg) on immobility time in the forced swim test.....79
- Figure 3.2: Timeline for Experiment 2.2.....80

Figure 3.3:	Effects of repeated ketamine treatment (10 mg/kg) and CUS on neurogenesis, as indicated by DCX staining, in the DG.....	81
Figure 3.4:	Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on plasma corticosterone.....	82
Figure 3.5:	Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on monoamine levels.....	83
Figure 3.6:	Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on open field performance, novel object exploration, and social preference-avoidance.....	84
Figure 3.7:	Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on elevated plus maze performance.....	85
Figure 3.8:	Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on performance in a two-trial Y-maze.....	86
Figure 3.9:	CUS and repeated ketamine (10 mg/kg) treatment time-dependently altered sucrose preference.....	87

Chapter 4

Figure 4.1:	Timeline for Experiment 3.1a.....	112
Figure 4.2:	Effects of JNK antagonism and acute stress in the open field test.....	113
Figure 4.3:	Effects of acute stress and JNK inhibition on performance in the elevated plus maze.....	114
Figure 4.4:	Effects of the JNK antagonism and acute stress on corticosterone levels.....	115
Figure 4.5:	Effects of JNK antagonism and acute stress on levels of central monoamines.....	116
Figure 4.6:	Effects of JNK and p38 inhibition on plasma corticosterone in the context of acute stress.....	117
Figure 4.7:	Timeline for Experiment 3.2.....	118
Figure 4.8:	Effects of ketamine and acute restraint stress on nestlet behavior.....	119

Figure 4.9: Effects of ketamine and restraint stress on JNK activity in the PFC and hippocampus.....120

Figure 4.10: Effects of ketamine and restraint stress on GR activity in the PFC and hippocampus.....121

Appendix A

Figure A.1: Representative images of the effects of ketamine and CUS on DCX+ cells in the hippocampal DG.....162

Figure A.2: Representative immunoblots for the effects of ketamine and restraint stress on levels of JNK and pJNK.....163

Figure A.3: Representative immunoblots for the effects of ketamine and restraint stress on levels of GR and pGR.....164

List of Abbreviations

4E-BP1: eukaryotic initiation factor 4E binding protein
5-HIAA: 5-hydroxyindoleacetic acid
5-HT: 5-hydroxytryptamine (serotonin)
ACE: adverse childhood experience
ACTH: adrenocorticotrophic hormone
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA: analysis of variance
AP-1: activator protein-1
ATF: activating transcription factor
BCA: bicinchoninic acid assay
BDNF: brain-derived neurotrophic factor
CNS: central nervous system
CRH: corticotropin-releasing hormone
CSF: cerebrospinal fluid
CUS: chronic unpredictable stress
DA: dopamine
DCX: doublecortin
DG: dentate gyrus
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
eEF2: eukaryotic elongation factor 2
ERK: extracellular signal-regulated kinase
FST: forced swim test
GABA: gamma-aminobutyric acid
GM-CSF: granulocyte-macrophage colony stimulating factor
GR: glucocorticoid receptor
GRE: glucocorticoid-responsive element
HC: hippocampus
hGR: human glucocorticoid receptor
HPA: hypothalamic-pituitary-adrenal
HPLC: high-performance liquid chromatography
HRP: horseradish peroxidase
HSP: heat shock protein
IL: interleukin
IFN: interferon
i.p.: intraperitoneal
JNK: c-Jun N-terminal kinase
LPS: lipopolysaccharide
LTP: long-term potentiation
MAOI: monoamine oxidase inhibitor
MAPK: mitogen-activated protein kinase
mGR: mouse glucocorticoid receptor
MHPG: 3-methoxy-4-hydroxyphenylglycol

MR: mineralocorticoid receptor
mTOR: mammalian target of rapamycin
NBQX: 2,3-dihydroxy-6-nitro-7-sulfoamoylbenzo(f)-quinoxaline
NE: norepinephrine
NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA: N-methyl-D-aspartate
NSAID: nonsteroidal anti-inflammatory drug
P70S6K: p70S6 kinase
PBS: phosphate-buffered saline
PFC: prefrontal cortex
PKB: protein kinase B
PLSD: protected least significant difference
PP2A: protein phosphatase 2
PSD95: postsynaptic density protein 95
PVN: paraventricular nucleus
rGR: rat glucocorticoid receptor
RIA: radioimmunoassay
RIPA: radio immuno-precipitation assay
S: serine
SAPK: stress-activated protein kinase
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGZ: subgranular zone
SLE: stressful life event
SNRI: selective norepinephrine reuptake inhibitor
SSRI: selective serotonin reuptake inhibitor
SVZ: subventricular zone
TBS: tris-buffered saline
TBS-T: tris-buffered saline with tween
TCA: tricyclic antidepressant
TNF: tumor necrosis factor
TRD: treatment-resistant depression
TrkB: tyrosine receptor kinase B
TRP: tryptophan
UV: ultraviolet
VTA: ventral tegmental area
WHO: world health organization

Preface

This thesis is largely based on previously published manuscripts. Chapters 2 and 3 are based on two separately prepared manuscripts that were eventually combined into one manuscript for publication. Chapters 2 and 3 also contain behavioral data that was not published. Chapter 4 is based on one manuscript, however an additional experiment was added as we expanded our investigations. In each case, the methods, results, figures, and portions of the discussion were reproduced in this document. The use, with permission, of copyrighted material is hereby acknowledged.

Manuscript 1 (Chapters 2 and 3):

Clarke M, Razmjou S, Prowse N, Dwyer Z, Litteljohn D, Pentz R, Anisman H, Hayley S (2017) Ketamine modulates hippocampal neurogenesis and proinflammatory cytokines but not stressor induced neurochemical changes. *Neuropharmacology* 112(A): 210-220.

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Chapter 1. General Introduction

1.1 Depression Background

Depression is one of the most common mental health conditions worldwide, with estimates of lifetime prevalence ranging from 8–20% (Nestler et al. 2002; Kessler and Bromet, 2013). It is a debilitating disease that is characterized by a wide range of symptoms, including distinct changes in mood (sadness or irritability), impaired cognitive function, diminished interest, altered psychomotor activity (slowing of speech and action), and disturbed neurovegetative functions such as altered sleep and appetite patterns (Fava and Kendler, 2000; Belmaker and Agam, 2008; Otte et al. 2016). The course of depression is varied, with many experiencing their first depressive episode in childhood or adolescence, and others not experiencing a first depressive episode until adulthood (Fava and Kendler, 2000). Regardless of age of onset, for most people depression is a life-long, episodic disorder with depressive episodes recurring multiple times. Additionally, approximately 20–25% of patients with depression experience a course that is chronic and continuous, leaving them without any periods of remission (Meuller and Leon, 1996).

The toll of depression on quality of life and global functioning is enormous, and due to the risk of suicide, it is life threatening (Blazer, 2000). Of the estimated 800,000 suicides worldwide every year, up to 50% are thought to occur within a depressive episode (WHO, 2016), and there is a 20% greater risk of suicide among depressed patients than in the general population (Chesney et al., 2014). Furthermore, depression increases the mortality risk in both the general population and populations with medical illnesses by 60–80% (Cuijpers, et al., 2014; Walker et al., 2014). In fact, according to the world health organization (WHO), depression is the leading contributor to global disease burden, as

expressed in disability-adjusted life years, out of all medical conditions (Vos et al., 2013; WHO, 2017) and has been associated with the increased risk of other medical conditions including diabetes, heart disease, stroke, hypertension, obesity, cancer, cognitive impairment, and Alzheimer disease (Whooley and Wong, 2013; Penninx et al., 2013).

Depression is clearly a major clinical issue, and unfortunately, currently available treatments are lacking in both speed and efficacy (Thase et al., 2005; Trivedi et al., 2006). This thesis will focus on novel treatment strategies for depression, and our work will employ stressor-based animal models of depression given the strong association between stress and depression (Fava and Kendler, 2000; Keers and Uher, 2012). Before discussing traditional antidepressant medications, their limitations, and the exciting role of ketamine [an anesthetic and N-methyl-D-aspartate (NMDA) receptor antagonist] as a new form of treatment in depression, an overview of the risk factors and pathophysiology hypothesized to be underlying the condition will be presented.

1.1.1 Risk: Genetic and Environmental

It is well documented that depressive illnesses tend to run in families, and it has been estimated that first-degree relatives of patients with depression have a threefold greater risk of developing the condition than those in the general population (Geschwind and Flint, 2015). We know that depression is highly heritable, with genetic contribution accounting for roughly 35–40% of risk (Sullivan et al., 2000; Flint and Kendler, 2014). However, despite the high degree of heritability, genetic effects have not been consistently or reproducibly found (Bosker et al., 2011). This is most likely because depression is a complex condition involving multiple genetic factors (rather than a single gene), which

culminate to put one at risk for the disorder. Furthermore, the difficulty in finding significant genetic associations is further exacerbated by the fact that depression displays such a high degree of heterogeneity (Hyman, 2014; Dunn et al., 2015). Nevertheless, numerous studies have indicated that genetic risk alone is not sufficient or necessary for depression anyway, and that it is the result of multiple, interdependent genetic and environmental risk factors (Keers and Uher, 2012; Mullins et al., 2016; Otte et al., 2016; Gonda et al., 2018).

Studies of environmental risk factors have found several forms of psychosocial adversity to be associated with depression, and the link between stress and depression is now one of the most reliable and robust findings in the psychiatric literature (Sullivan et al., 2000; Keers and Uher, 2012; Frodl and O’Keane, 2013). Notably, there is strong evidence that at least some of this association is causative (Fava and Kendler, 2000).

Much early depression research focused on stressful life events (SLEs) that occurred recently (within weeks or months of depression onset), such as job loss, relationship break-up, death of a loved one, and significant health problems. Indeed, it has been reported that severe SLEs more than double the risk for depression (Kessler, 1997). However, further evidence has implicated more distal (occurring long before the onset of depression) stressful events, namely adverse childhood experiences (ACEs), which include physical, emotional, and sexual abuse, neglect, domestic violence, and early separation from parents (Li et al., 2016). ACEs have been found to increase the risk for depression in adulthood, and this effect has been shown to persist throughout the lifetime (Clark et al., 2010; Scott et al., 2010; Norman et al., 2012). Taken together, it is clear that enduring, pathological effects of stress can occur regardless of when the stress was experienced.

Indeed, animal studies have established that exposure to both acute and chronic stress leads to lasting behavioral changes that mirror those seen in depression, including decreased motivation, anhedonia, reduced grooming, and altered sleep patterns (Willner, 2005; Luo et al., 2008; Wang et al., 2018; Zhang et al., 2018). Furthermore, stressor exposure causes neurochemical, hormonal, and brain-region specific structural changes that are also associated with the condition (Anisman, 2009; McEwen et al., 2012). There are a number of neurological systems that have been implicated in the stress-induced vulnerability to depression, and these will be introduced in the following section.

1.2 Pathophysiology

Although the pathophysiology underlying depression has not been fully established, there has certainly been much progress in this area over the past 50 years, and multiple mechanisms have been implicated. These include, but are not limited to, monoamine transmission, neurogenesis and neuroplasticity, inflammation, and hypothalamic-pituitary-adrenal (HPA) axis function (Dean and Keshavan, 2017). It is worth mentioning here that these systems do not operate in isolation, and in fact are interconnected and interdependent, with changes to one system effecting changes in the others.

1.2.1 Monoamines

The monoamine hypothesis of depression, which is still the most prevailing hypothesis, proposes that depression is caused by abnormalities in the levels of one or more of the monoamines, and that antidepressant effects can be achieved by enhancing monoamine function in the synapse (McLeod and McLeod, 1971; Mulinari, 2012; Liu et al., 2017). The monoamines are a group of neurotransmitters that include serotonin (5-HT), norepinephrine (NE), and dopamine (DA). It was first recognized that monoamines might play a role in the pathophysiology of depression when the antihypertensive drug, reserpine, was found to reduce monoamine levels, and that some patients taking this medication developed depression (Schildkraut, 1965). All first generation antidepressants, the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), and newer antidepressants, including selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs), enhance the effects of monoamines (Mulinari, 2012; Otte et al., 2016; Belujon and Grace, 2017).

There is undeniably a large body of evidence linking altered monoamine levels and depression, and in this regard, reduced levels of monoamines, their metabolites, and their precursors have been observed in depressed patients (Tekes et al., 1988; Der-Avakian and Markou, 2012; Yoon et al., 2017). For example, patients with untreated depression have been reported to have lower plasma levels of tryptophan (TRP; the precursor to 5-HT) (Coppen et al., 1973), and suicide has been correlated with lower cerebrospinal fluid (CSF) levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Nordstrom and Asberg, 1992). When levels of 5-HT and NE were depleted in patients who had responded to 5-HT- and NE-targeting antidepressants, respectively, the antidepressant effects were

reversed and depression scores increased (Shopsin et al., 1975; Delgado et al., 1990; Delgado et al., 1993). Furthermore, animal studies using chronic unpredictable stress [(CUS); one of the most frequently-utilized animal models of depression, generally capable of inducing a depressive-like phenotype] reported stress-induced reductions in 5-HT, 5-HIAA, NE, and DA that were associated with depressive-like behaviors (Wang et al., 2018).

Despite these findings, the vast majority of studies measuring 5-HT, NE, and DA metabolites in the plasma, urine, and CSF of depressed patients, as well as those examining postmortem brain tissue from depressed patients, have had mixed results at best (Belmaker, 2004; Pech et al., 2018). It has also been reported that depletion of 5-HT and NE in healthy subjects fails to induce depressive symptoms, or even worsen symptoms in depressed patients (Benkelfat et al., 1994; Delgado and Moreno, 2000). So, although altered monoamine functioning is clearly involved in depression pathology, it is now obvious that the monoamine hypothesis is oversimplified. Since antidepressants have robust effects on neurotransmission within hours, and yet the clinical antidepressant effects of these medications take weeks to appear (Trivedi et al., 2006), it is currently theorized that depression is caused by dysfunction in brain areas and neuronal systems that are modulated by monoamines (Delgado and Moreno 2000). In this regard, we know that chronic treatment with monoamine-targeting antidepressants leads to multiple downstream effects, including altered gene expression and increased neural and synaptic plasticity, and it is these changes that are now believed to underlie their clinical effects (Wong and Licinio, 2001; Otte et al., 2016).

1.2.2 Neurogenesis and Neuroplasticity

Neuroplasticity (the ability of neurons to change in form and function in response to their environment) and neurogenesis (the generation of new neurons from pluripotent stem cells) are both processes that are consistently disturbed in patients with depression (Mineur et al., 2007; Pittenger and Duman, 2008; Otte et al., 2016; Dean and Keshavan, 2017). Adult neurogenesis has been found to occur in the subventricular zone (SVZ) of the lateral ventricles, and also in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Altman, 1965; Gates et al., 1995; Couillard-Despres, 2005; Vadodaria and Gage, 2014). It is hippocampal neurogenesis that has received the most attention for its role in the pathophysiology and treatment of depression, and further discussion of neurogenesis will refer to neurogenesis in the DG.

It is well established that many acute and chronic stressors profoundly reduce neurogenesis (Cameron and Gould, 1994; Gould et al., 1997; Yap et al., 2006; Egeland et al., 2015), and neurogenesis can in turn modulate stress responsiveness (Snyder et al., 2010; Schoenfeld and Gould, 2012). Indeed, animals exposed to CUS tend to have reduced neurogenesis, display an impaired ability to cope with future stressors, and develop depressive-like behaviors (Mineur et al., 2007). Interestingly, reductions in neurogenesis in the absence of stress do not necessarily lead to a depressive-like phenotype; however in the presence of stress, reduced neurogenesis can promote depressive-like symptoms (Egeland et al., 2015), suggesting that reduced neurogenesis can lead to depression through impaired stress modulation (Petrik et al., 2011). Evidence is also found in postmortem human brain tissue from patients with depression, which displays reduced hippocampal neurogenesis (Lucassen et al., 2010). Moreover, neurogenesis is associated with

antidepressant efficacy (Boldrini and Hen, 2012; Miller and Hen, 2015), and it is believed that the clinical effects of antidepressants are, at least in part, mediated through this mechanism (Egeland et al., 2015; Miller and Hen, 2015).

The prefrontal cortex (PFC) is important for higher-order cognitive functions, and plays a role in the neuroendocrine, autonomic, and behavioral response to stress (McKleeven et al., 2015; Csabai et al., 2018). Accordingly, PFC neurons are highly sensitive to stress. Postmortem studies have discovered atrophy of PFC pyramidal neurons in the brain tissue of depressed patients (Rajkowska et al., 1999), and animal studies using chronic stress models have implicated stress as a causative factor in such neuronal atrophy (Liu and Aghajanian, 2008). Furthermore, evidence exists that even short periods of intense stress can cause significant structural remodeling (such as reduced density, length, and complexity of apical dendrites) of pyramidal neurons within the PFC, and that this can lead to impaired cognitive function and emotional regulation (Cook and Wellman, 2004; Radley et al., 2004; Homes and Wellman, 2009). Functional abnormalities (Baxter et al., 1989; Mayberg et al., 1999) and volume shrinkage (Drevets et al., 2008) have also been found in the PFC of depressed patients, and it is believed that stress-induced structural and functional changes to excitatory synapses within the PFC are key contributors to depression (Duman et al., 2016).

Brain-derived neurotrophic factor (BDNF) is a growth factor that supports the survival of neurons and also promotes neurogenesis and synaptogenesis (Huang and Reichardt, 2001). Not surprisingly, depressed patients have lower levels of BDNF in their blood serum, and lower *BDNF* mRNA levels in their leukocytes (Molendijk et al., 2014). Animal studies have shown that stressors reduce BDNF (Post, 2007), and decreasing

BDNF using animal knockout models resulted in reduced neurogenesis (Lee et al., 2002) and induced depressive-like behaviors (Taliaz, 2010). The current thinking is that stress reduces levels of neurotrophins like BDNF, which in turn reduce neurogenesis and hippocampal functioning, ultimately contributing to the onset of depression (Keers and Uher, 2012). Additionally, many antidepressants can prevent the reductions in BDNF that are caused by stressors, and in fact, BDNF levels have been shown to be normalized by both pharmacological and non-pharmacological antidepressant treatments (Post 2007; Molendijk et al., 2014). For instance, the SSRI, Fluoxetine, increases the expression of *BDNF* mRNA in the DG of the hippocampus, the ventral tegmental area (VTA), and the nucleus accumbens (Molteni et al., 2006). The hippocampus is important for learning and memory, while the VTA and nucleus accumbens are integral parts of the reward pathway, dysfunction of which can produce the anhedonia seen in depression. This suggests that BDNF may play a role in the adaptation of both limbic and cortical circuits to changes in the environment (Castren and Rantamaki, 2010).

1.2.3 Inflammation

There is now an extensive amount of research supporting the role of inflammation in depression (Anisman, 2009; Miller and Raison, 2015; Martin-Hernandez et al., 2018). Increased levels of inflammatory cytokines, such as interleukin (IL)-1 β , IL-2, IL-6 and tumor necrosis factor (TNF)- α , have been found in depressed patients (Dowlati et al., 2010; Haapakoski et al., 2015). Depressed patients have a higher incidence of autoimmune disorders, and patients with inflammatory diseases have increased rates of depression (Pasco et al., 2010). In addition to autoimmune diseases, prior serious infections also

increase the risk of subsequent diagnosis of depression (Benros et al., 2013). Interferon- α (INF- α) treatment of patients with hepatitis C leads to depressive features that are related to increased levels of IL-2, IL-6 and TNF- α (Wichers et al., 2007), and both cancer and hepatitis patients treated with IL-2 or IFN- γ often develop depression (Myint et al., 2009). Likewise, subjects who received typhoid vaccinations with *Salmonella Typhi* developed depressed mood in direct proportion to increases in IL-6 (Weight et al., 2005). Furthermore, inducing inflammation in research subjects provokes symptoms of depression. For instance, it was reported by Engler et al. (2017) that in healthy volunteers, low-dose treatment with the endotoxin, lipopolysaccharide (LPS), increased peripheral blood cytokine levels, increased CSF levels of IL-6, and also scores of dysthymia (low mood).

Inflammatory cytokines derived from the brains' resident immune cells (microglia) play a role in synaptic plasticity and spine synapse formation (Khairova et al., 2009; Duman et al., 2016). In fact, low levels of inflammatory cytokines, such as TNF- α and IL-1 β , are necessary for normal brain function, and support neuroplasticity. However, significantly elevated levels of these proteins, due to stress and/or inflammation, appear to have the opposite effect, contributing to damage, atrophy and loss of spine synapses (Duman et al., 2016), possibly contributing to the pathophysiology of depression.

The use of anti-inflammatory agents, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and cytokine inhibitors (both of which reduce inflammation by inhibiting proinflammatory cytokines), as adjunct antidepressant treatments has been investigated, and some of the results look promising (Kohler et al., 2014; Zuzarte et al., 2018). For example, psoriasis patients treated with anti-inflammatories had reduced depression symptoms, and this was independent of any reduction in psoriasis symptoms (Tyring et al.,

2006). Unfortunately, there have been conflicting results as to their usefulness, with some trials even associating NSAIDs with diminished antidepressant effects, and the risk of severe side effects is well documented (Kohler et al., 2014).

1.2.4 HPA Axis

The HPA axis is one of the most studied biological systems in the context of depression, and its dysregulation has long been associated with the disorder (Pariante and Lightman, 2008). Its purpose is to mount an immediate response to stress when a threat is perceived, and then to return the organism to a baseline state afterward. Briefly, when the cortical brain region perceives stress, it transmits this information to the paraventricular nucleus (PVN) of the hypothalamus, which then releases corticotropin-releasing hormone (CRH) onto the pituitary. The pituitary then releases adrenocorticotropic hormone (ACTH) into the plasma, which stimulates the cortex of the adrenal glands to release glucocorticoids (cortisol in humans, corticosterone in rodents) into the blood (Nestler et al., 2002; Juruena et al., 2017). Glucocorticoids are responsible for a range of functions, such as promoting gluconeogenesis, catabolic activity, suppression of innate immunity, and insulin resistance (Juruena et al., 2017). They also act at the hypothalamus and pituitary to inhibit further release of CRH and ACTH, respectively, by binding to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), in a negative feedback loop (Herman et al., 1989; Swanson and Simmons, 1989; Fischer et al., 2017).

There is considerable evidence linking early and recent life stress to long term alterations in the HPA axis, which in turn may promote vulnerability to depression (Shea et al., 2005; Rao et al., 2008; Von Werne Baes et al., 2012). Changes to the HPA axis that

are reported in patients with depression include: increased cortisol, CRH, and ACTH levels, an exaggerated response to ACTH, and enlargement of both the pituitary and adrenal glands (Sachar et al., 1970; Carroll et al., 2007; Knorr et al., 2010; Von Werne Baes et al., 2012). Prospective studies of at-risk populations have found increased cortisol to be a risk factor for subsequent depression diagnosis (Harris et al, 2000). Additional evidence of a causative relationship is provided by studies showing that treatment with synthetic glucocorticoids is associated with increased risk of both suicide and depression (Fardet et al., 2012).

Patients with depression are also reported to have reduced glucocorticoid sensitivity, or ‘glucocorticoid resistance’, and often display decreased suppression of cortisol in response to dexamethasone (a synthetic adrenal steroid with glucocorticoid activity) in what is termed dexamethasone non-suppression (Pariante and Miller, 2001). Since glucocorticoids are potent anti-inflammatory hormones, pathways linked with glucocorticoid resistance could contribute to the chronic inflammation seen in depression (Silverman and Sternberg, 2012).

Long-term glucocorticoid exposure causes alterations to many regions of the brain, including the PFC, the hippocampus, and the amygdala (Dean and Keshavan, 2017). One functional consequence of extended glucocorticoid exposure is reduced activity in the PFC, which is due to decreased dendritic complexity of pyramidal neurons, and increased activity of gamma-aminobutyric acid (GABA) interneurons (which are inhibitory) (Cerqueira et al., 2005). Since this region is important for the processing of emotions generated by subcortical areas, such as the amygdala, reduced PFC activity leads to inadequate processing of affective information (Cerqueira et al., 2005). Chronically high

levels of cortisol also impair hippocampal adaptability. Animal studies have shown that chronic stress decreases plasticity and long-term potentiation (LTP) in the CA1 region of the hippocampus, which in turn reduces learning and adaptation (Alvarez et al., 2002).

Given the strong association between HPA axis dysregulation and depression, there has been a lot of inquiry into the use of HPA axis modulating compounds as therapeutic agents. Unfortunately, these investigations have failed to show much promise. For example, although increased CRH levels have been found in the CSF of depressed patients (Nemeroff et al., 1984), and numerous animal studies appeared encouraging (Overstreet and Griebel, 2004; Hodgson et al., 2007;), the majority of randomized controlled trials using CRH antagonists failed to find them useful in the treatment of depression (Aubry et al., 2013). There have also been mixed results from clinical trials utilizing glucocorticoid-lowering agents (Jahn et al., 2004; McAllister-Williams et al., 2016). At this point, no new antidepressant treatments have been discovered by directly targeting the HPA axis (Otte et al., 2016).

1.2.5 Intracellular Pathways

1.2.5.1 Glucocorticoid Receptor

Many glucocorticoid effects, including HPA axis regulation, are mediated through the GR, and impairment of GR signaling is hypothesized to play a key role in HPA axis dysregulation (Claes 2009; Jovicic et al., 2015). Indeed, both *in vitro* and *in vivo* studies have shown GR sensitivity to be reduced in depression (Pariante, 2004; Carvalho et al., 2008; Claes 2009).

The GR is a transcription factor and member of the nuclear hormone receptor subfamily. It is widely expressed throughout the body and plays an important role in physiology and development (Yamamoto, 1985; Cato and Wade, 1996; Davies et al., 2008). Its target genes include ones that are implicated in metabolism, neuronal survival, neurogenesis, inflammation, and HPA axis regulation (Yamamoto, 1985; Munck, 2005; Kadmiel and Cidlowski, 2013). Specifically, GR activation inhibits cell growth and inflammation, and mediates the majority of glucocorticoid negative feedback mechanisms (Dallman et al., 1989; De Kloet et al., 1998; Rogatski et al., 1998).

In the absence of a ligand, the GR resides in the cytosol, complexed with chaperone proteins such as heat shock proteins (HSPs) 90 and 70 (Pratt et al., 2006; Petta et al., 2016). Glucocorticoid (or ligand) binding to GR leads to its dissociation from chaperones and subsequent translocation into the nucleus, where it modulates the expression of GR-dependent target genes both positively and negatively (Davies et al., 2008; Petta et al., 2016). In a mechanism known as transactivation, GR homodimers bind to deoxyribonucleic acid (DNA) sequences called glucocorticoid-responsive elements (GREs), leading to transcriptional activation. Conversely, in a mechanism known as transrepression, the GR interacts with DNA-bound transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and activator protein-1 (AP-1), preventing them from binding their target genes and thus repressing their respective signaling cascades (Rao et al., 2011). Interestingly, Herr et al. (2003) reported that many antidepressants enhance steroid-induced GR-mediated gene transcription.

Phosphorylation of the GR in the N-terminal domain is an important modification, and affects its stability, subcellular trafficking, and transcriptional activity (Bodwell, 1998;

Ismaili and Garabedian, 2006; Davies, 2008). Multiple amino acids on the human GR (hGR) can be phosphorylated, and the most studied are serines 203, 211, 226, and 404 (Gallagher-Beckley and Cidlowski, 2009; Kadmiel and Cidlowski, 20013). Of these, phosphorylation of hGR at serine 226 (S226) has been implicated in glucocorticoid resistance, and was found to reduce hormone signaling by increasing nuclear export of the GR (Chen et al., 2008). Moreover, it has been reported that depressed patients have increased phosphorylation of the GR at S226 when compared to healthy controls (Simic et al., 2013), and chronic stress in rats increased levels of phosphorylated rat GR (rGR) at serine 246 (analogous to S226 in humans) (Mitic et al., 2013). We now know that the kinase responsible for phosphorylation of the hGR at S226 is the mitogen-activated protein kinase (MAPK) family member, c-Jun N-terminal kinase (JNK) (Itoh et al., 2002).

1.2.5.2 c-Jun N-Terminal Kinase

JNK, [originally named stress-activated protein kinase (SAPK), but renamed due to the discovery that it phosphorylates c-Jun (Hibi et al., 1993; Karin and Gallagher 2005)], is a protein kinase that is activated in response to cellular stressors including DNA damage, oxidative stress, inflammatory cytokines, ultraviolet (UV) radiation, heat, and osmotic shock (Hibi et al., 1993; Westwick et al., 1994; Kyriakis and Avruch 2012). The JNK pathway is involved in a broad range of biological processes including cytokine signaling, cell death, proliferation, and survival. Ten isoforms of JNK have been identified, and are encoded by the three genes: *JNK1*, *JNK2*, and *JNK3* (Davis 2000; Guo et al., 2018).

It has recently been acknowledged that JNK signaling likely plays a role in stress-related pathology, such as that seen in depression (Hollo et al., 2018). In this regard, it has

been reported (Martin-Hernandez et al., 2018) that post mortem PFC tissue from patients with depression showed elevated levels of activated JNK. In animals, acute stress increased active forms of JNK in the PFC and hippocampus, and JNK inhibition had antidepressant-like effects in tests of behavioral despair (Galeotti and Ghelardini, 2012). Likewise, chronic stress also increased active JNK in the PFC and hippocampus (Espinosa-Oliva et al., 2011; Li et al., 2014; Rosa et al., 2018), and treatment with an antidepressant agent not only prevented this effect, but also reduced depressive-like behavior (Li et al., 2014).

Concerning neurogenesis, Mohammad et al. (2018) reported that mice either lacking *JNK1*, or treated with a JNK inhibitor, displayed increased neurogenesis in the hippocampal neurogenic niche, and this effect was accompanied by a reduction in anxiety-like and depressive-like behaviors. Interestingly, they found that restricting JNK inhibition to adult born granule cells was also sufficient to reduce the anxiety-like and depressive-like behaviors, suggesting that JNK may be able to affect mood through its impact on this area. There is also some evidence linking JNK to the inflammatory mechanisms underlying depression, which may seem intuitive since depression is associated with increased levels of proinflammatory cytokines, which are capable of activating JNK (Saklatvala et al., 1999). For example, an *in vitro* study reported that the antidepressant, trazadone, counteracted the activation of JNK in response to LPS, and this effect was associated with neuroprotection (Daniele, 2015).

JNK activation has been shown to increase levels of phosphorylated hGR at S226, rGR at S246, and mouse GR (mGR) at S234 (Rogatsky et al., 1998; Davies et al., 2008), the same phosphorylation sites that have been associated with depression and stressor-induced changes in animal models (Chen et al., 2008; Simic et al., 2013; Mitic et al., 2013).

Multiple studies have shown that activation of JNK inhibits GR-mediated transcriptional activity, and likewise, JNK inhibition leads to increased GR transcriptional activity (Wang et al., 2005; Davies et al., 2008), demonstrating that JNK negatively regulates the GR. Accordingly, given the strong role of the GR in negative feedback of the HPA axis, JNK-mediated influences on GR may play a role in the HPA axis dysregulation observed in depressed patients (Holloos et al., 2018). Indeed, there is evidence that JNK activation may contribute to glucocorticoid resistance (Wang et al., 2005). For example, dexamethasone non-suppression has been seen after rodents were chronically treated with the JNK activator, LPS (Yirmiya et al., 1996; Weidenfeld and Yirmiya, 1999).

There is clear evidence supporting a role for JNK in depressive pathology, and it has been hypothesized that inhibiting the phosphorylation of GR at S226 by inhibiting JNK could improve depressive symptoms (Jovicic et al., 2015). Nevertheless, at this time, much is still unknown regarding the effects of JNK inhibition on monoamines, neurogenesis, inflammation, and HPA axis functioning in the context of depression-relevant stressor models and is an area that ought to be explored.

Naturally, there are other mechanisms that have been implicated in depressive pathology. This includes the glutamate system, which will be discussed in the next section when we introduce ketamine, an NMDA receptor antagonist with exciting antidepressant properties.

1.3 Treatment

1.3.1 Current Treatments

Recommended treatment for depression is either pharmacological or non-pharmacological, depending on symptom severity. For mild depressive episodes, the recommended first-line treatment strategies include psychotherapy, self-management, and psychoeducation (Kennedy et al., 2016). However, for depressive episodes of moderate or greater severity, medication is recommended, and newer antidepressants such as SSRIs and SNRIs are used as first-line treatments. The older (first-generation) TCAs and MAOIs are recommended as second and third-line treatments, respectively, due to their potential for severe side effects and their limited safety profile (Kennedy et al., 2016).

Despite their wide use, currently available antidepressants are not without substantial limitations. The two most significant concerns are their latency to produce desired antidepressant effects, and their lack of efficacy in a large percentage of the depressed population (Rush et al., 2006; Kennedy et al., 2016). Unfortunately, monoamine-based antidepressants take weeks or even months to become effective in reducing depressive symptoms (Trivedi et al., 2006). Accordingly, the Canadian Network for Mood and Anxiety Treatments (CANMAT) defines “early response” as a 20–30% reduction in depression scale scores at 2–4 weeks into the antidepressant treatment, with response and remission often only considered at 6–12 weeks (Kudlow et al., 2014; Kennedy et al., 2016). Given the urgency with which depression ought to be treated, this seems like a long time for patients to wait. Not only is there a profound impairment to social, physical, and occupational functioning for the depressed patient, but the risk of suicide makes time a particularly critical factor.

There has also been concern regarding the efficacy of traditional antidepressants. Meta-analyses of both published and unpublished clinical trials with SSRIs have indicated that their benefit may be significant, but only marginally (Kirsch et al., 2008). Indeed, it has been reported that SSRIs only reduced depression scores by an average of 1.8 points over placebo, when a reduction of 3 or more points is considered to be clinically significant (Kirsch et al., 2002). In addition to the modest effect size reported, there are undeniably a large number of depressed patients who do not respond to antidepressants at all. In fact, antidepressants are completely ineffective in at least 30–40% of patients, leaving a large number of patients with treatment-resistant depression (TRD) (Thase et al., 2005; Rush et al., 2006). Given these shortcomings, it is important to look beyond traditional monoamine-based drugs in the development of novel, more rapid and efficacious treatment strategies.

1.3.2 Ketamine

1.3.2.1 Background

As discussed, current antidepressant research has begun to move away from the monoamine hypothesis, and toward alternate pathological mechanisms, including neuroplasticity, inflammation, and HPA axis dysfunction. Another area that has recently gained a lot of attention for its involvement in depression is the glutamatergic neurotransmitter system. In this regard, ketamine, a non-competitive NMDA receptor antagonist widely used as a dissociative anesthetic, has been found to have antidepressant properties. What makes ketamine particularly exciting is that it appears to address the two major limitations of traditional monoamine-based drugs.

While traditional antidepressants take a minimum of weeks to be effective, a single intravenous infusion of ketamine, given at a sub-anesthetic dose, can produce clinically significant antidepressant effects within hours (Berman et al., 2000; Zarate et al., 2006). Meta-analysis revealed large effect sizes at 4 and 24 hours after treatment (Coyle and Laws, 2015), and the effects of a single infusion have been reported to persist for up to two weeks (Newport et al., 2015). Addressing the limited efficacy displayed by traditional antidepressants, ketamine has been found to be effective in historically treatment-resistant patients (Liebrenz et al., 2007; Machado-Vieira et al., 2009; Murrough et al., 2013). In patients with TRD, response rates at 24 hours averaged 52% for a single infusion (Newport et al., 2015) and 70.8% for repeated infusions (Murrough et al., 2013). Ketamine has also been shown to reduce suicidal ideation in suicidal patients (Diazgranados et al., 2010; Price et al., 2014), making it a strong candidate for use in emergency situations.

Unfortunately, there are limitations to using ketamine for depression treatment. For instance, ketamine's effects are transient, with high relapse rates in the weeks following treatment (Murrough et al., 2013; Rasmussen et al., 2013). Limited attempts to prolong its actions using serial dosing schedules have been inconsistent, and almost always lack a single-infusion group for adequate comparison. There are, however, some reported cases of its longer-term use in patients with severe TRD (Kwon et al., 2018). For example, a patient with TRD was given ketamine once every 1-2 weeks over a period of 10 months, for a total of 36 doses. The patient showed mood stabilization and a 50% reduction in symptom severity and the effects persisted for 4 weeks post treatment, however relapse did occur at 5 weeks (Kwon et al., 2018).

In spite of the issues surrounding the length of its clinically significant effects, the discovery of ketamine's rapid antidepressant effects, even in patients with TRD, has led to much enthusiasm. Nevertheless, there are also undeniable concerns with its use in the clinical setting, principally its dissociative properties and abuse potential. Therefore, there has been a considerable amount of inquiry into the mechanisms underlying its antidepressant actions in the name of developing pharmacological treatments that are safer and offer a more practical clinical profile.

1.3.2.2 Mechanisms of action

It is now believed that rapid changes in synaptic plasticity and function are likely involved in the fast-acting effects of ketamine (Duman et al., 2016). Synaptic plasticity is achieved through the expression of synaptic proteins, both in the soma and in the dendrites (Hoeffler and Klann, 2010). The mammalian target of rapamycin (mTOR) signaling pathway is associated with the synthesis of synaptic proteins, which are required for the formation, maturation and function of new spine synapses (Hoeffler and Klann, 2010). Ketamine has been found to rapidly activate mTOR signaling in the PFC by increasing levels of phosphorylated and activated forms of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB; more commonly called Akt) (Li et al., 2010; Miller et al., 2014). Ketamine-mediated mTOR activation was similarly found to be accompanied by increased levels of phosphorylated forms of eukaryotic initiation factor 4E binding protein (4E-BP1) and p70S6 kinase (p70S6K), which is required for synapse formation and maturation (Li et al., 2010; Li et al., 2011; Miller et al., 2014). Additionally, ketamine administration has been shown to increase levels of synaptic proteins [specifically

postsynaptic density protein 95 (PSD95), GluA1, and synapsin I], and pre-treatment with the mTOR inhibitor, rapamycin, abolished this effect (Li et al., 2010; Li et al., 2011). Moreover, mTOR inhibition also blocked the antidepressant effects of ketamine on depressive-like behavior in two established behavioral models of despair [the forced swim test (FST) and learned helplessness in response to inescapable stress] (Li et al., 2010), further supporting a role of mTOR activation in ketamine's effects.

The antagonism of NMDA receptors by ketamine is believed to induce mTOR and synaptogenesis through indirect pathways (Zanos and Gould, 2018), and many studies have highlighted the importance of glutamate transmission and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor potentiation in ketamine's antidepressant effects (Kavalali and Monteggia, 2015; Scheuing et al., 2015). Indeed, it has been reported that blocking AMPA receptors prevented the behavioral and molecular effects of ketamine that are believed to be associated with its antidepressant properties. For example, Maeng et al. (2008) found that the antidepressant-like behavioral effects of ketamine were attenuated by blockage of AMPA receptors with the AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfoamoylbenzo(f)-quinoxaline (NBQX). Furthermore, blocking AMPA receptors prevented the ketamine-induced activation of 4E-BP1, p70S6K and mTOR, as well as ERK and Akt in the PFC (Li et al., 2010).

NMDA receptor blockade is assumed to increase glutamate transmission and AMPA receptor potentiation by blocking the NMDA receptors on inhibitory GABAergic interneurons, leading to reduced inhibition (Homayoun and Moghaddam, 2007). In fact, it is thought that disinhibition of pyramidal cells, caused by decreased output of fast-spiking GABAergic interneurons, is responsible for the antidepressant effects of ketamine.

Windman and McMahon (2018) tested this hypothesis using electrophysiology in slices of dorsal hippocampus tissue, and found that low doses of ketamine did reduce the inhibitory input onto pyramidal cells, leading to increased pyramidal cell excitability, supporting the disinhibition hypothesis. For a simplified illustration of the proposed mechanisms underlying ketamine's antidepressant effects, see Figure 1.1.

The long-term goal of ketamine research, in the context of depression, is the creation of novel antidepressants that have the benefits of ketamine without the safety or practical concerns. Therefore, one of the aims of the current thesis is to find out more about how ketamine exerts its antidepressant effects. In particular, we are interested in how ketamine might influence some of the pathological mechanisms that are believed to underlie depression (namely monoamine neurotransmission, neurogenesis, inflammation, and HPA axis activity) within the context of stressor-based animal models of depression. We are also interested in the pathways that ketamine acts upon.

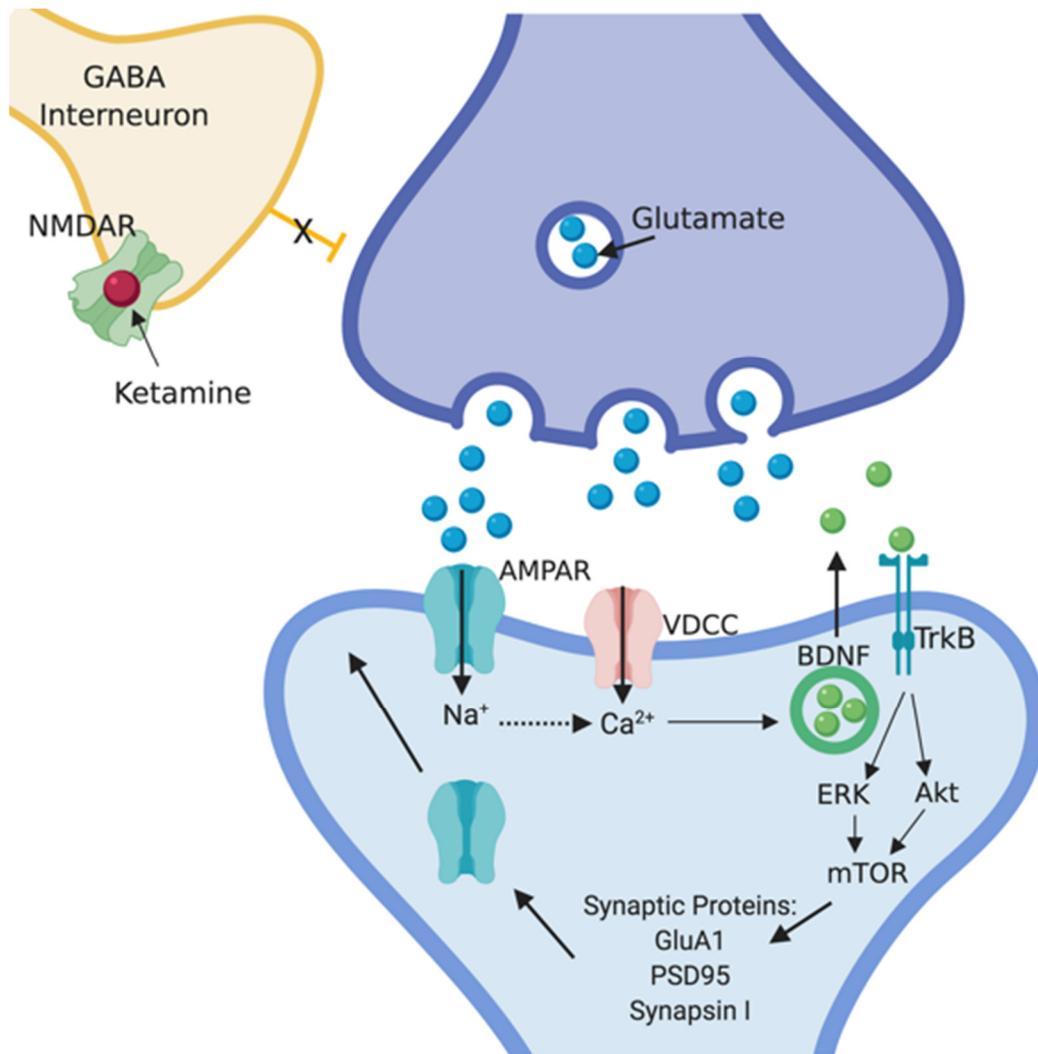


Figure 1.1. Simplified diagram of the proposed mechanisms underlying ketamine's antidepressant effects. Ketamine is believed to block NMDA receptors on tonic firing GABA interneurons, leading to disinhibition of excitatory pyramidal neurons and consequent glutamate transmission. The resulting burst of glutamate acts postsynaptically at AMPA receptors, which leads to Ca^{2+} influx via VDCCs and subsequently Ca^{2+} induced BDNF release. BDNF then stimulates TrkB receptors, thereby activating ERK and Akt, which activate the mTOR pathway, ultimately increasing synaptic protein synthesis and synaptogenesis. Figure created with BioRender.

1.4 Research objectives

Over the past two decades, since the first clinical trial was published, much has been realized about the usefulness of ketamine as a fast-acting and efficacious antidepressant. There has also been a great deal of investigation into the mechanisms underlying its antidepressant effects. However, at the time of the following animal studies, little was known about the effects of ketamine on corticosterone, central monoamine, and cytokine levels (corresponding to systems that have been implicated in the pathophysiology of depression) in the context of stressor exposure. We also did not know if repeated ketamine treatment could prolong its antidepressant-like effects in animal models, and many questions remained about the pathways involved.

Through a series of experiments presented in Chapters 2, 3, and 4, this thesis will test the following three hypotheses: 1. Ketamine treatment will modulate at least some of the stress-induced behavioral and neurochemical changes induced by both acute and chronic stressors, 2. Repeated ketamine treatment will prolong antidepressant-like effects, and 3. The JNK pathway is involved in the neurochemical and behavioral response to stressors, and ketamine may interact with this pathway.

Addressing our first hypothesis, in Chapter 2 we set out to determine how a single ketamine treatment would influence the behavioral and neurochemical consequences of two separate depression-relevant acute stressors: a restraint stress, and an immune challenge with the endotoxin, LPS. In Chapter 3, we also addressed the first hypothesis by examining the influence of repeated ketamine treatment on the behavioral and neurochemical outcomes of CUS. Here we also addressed the question of whether repeated ketamine treatment, compared to a single ketamine treatment, could prolong its

antidepressant-like effects in an animal model of antidepressant efficacy. Chapter 4 is where we introduce studies looking at the JNK pathway. Specifically, we were curious about its role in the pathophysiology of depression, and its possible involvement in the mechanisms underlying the response to antidepressant agents such as ketamine. As a result, in Chapter 4 we investigated the role of JNK in the behavioral and neurochemical response to a stressor model, and also assessed whether ketamine might be interacting with the JNK pathway. These experiments have paved the way for future studies to directly test the hypothesis that ketamine's antidepressant effects depend, at least in part, upon its interaction with the JNK pathway.

Chapter 2. Ketamine modulates pro-inflammatory cytokines, but not stressor-induced neurochemical changes

2.1 Abstract

Recently, considerable attention has focused on the rapid antidepressant effects observed in treatment-resistant patients that is produced by the NMDA receptor antagonist, ketamine. Surprisingly, the neurochemical and behavioral effects of ketamine in the context of stressor exposure are unclear. Thus, we assessed the impact of a single ketamine treatment together with acute (restraint or LPS) stressor exposure. Although acute restraint and LPS individually provoked the expected elevation of plasma corticosterone and brain-region specific monoamine variations, ketamine had no influence on corticosterone and had, at best, sparse effects on the monoamine changes. Ketamine did however dose-dependently reverse the LPS-induced elevation of the pro-inflammatory cytokines, IL-1 β and TNF- α . These data indicate that ketamine might be imparting antidepressant effects through inflammatory processes rather than the typical neurochemical/hormonal factors affected by stressors.

2.2 Introduction

Depression is a chronic, relapsing psychiatric condition estimated to affect over 15% percent of the population in developed countries (Kessler et al., 2003; Koo et al., 2009). Although currently available antidepressant medications are widely used, they are not without substantial limitations. Traditional pharmacological antidepressants are ineffective in 30-40% of depressed patients, leaving a large treatment-resistant population (Thase et al, 2005). Furthermore, even treatment responders often still display some degree

of symptomology and are at risk of relapse (Ghaemi, 2008). Another issue is that when effective, available treatments typically require weeks to months to provide relief (Trivedi et al., 2006). Such a long latency to therapeutic action is a major concern given the high risk of suicide in certain populations of depressed patients (Duman et al., 2012). Hence, it is of utmost importance to develop novel more efficacious and rapid treatment strategies. Such efforts should undoubtedly look beyond conventional monoamine-based drugs and begin to consider alternate mechanisms of depression pathology.

Emerging findings have recently indicated that ketamine, a non-competitive NMDA receptor antagonist widely used for its anesthetic properties, shows promise as a novel treatment for depression (Murrough, 2011). When given at a sub-anesthetic dose, a single ketamine injection has been reported to not only promote fast-acting antidepressant effects, often within hours (Berman et al., 2000; Zarate et al., 2006; Phelps et al., 2009), but also to be effective in historically treatment-resistant patients (Liebrenz et al, 2007; Machado-Vieira et al., 2009; Mathew et al, 2010). Furthermore, the antidepressant effects following a single dose have been reported to persist for several days (Liebrenz et al., 2007) to even weeks (Correll and Futter, 2006; Irwin and Iglewicz 2010; Mathew et al, 2010). Hence, ketamine is particularly unique in its clinical profile and of potentially enormous significance.

Given that stressor exposure is a leading risk factor in the development of depression (Fava and Kendler, 2000; Harro, 2012), it is important to evaluate the actions of ketamine in stressor-based models of the disorder. Indeed, recent studies have reported that ketamine reversed the behavioral and biochemical changes induced by CUS exposure (Garcia et al., 2009; Li et al., 2011). In addition to psychogenic stress, there is substantial

evidence that immune system activation (i.e. systemic stress) and subsequent cytokine release is involved in the development of depression (Dunn et al., 2005). Indeed, elevated cytokine levels have been reported in depressed patients, cytokines have been shown to promote activation of the HPA axis, and pro-inflammatory cytokines can induce depressive-like sickness behavior (Dunn et al., 2005; Zhang et al., 2014). Furthermore, a single dose of the bacterial endotoxin, LPS, can induce sickness behavior in animals that resembles those seen in depression (Hosseini et al., 2012; Zhang et al., 2014). Interestingly, ketamine was found to modulate cytokine levels in rodents (Takenaka et al., 1994; Taniguchi et al., 2001). Little is known, however, about its potential effects on inflammatory cytokine levels involved in the pathogenesis of mood disorders such as depression.

The data concerning ketamine are still rather sparse, and the nature of the brain-region specific monoamine changes that ketamine might induce in the context of various stressors are still unknown. It also remains to be determined whether ketamine might blunt neuroendocrine responses aligned with depressive-like behaviors. To this end, we evaluated the impact of a single ketamine exposure in mice that subsequently received an acute stressor in the form of either a psychogenic stressor (restraint) or an immune challenge (LPS) to elucidate whether ketamine could prevent the corticoid, central monoamine, immune, and behavioral changes induced by the stressor.

2.3 Methods

2.3.1 Animals

Male CD1 mice (8-10 weeks) were purchased from Charles River Laboratories (Laprairie, Quebec, Canada) and were acclimatized for 1 week before experimental procedures began. Animals were singly housed in standard polypropylene cages (27 × 21 × 14 cm), and maintained on a 12-hour light/dark cycle. Food and water were provided *ad libitum* and room temperature was maintained at 21 °C. All experiments were approved by the Carleton University Committee for Animal Care and adhered to the guidelines outlined by the Canadian Council for the Use and Care of Animals in Research.

2.3.2 Experiment 1.1: Ketamine treatment prior to an acute restraint stress

The aim of experiment 1.1 was to test our hypothesis that a single injection of ketamine, given prior to an acute psychogenic stressor, would modulate at least some of the effects of the stressor on behavioral and biochemical outcomes. To this end, mice were given an intraperitoneal (i.p.) injection of either saline or ketamine (5 mg/kg), and thirty minutes later, were further subdivided with one half being subjected to an acute restraint stress while the other half remained undisturbed in their home cage. The restraint stress, which itself lasted for 30 minutes, consisted of placing mice into a cone shaped plastic bag with an opening at the end to allow for breathing, and their tails were taped down to prevent excessive movement. Two cohorts were used, and five minutes after the stressor ended, one cohort was subjected to behavioral testing (open field/novel object exploration/social preference-avoidance, elevated plus maze) and the other cohort was rapidly decapitated

with their trunk blood and brain tissue collected for analysis of corticosterone and central monoamines. (See Figure 2.1; N = 40 per cohort; n = 10 per group).

2.3.2.1 Open field test, novel object exploration, and social preference-avoidance test

Open field test

Three separate behavioral tests were conducted in succession, all taking place within the open field arena. The open field test is used to assess, among other things, anxiety-like behavior, or “emotional reactivity” (Denenberg, 1969). When mammals encounter strange or noxious situations such as novel open spaces, they often freeze, and their exploratory behavior is reduced. The more anxious, or emotional, an animal is, the less they will explore this novel environment (Denenberg, 1969). Five minutes after the stressor exposure, mice were placed into a clear, Plexiglas open-field arena (50 × 50 cm), which was illuminated by ambient fluorescent ceiling lights. Mice were placed in the bottom left corner of the arena and allowed to freely explore the space for 5 minutes. All activity was tracked through a video camera that was mounted directly above the apparatus and was connected to tracking software (EthoVision, Noldus, Netherlands). For the open field test, the software scored latency to enter, frequency of entries and time spent in the center, as well as total distance traveled and time spent in corners.

Novel object exploration and social preference-avoidance test

In the novel object exploration portion of this test, modified from Litteljohn et al. (2010), the response of mice to a novel object in a familiar environment was assessed. After the open field test, mice were removed and returned to their home cages while an empty wire mesh cage was placed against one wall of the arena. After one minute mice were

placed back into the arena and allowed to freely explore the novel object for 2.5 minutes. For the social preference-avoidance test portion, described previously by Toth and Neumann (2013) and Berton et al. (2006), mice were again removed and a conspecific was placed into the wire mesh cage. After one minute in their home cages, mice were returned to the arena and allowed 2.5 minutes of free exploration. For the tests involving the wire mesh cage, latency to enter, frequency of entries, and time spent within an 8 cm-wide perimeter around the cage was scored (by the same tracking software used in the open field test).

2.3.2.2 Elevated plus maze test

The elevated plus maze test is also used to assess anxiety-like behavior based on the principle that rodents often find open spaces noxious. Immediately after the open field procedure, mice were subjected to an elevated plus maze test for 5 minutes. Specifically, they were placed into the center of a Plexiglas apparatus (30 cm high), which was composed of four arms (6 × 30 cm), two that were enclosed by walls, and two that were open. Time spent in and number of entries into each of the four arms was recorded (by an experimenter who was blind to the animal groups). To be scored as an entry into an arm, all four paws had to be placed into that arm.

2.3.2.3 Corticosterone analyses

Five minutes after stressor exposure, mice were rapidly decapitated and trunk blood was taken. Trunk blood, collected in tubes containing 10 µg of ethylenediaminetetraacetic acid (EDTA), was centrifuged at 3600 rpm for 8 minutes. Plasma was then collected and

immediately stored at -80°C until analyzed. Corticosterone levels were measured by a commercial radioimmunoassay (RIA) kit (ICN Biomedicals, CA, USA). Inter-assay variability was avoided by assaying all samples (in duplicate) within a single run.

2.3.2.4 Monoamine detection

Mice were rapidly decapitated and their brains were collected for monoamine detection. Brains were sliced into coronal sections using a chilled microdissecting block containing slots (0.5mm apart) for single-edged razor blades. The hypothalamus, PFC and hippocampus were collected bilaterally using razor blades and immediately placed on dry ice. The tissue was stored at -80 °C until analyzed using high-performance liquid chromatography (HPLC).

HPLC was performed as previously reported by Liu et al. (2014), to determine the levels of NE and 5-HT, and their metabolites, 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-HIAA. Briefly, brain tissue was homogenized in a solution of 0.3 M monochloroacetic acid, 0.1 mM NaEDTA, and 1% methanol in H₂O, and centrifuged at 10 000 rpm at 4°C. Supernatants were then injected at a flow rate of 1 ml/min into the HPLC system (Agilent 1100) with an electrochemical detector (DECADE SDC) and Eclipse XDB-C-8 (4.6–150 mm column). Each liter of the mobile phase used for the separation comprised 90 mM sodium phosphate monobasic, 1.1 mM 1-octane sulfuric acid, 50 mM EDTA, 10% acetonitrile, 50 mM citric acid, 5 mM potassium chloride, and HPLC-grade water. Determination of the area and height of the peaks was carried out with the aid of a Hewlett-Packard integrator. The protein concentration of each sample was determined

using a bicinchoninic acid assay (BCA) protein analysis kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkman, PC800 colorimeter).

2.3.3 Experiment 1.2: Ketamine treatment prior to LPS

The aim of this study was to test our hypothesis that a single ketamine injection would modulate the effects of a later immune challenge on sickness behavior, as well as corticosterone, central monoamine, and plasma cytokine levels. To this end, mice (n = 8/group) were given a single injection of either saline or ketamine (5 or 10 mg/kg, i.p.) 30 minutes prior to an injection of saline or the bacterial endotoxin, LPS (10 µg, i.p.). Mice were assessed for sickness behavior 2 hours after the LPS injection, and then rapidly decapitated with their trunk blood and brain tissue collected for analysis (See Figure 2.7).

2.3.3.1 Sickness behavior

The overall appearance of each animal was rated to assess the degree of sickness exhibited. Sickness measurements were scored on a four-point scale (0 = no symptom, 1 = one symptom present, 2 = two symptoms present, 3 = three or more symptoms) with respect to lethargy (diminished exploration and locomotion), curled body posture, ptosis (drooping eyelids), ragged fur, pilo erection, and overall nonresponsiveness. We previously observed that this procedure yielded better than 90% agreement between two raters who were blind to the treatment mice received.

2.3.3.2 Corticosterone analysis

Corticosterone analysis was conducted as in Experiment 1.1.

2.3.3.3 Monoamine detection

Monoamine detection was conducted as in Experiment 1.1.

2.3.3.4 Plasma cytokine analysis

Levels of circulating cytokines [granulocyte-macrophage colony stimulating factor (GM-CSF), IFN- γ , IL-10, IL-1 β , IL-6 and TNF- α] were determined by multiplex analysis using the Luminex 100 suspension-based bead array system (Luminex Corp., Austin, TX, USA) with a custom multiple cytokine detection kit (MILLIPLEX MAP Mouse Cytokine/Chemokine Kit, Millipore, Cat. #MPXMCYTO-70K). The assay was performed as previously reported by Gibb et al. (2011) according to the manufacturer's instructions (see www.millipore.com/userguides).

Briefly, a standard curve was generated in order to determine protein concentrations in plasma samples. Next, 25 μ l of prepared standards or controls was added to a pre-wet 96-well filter plate. For unknown wells, 25 μ l of assay buffer and 25 μ l of plasma sample were added to each well. The pre-mixed anti-mouse cytokine beads were vortexed and sonicated, and 25 μ l of the bead solution was added to each well. Plates were then incubated on a plate shaker overnight at 4°C. Following incubation samples were washed twice and removed by vacuum filtration. Subsequently, 25 μ l of detection antibody beads were added to each well and incubated, again on a plate shaker, for 90 minutes at room temperature. This was followed by 25 μ l of streptavidin-PE being added to each well containing the detection antibodies and samples, and this mixture was again incubated with agitation for 30 minutes. Finally, 25 μ l of Beadlyte stop solution was added and samples were then resuspended in sheath fluid. The filter plate was analyzed using the Luminex

100 instrument and the data fitted with a five-parameter logistic regression curve using Analyst software (Millipore). In cases where cytokine levels were too low to be detected, samples were assigned a value of one-half the lower limit of quantification.

2.3.4 Statistical analysis

Data were analyzed by analysis of variance (ANOVA). For ANOVAs exhibiting a significant interaction, post hoc analyses using Fisher's protected least significant difference (Fisher's PLSD) were conducted to determine the exact nature of the relationship. Data were evaluated using a StatView (version 6.0) statistical software package available from the SAS Institute, Inc.

2.4 Results

2.4.1 Experiment 1.1: Ketamine treatment prior to an acute restraint stress

2.4.1.1 Open field /novel object/social avoidance testing

Open field

The open field test revealed a significant stress x ketamine interaction ($F_{1,35} = 4.14$, $p < 0.05$) for the number of entries into the center square of the arena. As shown in Figure 2.2A, post hoc analysis indicated that the combined stress and ketamine treatments resulted in significantly fewer entries into the center square than in any other group ($p < 0.05$). Furthermore, a stress x ketamine interaction was also apparent with respect to the time spent in the four corners of the arena ($F_{1,35} = 11.51$, $p < 0.05$; Figure 2.2B). Post hoc analysis again revealed that the stress and ketamine effects were limited to the group that received the combined treatment, such that this group displayed increased time spent in the

corners when compared to all other groups ($p < 0.05$). There were no effects of either stress or ketamine treatment on total distance moved ($p > 0.05$; data not shown).

Novel object exploration

When mice were placed into an open field arena in the presence of an empty wire mesh cage, a significant main effect of stress was found for the duration of time spent in a predetermined perimeter (the interaction zone) around the cage ($F_{1, 35} = 8.07, p < 0.01$; Figure 2.3A). Specifically, mice that were exposed to the acute restraint stress spent less time around the cage than the controls ($p < 0.01$). There was no effect of ketamine observed ($p > 0.05$).

Social preference-avoidance test

In a measure of social preference-avoidance behavior, there was a significant effect of stress found for the duration of time spent in the interaction zone surrounding the wire mesh cage when it contained another mouse ($F_{1, 35} = 6.96; p < 0.05$; data not shown), such that the time spent in the zone was decreased in the stressed mice ($p < 0.05$). In addition, when the time spent in the interaction zone in the presence of the target mouse was compared to the time spent in the perimeter when the cage was empty, there was another significant effect of stress ($F_{1, 35} = 6.96; p < 0.05$; Figure 2.3B). Specifically, the stressed mice spent even less time in the interaction zone when there was a mouse in the cage, than they did when the cage was empty ($p < 0.05$).

2.4.1.2 Elevated plus maze

The elevated plus maze was used as an additional measure of anxiety-like behavior and locomotor activity. There was a significant effect of ketamine on duration in the open arms ($F_{1,34} = 6.38, p < 0.05$; Figure 2.4A), which resulted in the ketamine-treated mice spending less time in the open arms than the controls. The ketamine-treated mice also had fewer entries into the open arms compared to all arm entries than did the saline-treated mice ($F_{1,34} = 7.31, p < 0.05$; Figure 2.4B). The number of entries and time spent in the closed arms was also examined and while there was no effect of either treatment on time spent in the closed arms ($p > 0.05$; data not shown), there was a modest but significant increase in the number of entries into the closed arms compared to all arm entries made by the ketamine-treated mice ($F_{1,34} = 7.31; p < 0.05$; Figure 2.4C). Finally, there were no effects of either treatment on the total number of arm entries ($p > 0.05$; Figure 2.4D).

2.4.1.3 Corticosterone analysis

The restraint stressor significantly increased plasma corticosterone levels relative to non-stressed animals ($F_{1,28} = 104.53, p < 0.001$; Figure 2.5). There was no influence of ketamine administration on corticosterone levels ($p > 0.05$).

2.4.1.4 Monoamine detection

Although there were no significant interaction effects, hypothalamic levels of 5-HT were increased by the stressor ($F_{1,27} = 12.86, p < 0.05$; data not shown), whereas ketamine treatment was without effect. In contrast, there were no significant differences in hypothalamic NE, MHPG or 5-HIAA concentrations among the groups.

Within the PFC, a significant stress x ketamine interaction was present with regard to 5-HT levels within the PFC ($F_{1,26} = 4.70, p < 0.05$). As shown in Figure 2.6A, the restraint stress decreased 5-HT levels ($p < 0.05$) but this effect was attenuated by ketamine treatment ($p < 0.05$). Furthermore, 5-HIAA levels were reduced by ketamine treatment ($F_{1,26} = 5.63, p < 0.05$). Finally, PFC MHPG was increased by the stressor ($F_{1,27} = 7.89, p < 0.01$; Figure 2.6A), in the absence of any effect of ketamine or any effects on NE.

In the absence of interaction effects, significant main effects for the stressor and ketamine treatments were reported within the hippocampus, with both treatments reducing NE levels ($F_{S1,26} = 5.47$ and 5.46 respectively, $p < 0.05$) along with promoting elevations of hippocampal 5-HIAA levels ($F_{S1,27} = 7.76$ and 9.07 respectively, $p < 0.01$; Figure 2.6B). Finally, levels of 5-HT were increased by the stressor ($F_{1,26} = 5.43, p < 0.05$; Figure 2.6B) whereas no significant variations in hippocampal MHPG were observed.

2.4.2 Experiment 1.2: Ketamine treatment prior to LPS

2.4.2.1 Sickness behavior

The effects of a single ketamine injection, at either a high (10mg/kg) or low (5mg/kg) dose, on the sickness behavior induced by LPS were assessed. A significant main effect was observed with regards to the impact of LPS on sickness symptom presentation ($F_{1,42} = 111.79, p < 0.001$; Figure 2.8). The follow up comparisons confirmed that LPS markedly promoted sickness behavior ($p < 0.05$), with average composite sickness score among the non-LPS-treated (saline alone, or ketamine alone at 5 or 10 mg/kg) mice being 1.1 ± 0.07 , while that of the LPS-treated (LPS alone or LPS with the either of the two ketamine doses) mice was 2.43 ± 0.13 . However, there was no significant main effect for

ketamine nor was there any significant interaction with LPS. In fact, the ketamine-treated mice had scores virtually identical to those that received saline.

2.4.2.2 Corticosterone analysis

LPS treatment provoked a large elevation in plasma corticosterone ($F_{1,42} = 68.81$, $p < 0.001$; Figure 2.9). However, no effect of ketamine administration at either of the doses was observed.

2.4.2.3 Monoamine detection

Although no differences were evident for hypothalamic NE, LPS and ketamine both increased hypothalamic levels of the NE metabolite, MHPG, within the hypothalamus ($F_{1,40} = 8.73$, $p < 0.01$ and $F_{2,40} = 3.46$, $p < 0.05$, respectively; data not shown). In contrast, in the absence of any LPS effects or changes in 5-HT, both ketamine doses significantly reduced hypothalamic levels of 5-HIAA ($F_{S2,40} = 3.71$, $p < 0.05$; data not shown).

Within the PFC, a significant main effect was apparent for LPS with respect to levels of MHPG ($F_{1,42} = 12.38$) but not NE. As shown in Figure 2.10A, LPS increased MHPG accumulation ($p < 0.01$), whereas ketamine had no effect. No significant variations in PFC 5-HT or 5-HIAA were apparent for either of the treatments.

Finally, within the hippocampus, LPS reduced NE while it increased levels of the metabolite, MHPG ($F_{1,42} = 7.73$, $p < 0.01$ and $F_{1,42} = 6.76$, $p < 0.05$, respectively; Figure 2.10B). However, hippocampal 5-HT levels varied as a function of an LPS x ketamine interaction ($F_{2,42} = 3.34$, $p < 0.05$). As shown in Figure 2.10B, ketamine attenuated the LPS induced elevation of 5-HT ($p < 0.01$). Similarly, 5-HIAA levels were significantly

increased by LPS ($F_{1,42} = 14.38, p < 0.001$; Figure 2.10B), though ketamine had no effect in this case.

2.4.2.4 Plasma cytokine analysis

The effects of ketamine on LPS-induced cytokine changes were assessed for the following cytokines: GM-CSF, IFN- γ , IL-10, IL-1 β , IL-6 and TNF- α . Levels of IFN- γ were generally below detection, however, LPS significantly increased circulating levels of GM-CSF, IL-10 and IL-6 ($F_{1,44} = 34.03, F_{1,42} = 45.07$ and $F_{1,43} = 229.46$, respectively, $p < 0.05$; data not shown), whereas ketamine was without effect. However, a significant LPS x ketamine interaction was apparent for IL-1 β and TNF- α ($F_{2,43} = 3.26$ and $F_{2,41} = 5.00$ respectively, $p < 0.05$). As shown in Figure 2.11A, high dose (10 mg/kg) ketamine prevented the LPS-induced elevation in IL-1 β . Similarly, ketamine dose-dependently attenuated LPS-induced elevations of TNF- α ($p < 0.05$; Figure 2.11B).

2.5 Discussion

Current antidepressant treatments are seriously limited, with remission rates usually only achieved after prolonged treatment, often with multiple different drug trials, and 30-40% of individuals not responding at all (Trivedi et al., 2006; Berlim and Turecki, 2007). The failure of artificial reductions in monoamine levels to produce depressive symptoms (Berton and Nestler, 2006; Ruhe et al., 2007), coupled with studies showing that antidepressants modulate glutamatergic imbalances in depressed patients (Prikhozhan et

al., 1990; Mauri et al., 1998), have contributed to the shift in focus towards non-monoaminergic mechanisms of depression. In this regard, ketamine has emerged as a specific NMDA receptor antagonist with very rapid and lasting antidepressant consequences (Berman et al., 2000; Zarate et al., 2006), even in previously treatment-resistant patients (Zarate et al., 2006; Krystal, 2007; Murrough et al., 2013).

Given that depressed individuals often display heightened stressor sensitivity and/or elevated basal neurochemical or HPA axis activity (Parker et al., 2003), it was of interest to assess whether ketamine might reduce basic biological stressor responses. In this regard, we found that both an acute restraint stressor and a single challenge with the bacterial endotoxin, LPS, markedly elevated plasma corticosterone levels, but neither of these elevations were affected by ketamine treatment. Similarly, the various stressor induced brain-region specific monoamine changes were generally unaffected by ketamine treatment; or certainly at the very least, there was no apparent pattern of ketamine effects. Thus, ketamine was clearly not affecting the immediate hormonal/neurochemical response induced by two different categories of stressors, and hence, its relatively rapid antidepressant-like effects do not appear to stem from any changes to acute stress reactivity. However, a caveat of these analyses is the fact that they represent a single static time point obtained from post-mortem tissue, and further studies should investigate other time points.

The behavioral data suggest that both ketamine and the restraint stress had anxiogenic-like effects, although how they were manifest varied as a function of the specific tests. Given that restraint stress has been reported to induce non-social (Hartmann et al., 2019) and social (Gehlert et al., 2005; Doremus-Fitzwater et al., 2009) anxiety-like

behavior, it was not surprising that it reduced exploration time in both the novel object exploration and the social preference-avoidance tests. Both of these tests are considered to be models of anxiety (Belzung and Le Pape, 1994; Toth and Neumann, 2013), and exploration time is typically increased by anxiolytic (i.e. benzodiazepines) and antidepressant (i.e. SSRIs) medications (Crawley, 1985; File, 1985; Griebel et al., 1993; Razzoli et al., 2011). The fact that ketamine did not modify the stress-induced reduction of exploration time in either test suggests that it does not possess anxiolytic properties, at least under the conditions of the present investigation.

Interestingly, while ketamine did not alter behavior in the aforementioned tests, the reduction of open arm entries displayed by ketamine-treated mice in the elevated plus maze actually suggests an anxiogenic effect of the drug. Moreover, when we analyzed the results from the open field test, we found that the combination of acute stress and ketamine together reduced the number of entries into the center of the arena, and increased the time that was spent in the corners, providing more evidence of an anxiogenic effect of ketamine. Other reports of ketamine's effects on anxiety-like behavior in rodents are sparse, and the results have been mixed, with some reporting anxiogenic effects (Baber et al., 2001; da Silva et al., 2010) and others reporting anxiolytic effects (Silvestre et al., 2002). Nevertheless, our findings are in line with reports that list anxiety as one of the most common side effects of ketamine treatment for depression (Short et al., 2017; Aust 2019). Considering that the human literature suggests this side effect is transient, lasting only a couple of hours (Bobo et al., 2016), it could be inferred that the effect would also be transient in mice, although testing behavior at alternate time-points would be necessary for

confirmation. This is an important factor, since inducing anxiety in depressed patients would clearly have unwanted consequences.

Beyond the neurochemical and behavioral changes, it has become increasingly clear that inflammatory processes are relevant for depression, and immunological or systemic stressors (much like psychogenic stressors) might promote the illness (Dantzer and Kelley, 2007; Sperner-Unterweger et al., 2014). We presently chose an LPS regimen consistent with our previous work (Hayley et al., 2001; Gibb et al., 2008) in order to assess whether ketamine would modulate the well known immediate sickness, neurochemical and inflammatory effects of this systemic stressor. As expected LPS-induced marked sickness symptoms, including curled body posture, lethargy, ptosis and piloerection, however, ketamine had no effect whatsoever on any of these symptoms.

Yet, one of the most striking findings of the present study was that ketamine dose-dependently antagonized the LPS induced rise of IL-1 β and TNF- α . This is not totally unprecedented (Chang et al., 2009; Ward et al., 2011), but the current study is the first to illustrate ketamine's highly selective actions, as it had no effect on the LPS induced elevations of IL-6, IL-10, IFN- γ or GM-CSF. This might be particularly important given that IL-1 β and TNF- α act primarily through NF- κ B signaling [which has been linked to depressive outcomes (Koo et al., 2010)], whereas the other cytokines are more aligned with the JAK-STAT signaling pathway (Mercurio and Manning, 1999; Kiu and Nicholson, 2012; Hayley et al., 2013; Lichtblau et al., 2013). Indeed, there is some evidence that ketamine can suppress endotoxin-induced NF- κ B activity (Sun et al., 2004; Yu et al., 2006).

Recent data have also raised the possibility that cytokines might be used as endogenous biomarkers of antidepressant efficacy, with one study showing that baseline levels of IL-6 but not TNF- α were predictive of who would be responders vs. non-responders to ketamine treatment (Yang et al., 2015). Furthermore, a recent multiplex study looking at 66 different factors (including over a dozen cytokines, numerous stress factors and peptides) found that ketamine, in the absence of an inflammatory stimulus, selectively altered TNF- α , fibroblast growth factor-9, and the anti-inflammatory cytokine, IL-4 (Wesseling et al., 2015). Hence, it appears that ketamine can alter the basal inflammatory cytokine milieu, as well as modify cytokine signaling in the face of an immune stressor (e.g. LPS). It remains to be determined whether specific cytokine profiles might offer predictive potential for gauging antidepressant response efficacy and disease trajectory.

2.6 Conclusions

In summary, ketamine had selective antagonistic effects for LPS induced IL-1 β and TNF- α , suggesting its potential importance in depressed individuals with altered pro-inflammatory cytokines (Hayley et al., 2013). However, the fact that ketamine did not affect LPS-induced sickness or other cytokines, indicates that ketamine's anti-inflammatory effects are highly selective and the drug is not influencing sickness symptom clusters that have previously been suggested to reflect neurovegetative features of depression (Dantzer and Kelley, 2007). One caveat here is that we did not test mice at a later time (24 h) when a recent study reported LPS-induced anhedonia, which was modified

by ketamine treatment (Walker et al., 2013). Intriguingly, ketamine did not appreciably influence the acute corticoid and central monoamine effects of a typical laboratory stressor (restraint) or an immunogenic stressor (LPS). Our data therefore indicates that ketamine is not likely exerting any effects through acute stress reactivity, and may be imparting antidepressant effects, at least in part, through inflammatory processes.

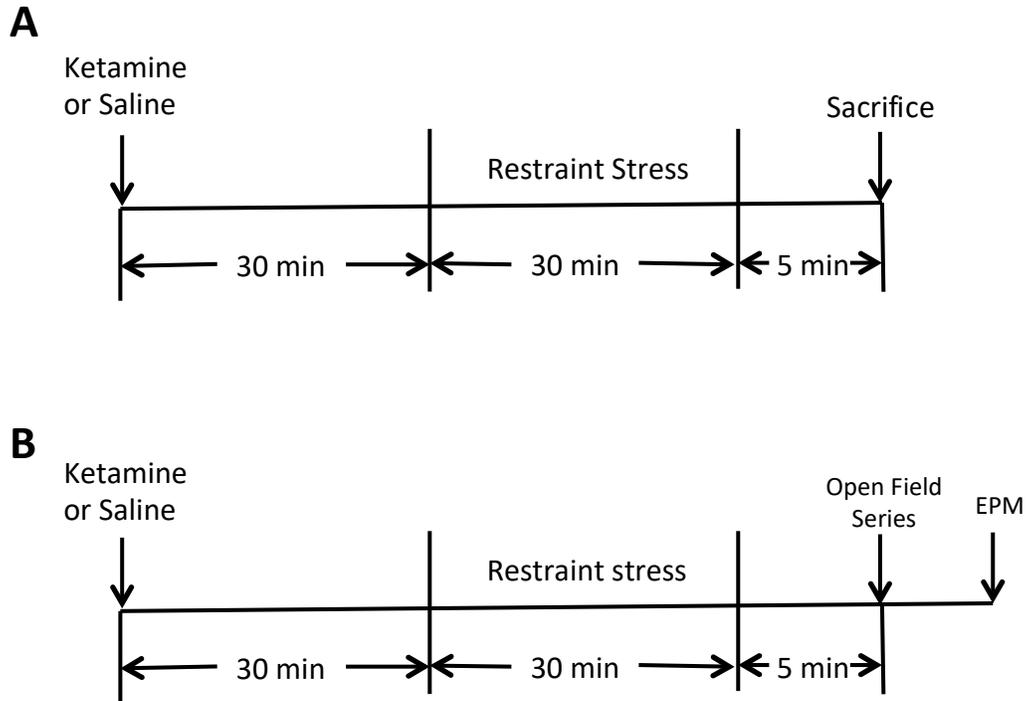


Figure 2.1. Timeline for Experiment 1.1. Mice were treated with ketamine (5 mg/kg, i.p.) or saline 30 minutes prior to an acute restraint stress. Five minutes after stressor exposure mice in cohort one (**A**) were rapidly decapitated with their trunk blood and brain tissue collected for analysis, and mice in cohort two (**B**) underwent behavioral testing. Open field series = open field test, novel object exploration, social preference-avoidance; EPM = elevated plus maze.

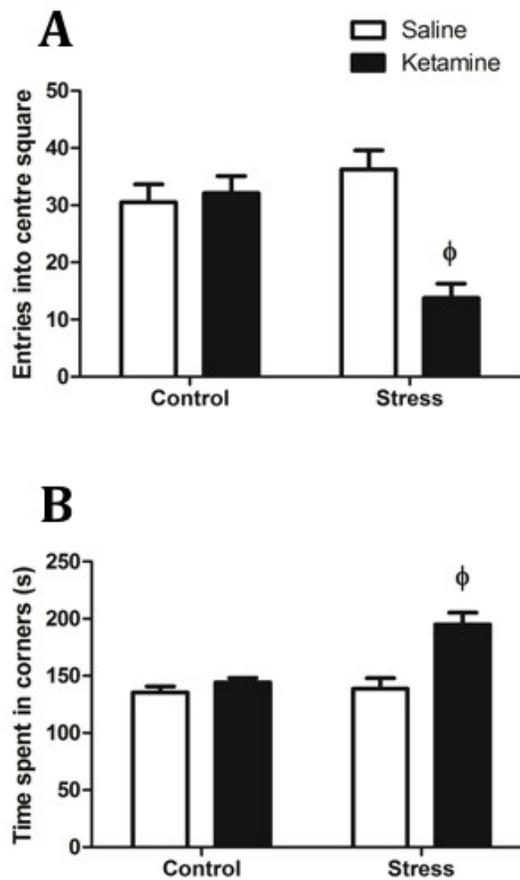


Figure 2.2. Effects of a single ketamine injection and restraint stress on open field performance. **A.** Ketamine-treated mice that were also exposed to an acute restraint stress displayed fewer entries into the center of an open field arena compared to all other groups. **B.** The stress plus ketamine treatment also resulted in more time spent in the corners with respect to all other treatment groups. Data expressed as means \pm SEM. ϕ $p < 0.05$, relative to all other groups.

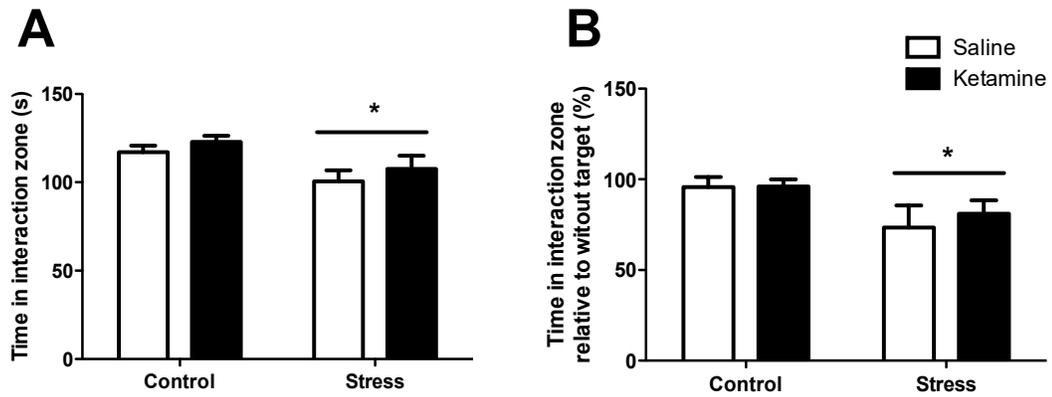


Figure 2.3. Effects of a single ketamine injection and restraint stress on novel exploration and social preference-avoidance. **A.** Mice that were subjected to the restraint stress spent less time in the interaction zone around a novel, empty cage. **B.** When mice were exposed to a cage containing another mouse, the stressed groups spent significantly less time in the interaction zone compared to when the cage was empty, than the control groups did. Data expressed as means \pm SEM. * $p < 0.05$, relative to non-stressed control mice.

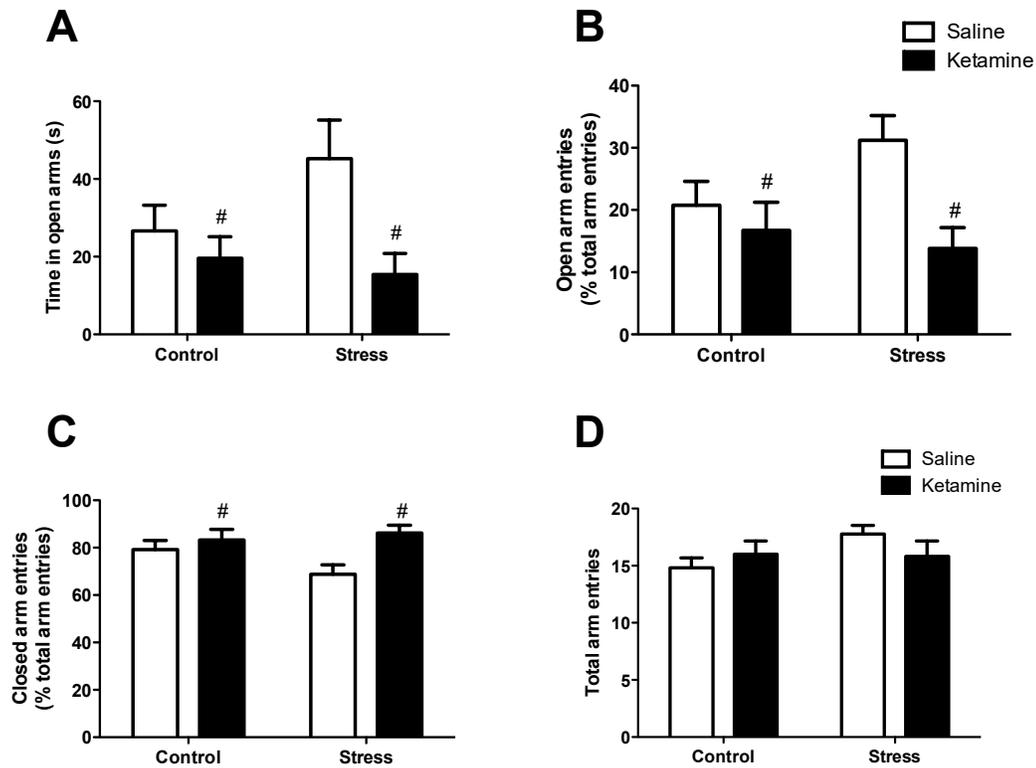


Figure 2.4. Effects of ketamine and restraint stress on elevated plus maze behavior. **A.** Mice that were treated with ketamine spent less time in the open arms of the plus maze compared to saline-treated mice. **B.** Ketamine-treated mice also had fewer entries into the open arms compared to all arm entries. **C.** The number of entries into the closed arms compared to all arm entries was modestly but significantly greater in the ketamine-treated mice. **D.** There were no effects of either treatment on total arm entries. Data expressed as means \pm SEM. # $p < 0.05$, relative to saline-treated mice.

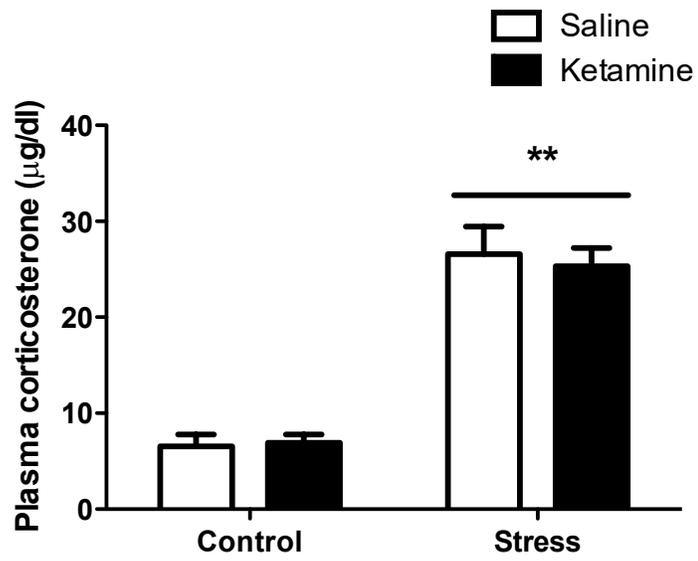


Figure 2.5. Effect of a single ketamine injection (5 mg/kg) and restraint stress on plasma corticosterone levels. Acute ketamine treatment did not influence the restraint stress-induced increase in levels of plasma corticosterone. Data expressed as means \pm SEM. ** $p < 0.001$, relative to non-stressed mice.

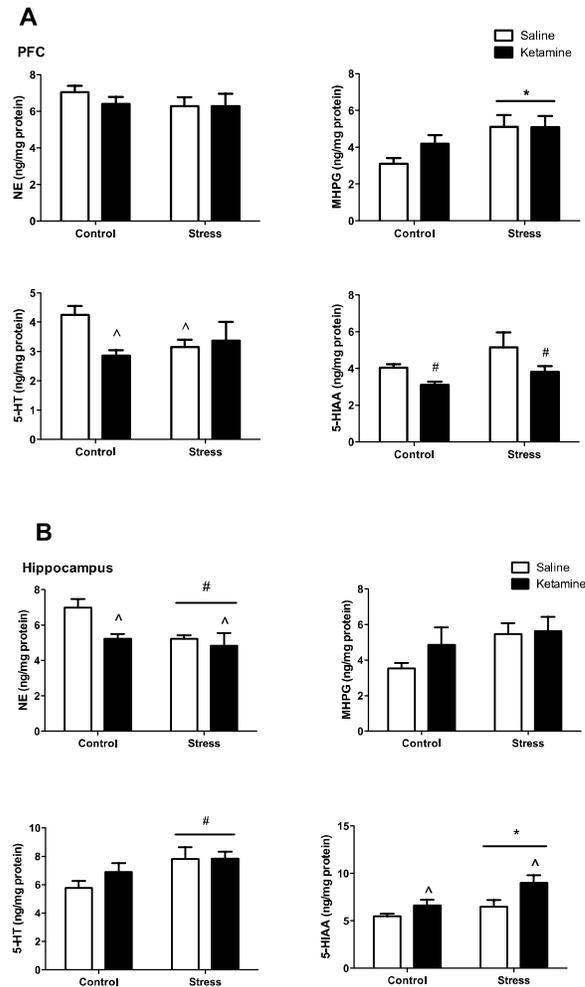


Figure 2.6. Effects of a single ketamine injection (5 mg/kg) and restraint stress on monoamine levels. **A.** Ketamine treatment prevented the stress-induced decrease in PFC 5-HT while it reduced levels of its metabolite, 5-HIAA. Restraint stress increased levels of MHPG. * $p < 0.01$, relative to non-stressed controls; ^ $p < 0.05$, relative to saline-treated controls; # $p < 0.05$, relative to saline-treated mice. **B.** Hippocampal levels of NE were reduced by both ketamine and the restraint stress, while levels of the 5-HT metabolite, 5-HIAA, were increased by both treatments. Restraint stress increased hippocampal 5-HT. # $p < 0.05$, relative to non-stressed controls; ^ $p < 0.05$, relative to saline-treated controls; * $p < 0.01$, relative to non-stressed controls. Data expressed as means \pm SEM.

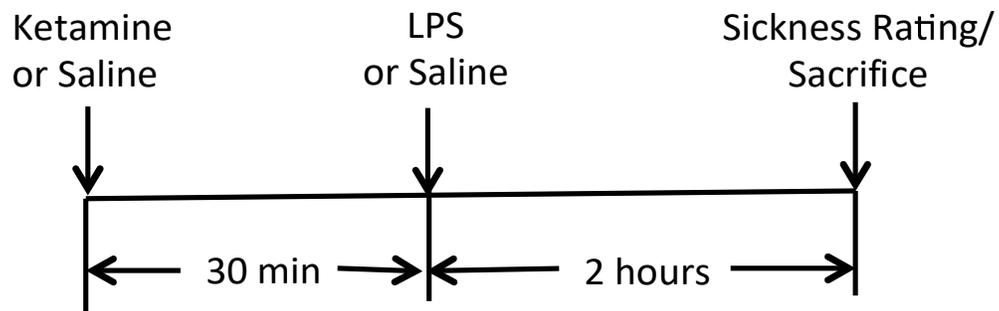


Figure 2.7. Timeline for Experiment 1.2. Mice were injected with ketamine (5 mg/kg or 10 mg/kg, i.p.) or saline 30 minutes prior to an acute LPS treatment (10 μ g, i.p.). Two hours later mice were rated for sickness behavior and subsequently decapitated with their trunk blood and brain tissue collected for analysis.

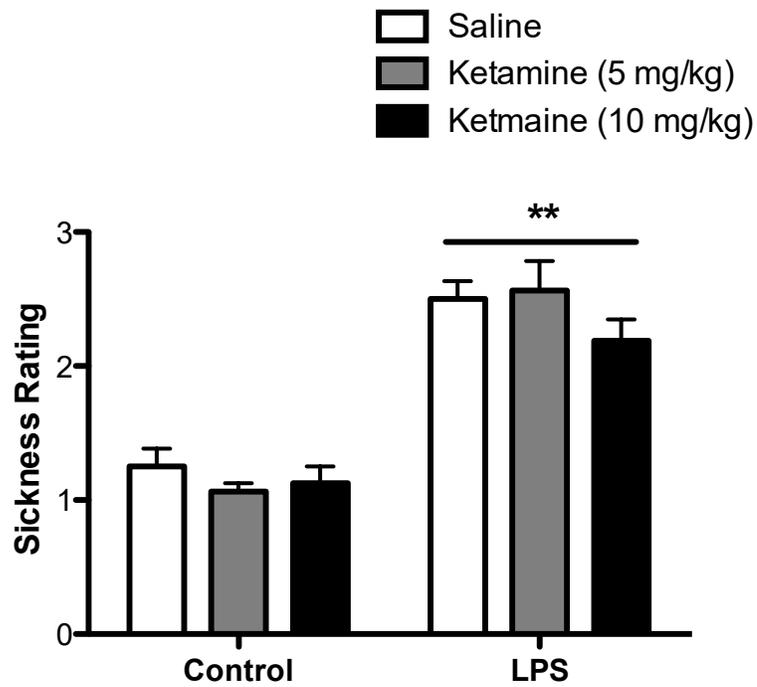


Figure 2.8. Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 μ g, i.p.) on sickness rating. Acute ketamine treatment did not influence the LPS-induced increase in sickness rating score. Data expressed as means \pm SEM. ** $p < 0.001$, relative to mice that did not receive LPS.

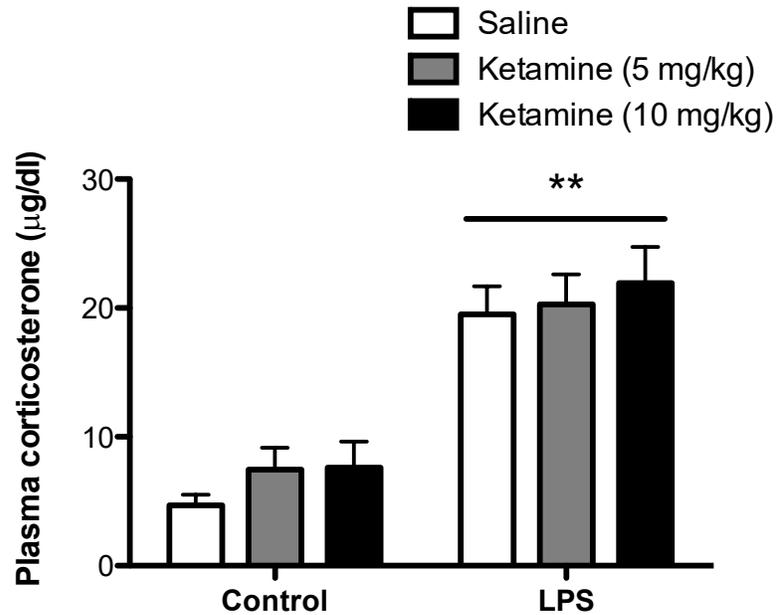


Figure 2.9. Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on plasma corticosterone. Acute ketamine treatment did not influence the LPS-induced increase in levels of plasma corticosterone. Data expressed as means \pm SEM. ** $p < 0.001$, relative to mice that did not receive LPS.

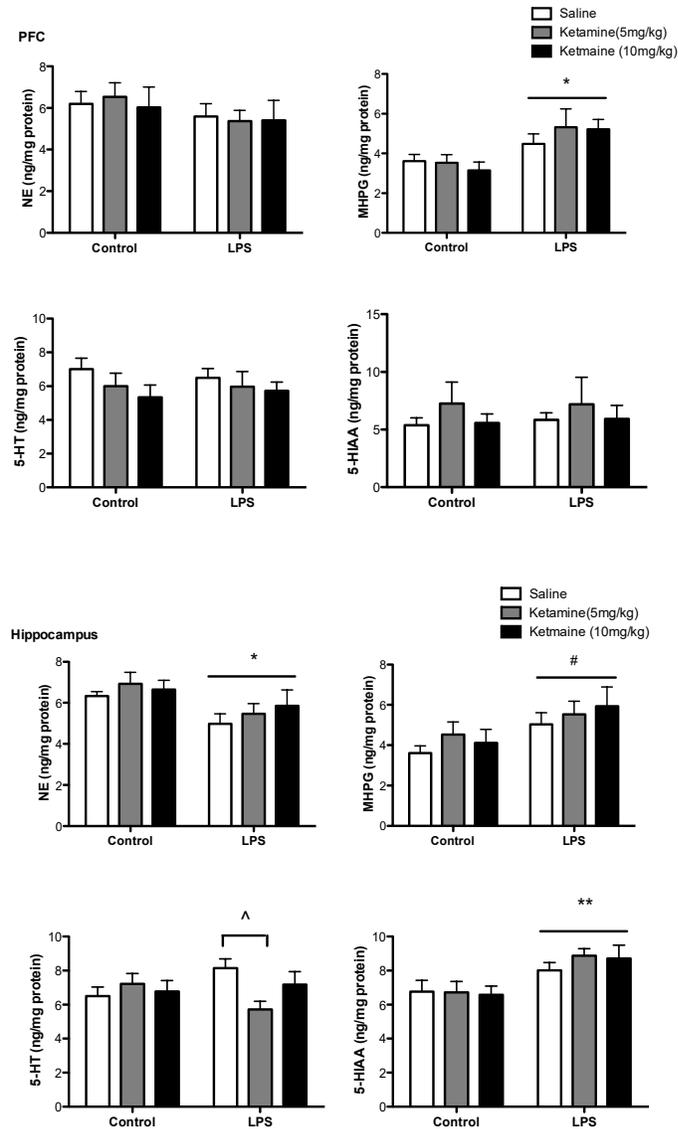


Figure 2.10. Effects of a single injection of ketamine (5 or 10mg/kg, i.p.) and LPS treatment (10 µg, i.p.) on monoamine levels. A. LPS increased levels of the NE metabolite, MHPG, within the PFC while ketamine was without effect. **B.** Ketamine attenuated the LPS-induced increase in hippocampal 5-HT, but did not influence the LPS-induced increase in NE, MHPG or 5-HIAA. Data expressed as means \pm SEM. \wedge $p < 0.01$, mice treated with LPS and low-dose ketamine relative to LPS only group; # $p < 0.05$, relative to mice that did not receive LPS; * $p < 0.01$, relative to mice that did not receive LPS; ** $p < 0.001$, relative to mice that did not receive LPS.

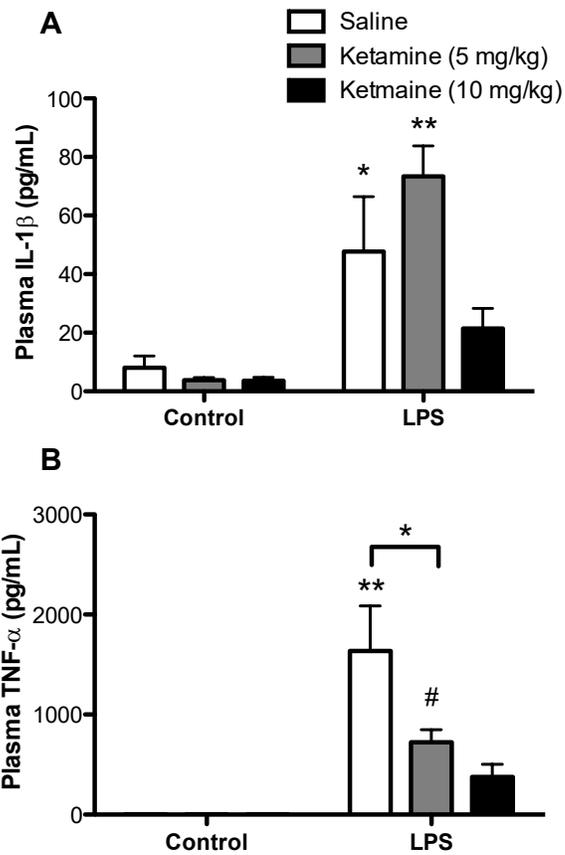


Figure 2.11. Effects of a single injection of ketamine (5 or 10 mg/kg, i.p.) and LPS treatment (10 μ g, i.p.) on plasma cytokines. A. The high dose of ketamine prevented the LPS-induced increase in IL-1 β . * $p < 0.01$, ** $p < 0.001$, both relative to non-LPS treated controls. **B.** Ketamine dose-dependently prevented the LPS-induced increase in TNF- α . # $p < 0.05$, relative to non-LPS treated controls; ** $p < 0.001$, relative to non-LPS treated controls; * $p < 0.01$, relative to LPS only. Data expressed as means \pm SEM.

Chapter 3. Ketamine increases hippocampal neurogenesis, but does not influence stressor-induced neurochemical changes.

3.1 Abstract

Treatment resistance, and the long effect latency associated with currently used antidepressants, are significant issues in the management of depression. There is therefore an urgent need for more efficacious and faster-acting medications. In this regard, ketamine has garnered a lot of attention over the past decade, however, the mechanisms through which ketamine acts are not well understood, nor are the outcomes of its chronic use. To this end, we assessed the effects of repeated ketamine treatment together with chronic unpredictable stressor exposure. Remarkably, the antidepressant-like effect of ketamine lasted for 8 days following repeated exposure to the drug. In the elevated plus maze and open field tests, repeated ketamine did not modify the CUS-induced changes, however, it did independently reduce the number of entries into the center of the open field arena, suggesting an anxiogenic-like effect in this test. While ketamine had only modest effects on central monoamine levels, it did increase adult hippocampal neurogenesis. The data presented here indicate that repeated ketamine administration may have more durable behavioral consequences than acute treatment alone, and ketamine might be imparting antidepressant effects, at least in part, through its effects on neuroplasticity.

3.2 Introduction

Depression is one of the most prevalent psychiatric disorders, estimated to affect over 300 million people worldwide (Kessler, 2007; WHO, 2018). A leading cause of disability, depression contributes greatly to the global burden of disease and is therefore a

major health concern (Reddy, 2010; WHO, 2018). Currently, monoaminergic-based medications, such as SSRIs, are recommended as first-line treatment by international guidelines (Bauer et al., 2007; Fogaca and Duman 2019). Despite the fact that these medications are widely available and frequently prescribed, they are limited in efficacy, relieving depressive symptoms in only 60-70% of patients (Thase et al., 2005; Trivedi et al., 2006). Additionally, patients often wait weeks, and in some cases months, before antidepressant effects are achieved (Derivan, 1995; Trivedi et al., 2006). Given the high risk of suicidality among depressed patients (Duman et al., 2012) and the personal suffering that depression induces, there is clearly an urgent need to discover novel pharmaceutical interventions that address the limitations of currently used antidepressants.

Ketamine, an NMDA receptor antagonist commonly used as an anesthetic and pain reliever, has recently been reported to have antidepressant properties (Berman et al., 2000; Zarate et al., 2006; Murrough, 2011). Importantly, it appears to address the two major limitations of traditional monoaminergic-based antidepressants. When given at a sub-anesthetic dose, a single infusion of ketamine produces antidepressant effects within hours (Berman et al., 2000; Liebrenz et al., 2007). Furthermore, it has been reported to be effective in a large percentage of treatment-resistant patients (Zarate et al., 2006; Machado-Vieira et al., 2009). While these findings are exciting, ketamine unfortunately has its own issues, such as abuse potential and undesirable dissociative side effects (Murrough et al., 2013b; Schatzberg, 2014). Learning exactly how ketamine is imparting antidepressant effects could lead to novel, more specific targets in the future, and away from the unwanted consequences of ketamine.

At this time, much is still unknown regarding the mechanisms underlying ketamine's antidepressant effects, and how they might be influenced by different dosing regimens. Moreover, relatively little is known about the consequences of repeated ketamine treatment, leaving many questions unanswered. For example, a single ketamine treatment typically relieves depressive symptoms for up to one week (Berman et al., 2000; Zarate et al., 2006), and although there is some evidence that repeated ketamine treatment may prolong its effects (Correll and Furrer, 2006; Aan Het Rot et al., 2010), the data supporting this finding are still quite limited. Currently, ketamine treatment for depression is done via infusion in a hospital setting, which is expensive and inconvenient, making prolonged intervals between treatments very desirable (Aan Het Rot et al., 2012).

Another important question is whether repeated ketamine treatment might influence hippocampal neurogenesis. Neurogenesis and neuroplasticity are frequently compromised in depression, with depressed patients displaying alterations such as reduced neurogenesis in the DG of the hippocampus, and atrophy in the PFC (Gould et al., 1997; Rajkowska et al., 1999; Perera et al., 2008; Pittenger and Duman, 2008; Tang et al., 2016). Chronic stress is considered the most important clinical risk factor for depression (Park, 2019) and is believed to be a key player in the aforementioned changes (Karrel, 1997; Czeh et al., 2001; Coe et al., 2003). Notably, virtually all monoamine-based antidepressants increase neurogenesis and synaptic plasticity and protect against stress-induced reductions in BDNF (Sheline et al., 2003; Czeh et al., 2007). There is mounting evidence that ketamine promotes synaptic plasticity in the PFC via the mTOR pathway, and that these changes contribute to its antidepressant actions (Li et al., 2011; Duman et al., 2012).

However, to the best of our knowledge there have been no reports of ketamine's influence on hippocampal neurogenesis in the context of depression-related pathology.

We previously found that a single injection of ketamine did not alter the neurochemical response to acute stressors (see Chapter 2), however, it remained to be seen if the neurochemical changes induced by CUS could be modulated by *repeated* ketamine treatment. Using the CUS model, we assessed the consequences of repeated ketamine administration in mice with the following two main objectives in mind: 1) to evaluate the ability of repeated ketamine treatment to modulate stress-induced changes in corticosterone levels, central monoamine levels, hippocampal neurogenesis, and a number of stress-sensitive behavioral tests, and 2) to determine if repeated ketamine treatment can prolong the antidepressant-like effects of ketamine in the FST, over those resulting from a single dose of ketamine.

3.3 Methods

3.3.1 Animals

Male CD1 mice (8-10 weeks) were purchased from Charles River Laboratories (Laprairie, Quebec, Canada) and acclimatized for 1 week before experimental procedures began. Animals were singly housed in standard polypropylene cages (27 x 21 x 14 cm), and maintained on a 12-hour light/dark cycle. Food and water were provided *ad libitum* and room temperature was maintained at 21°C. All experiments were approved by the Carleton University Committee for Animal Care and adhered to the guidelines outlined by the Canadian Council for the Use and Care of Animals in Research.

3.3.2 Experiment 2.1a: FST following a single ketamine treatment

The aim of Experiments 2.1a and 2.1b was to test our hypothesis that repeated ketamine treatments would promote longer-lasting antidepressant-like effects than a single ketamine treatment in the FST. In experiment 1a, to first establish that ketamine is indeed having antidepressant-like consequences, and to determine how long these effects can be detected following a single ketamine treatment, immobility was assessed in a FST following exposure to ketamine or vehicle. To this end, mice (n = 10/group) received a single i.p. injection of either saline or ketamine (10 mg/kg) and were subjected to the FST twice as follows: either 1 hour and 5 days later (cohort 1) or 2 days and 8 days later (cohort two).

3.3.2.1 FST procedure

The FST is a robust and well-accepted behavioral test for antidepressant efficacy, and has become a gold standard in antidepressant research (Li et al., 2010). The basic principal is that when animals are placed into a water-filled glass cylinder, from which they cannot escape, antidepressants will prolong escape behavior (swimming) and reduce the time spent immobile (Kitada et al., 1981). In a protocol that we have previously optimized, mice were placed into a 4 L glass beaker (height 25.1 cm, diameter 15.9 cm) filled to a depth of 15 cm with $22 \pm 1^\circ\text{C}$ water. Animals remained in the beaker for six minutes, during which time their activity was videotaped. The last four minutes of each video was scored for time spent immobile. Mobile behavior was defined as any motion beyond that which is required for the animal to remain afloat.

3.3.3 Experiment 2.1b: FST following repeated ketamine treatment

This experiment was identical to Expt. 1a, except that mice now received three separate injections of either ketamine (10 mg/kg, i.p.) or saline over the course of two weeks, on days 1, 8, and 15. Mice were assessed in the FST 2 days and 8 days after the last ketamine injection.

3.3.4 Experiment 2.2: Repeated ketamine and CUS

The aim of experiment 2.2 was to test the hypothesis that repeated ketamine treatment would modulate the behavioral and neurochemical changes induced by CUS. To this end, mice were randomly assigned to either a non-stress or a CUS (described below) condition, which lasted for 28 days. Two weeks into the stressor regimen, mice were further subdivided, and received either saline or ketamine (10 mg/kg, i.p.) on days 14, 21, and 28 (paralleling the repeated ketamine injection regimen used in Experiment 2.1b). Five cohorts were used to allow for multiple analyses and or endpoints. Cohort 1 was sacrificed by perfusion on day 29 (24 hours after the final ketamine injection), for immunohistological analysis to evaluate hippocampal neurogenesis (n = 8). Cohorts 2 and 3 were sacrificed by rapid decapitation on days 28 and 29 (at 2 and 24 hours after the final ketamine injection, respectively), and trunk blood and brain tissue were collected for corticosterone and monoamine determinations (n = 8). Cohort 4 was subjected to a number of behavioral tests commencing on day 29 (n = 10). Behavioral tests included the open field series (open field test, novel object exploration, social preference-avoidance test; day 29), elevated plus maze test (day 29), and two-trial Y-maze test (day 30). Cohort 5 was

used to assess sucrose preference during and immediately after the stressor regimen (see Figure 3.2).

3.3.4.1 CUS procedure

The CUS regimen involved twice daily exposure (given at unpredictable times; one in the morning and one in the afternoon) to a series of different stressors for 28 days. It included the following stressors:

Plexiglas restraint: mice were restrained in semicircular Plexiglas tubes (4 × 12 cm) with tails taped to prevent mice from turning (30 min)

Bag restraint: mice were restrained in tight-fitting conical bags with a nose-hole for breathing and tails taped to prevent mice from moving (30 min)

Social stress A: mice were placed in the cage of a non-experimental, aggressive retired breeder, and after the first hostile contact or submission by the experimental mouse, a mesh divider was placed between the two for the duration of the session (30 min)

Social stress B: mice were placed in a congener's soiled cage, with the latter having been removed (overnight)

Predator odor exposure: mice were placed in a cage containing rat feces and soiled bedding (overnight)

Wet bedding: mice had their bedding wetted with approximately 250 mL of room temperature water (overnight)

Tail hang: mice were suspended by their tails (60 sec)

Empty cage: mice were placed in an empty cage without sawdust or nesting (overnight)

Light during dark phase: mice were placed in an illuminated room (overnight)

Tail pinch: a small butterfly clamp, lined with gauze, was placed over the tail (5 min)

Cage tilt: the cage was propped at an angle of approximately 30° (overnight)

Due to the nature of the stressor paradigm, animals receiving the chronic stressor regimen were housed in holding rooms separate from, but otherwise identical to, their non-stressed counterparts.

3.3.4.2 Doublecortin (DCX) immunohistochemistry

DCX is a microtubule-associated protein that is expressed in all neuronal precursor cells, and is a reliable marker of adult neurogenesis (Couillard-Despres, 2005). Immunohistochemistry was used to assess levels of DCX expression in the hippocampal DG. Animals were perfused with saline followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde and then cryoprotected in a 30% w/v sucrose solution. Brains were then sliced on a cryostat into 40- μ m coronal sections containing the hippocampus. Hippocampal sections were operationally defined as early/rostral (bregma – 1.22 to – 1.82), middle (bregma – 1.82 to – 2.46) and late/caudal (bregma – 2.46 to – 2.92), as we previously reported (Seguin et al., 2009).

Slides were washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with a polyclonal goat anti-DCX antibody (Santa Cruz) at a concentration of 1:200. Slides were then rinsed with PBS and incubated overnight at 4°C with AlexaFluor-488 conjugated donkey anti-goat antibody (1:100; Invitrogen Life Technologies). Subsequently, slides were rinsed in PBS and cover-slipped using Fluoromount.

DCX⁺ staining was assessed using a Nikon C1 fluorescent microscope equipped with a Hamamatsu Orca camera. Stereology Investigator software was used to capture and

quantify images. Images of the DG were taken at representative z levels and exposure was limited to one minute. The images were taken at a constant exposure of 100.161, a constant gain of 2, a constant offset of 1, and a constant magnification of 30X. DCX+ neurons were counted using the Cell Counter extension of Image-J software (National Institutes of Health). Neurons were only counted if the soma was clearly visible and in line with the shape of the DG and a portion of the axonal processes was visible.

3.3.4.3 Corticosterone analyses

Mice were rapidly decapitated and trunk blood was taken. Trunk blood (collected in tubes containing 10 µg of EDTA) was centrifuged at 3600 rpm for 8 minutes. Plasma was then collected and immediately stored at -80°C until analyzed. Corticosterone levels were measured by a commercial RIA kit (ICN Biomedicals). Inter-assay variability was avoided by assaying all samples (in duplicate) within a single run.

3.3.4.4 Monoamine detection

Mice were rapidly decapitated and their brains were collected for monoamine detection. Brains were sliced into coronal sections using a chilled microdissecting block containing slots (0.5mm apart) for single-edged razor blades. The hypothalamus, PFC and hippocampus were dissected from the sections using razor blades. All tissue was collected bilaterally and immediately placed on dry ice. The tissue was stored at -80°C until analyzed using HPLC.

HPLC was performed as previously reported by Liu et al. (2014), to determine the levels of NE and 5-HT, and their respective metabolites, MHPG and 5-HIAA. Briefly,

brain tissue was homogenized in a solution of 0.3 M monochloroacetic acid, 0.1 mM NaEDTA, and 1% methanol in H₂O, and centrifuged at 10 000 rpm at 4°C. Supernatants were then injected at a flow rate of 1 mL/min into the HPLC system (Agilent 1100) with an electrochemical detector (DECADE SDC) and Eclipse XDB-C-8 (4.6-150 mm column). Each liter of the mobile phase used for the separation comprised 90 mM sodium phosphate monobasic, 1.1 mM 1-octane sulfuric acid, 50 mM EDTA, 10% acetonitrile, 50 mM citric acid, 5 mM potassium chloride, and HPLC-grade water. Determination of the area and height of the peaks was carried out with the aid of a Hewlett-Packard integrator. The protein concentration of each sample was determined using a BCA protein analysis kit (Pierce Scientific) and a spectrophotometer (Brinkman, PC800 colorimeter).

3.3.4.5 Open field test, novel object exploration, and social preference-avoidance test

Three separate behavioral tests were conducted in the open field arena in succession. For the open field test procedure, mice were placed into a clear, Plexiglas open-field arena (50 × 50 cm), which was illuminated by ambient fluorescent ceiling lights. Mice were placed in one corner of the arena and allowed to freely explore the space for 5 minutes. Activity was recorded by a video camera mounted directly above the open-field apparatus. The video camera was connected to tracking software (EthoVision, Noldus, The Netherlands), which scored latency to enter the center, time spent in and number of entries into the center square, middle region, and outer square.

In a procedure modified from Litteljohn et al., (2010), after the open field test, mice were removed and returned to their home cages while an empty wire mesh cage was placed against one wall of the arena. After one minute mice were placed back into the arena and

allowed to freely explore the novel object for 2.5 minutes. Subsequently, mice were returned to their home cages while a conspecific was placed into the wire mesh cage for the social preference-avoidance test, as described by Toth and Neumann (2013). Again after one minute in their home cages, mice were returned to the arena and allowed 2.5 minutes of free exploration. For the tests involving the wire mesh cage, latency to enter, frequency of entries, and time spent in an 8 cm-wide perimeter around the cage was scored (with the same tracking software used in the open field test).

3.3.4.6 Elevated plus maze test

The elevated plus-maze test is used to assess anxiety-like behavior based on the principle that rodents often find open spaces noxious. Mice were subjected to an elevated plus maze test for 5 minutes. Specifically, they were placed into the center of a Plexiglas apparatus (30 cm high), which was composed of four arms (6 × 30 cm), two that were enclosed by walls, and two that were open. Time spent in and number of entries into each of the four arms was recorded (by an experimenter who was blind to the animal groups). To be scored as an entry into an arm, all four paws had to be placed into that arm.

3.3.4.7 Two-trial Y-maze test

On day 30, mice underwent a two-trial Y-maze test (modified from Sarnyai et al., 2000). The y-maze consisted of a black Plexiglas maze, enclosed in a wooden shell, with three arms (each 30 cm long, 5 cm wide, and 15 cm tall) all at 120 degrees to each other. During the first trial, one arm was blocked off with a removable panel. Mice were placed in the far end of the ‘start arm’, facing the end-wall, and allowed to freely explore the other

two arms for five minutes. After a 30-minute rest in their home cages, mice were returned to the ‘start arm’, however in this trial all three arms were accessible. Mice were allowed to freely explore the maze for 5 minutes. Cues in the form of stickers were placed at the far end of each arm to help the mice distinguish between the arms. Activity was recorded by a video camera mounted directly above the maze. The total number of entries into each arm, defined as all four paws entering the arm, and time spent in each arm were scored by an experimenter who was blind to the groups.

3.3.4.8 Sucrose preference test

Anhedonia (the inability to experience pleasure from rewarding or enjoyable activities) is a core feature of depression (Liu et al., 2018). The sucrose preference test is used to measure anhedonia in animals by assessing their preference for sucrose over water (Liu et al., 2018). Mice were given free access to both a 2% sucrose solution and water for 2 days prior to baseline testing to acclimate them to the taste of sucrose. Initial baseline preference measures were taken the night before the start of the stress regimen. From 5pm to 8am, free access was provided to both a 2% sucrose solution and water, and the resulting consumption of both solutions was measured. Both sucrose and water consumption were calculated as a function of animal weight. Sucrose preference was measured starting at 5 pm on the same day as injections (which were delivered at 9am). Percentage sucrose preference was calculated as $\frac{\text{Sucrose Consumption}}{(\text{Sucrose Consumption} + \text{Water Consumption})} * 100$. Thereafter, the baseline percent sucrose was subtracted from that calculated during weeks 4 and 5 of the experiment.

3.3.5 Statistical analysis

Data from Experiments 2.1a and 2.1b were analyzed by two-tailed Student's *t*-test. For all other experiments, data were analyzed by ANOVA. For ANOVAs exhibiting a significant interaction, post hoc analyses using Fisher's PLSD were conducted to determine the exact nature of the relationship. Data were evaluated using a StatView (version 6.0) statistical software package available from the SAS Institute, Inc.

3.4 Results

3.4.1 Experiment 2.1a: FST following a single ketamine treatment

The effect of a single ketamine injection on FST performance 1 hour and again 5 days after, and 2 days and again 8 days after ketamine treatment was assessed. As shown in Figures 3.1A and 3.1B, ketamine treatment reduced forced swim time at the one-hour time point ($t = 2.45$, $df = 18$, $p < 0.05$) whereas no significant effects of ketamine treatment were found for any of the other time points ($p > 0.05$).

3.4.2 Experiment 2.1b: FST following repeated ketamine treatment

Repeated ketamine treatment significantly reduced immobility time at both 2 and 8 days after the last injection ($t = 2.66$, $df = 18$, $p < 0.05$ and $t = 5.43$, $df = 18$, $p < 0.001$, respectively; Figure 3.1C), indicating long-term antidepressant-like effects following three injections (over a two-week interval) of the drug.

3.4.3 Experiment 2.2: Repeated ketamine and CUS

3.4.3.1 DCX immunohistochemistry

No significant effects were apparent for the “early” (i.e. bregma – 1.22 to – 1.82) portion of the hippocampus (Figure 3.3A). Yet, although the interaction missed significance, there were significant main effects for both ketamine injection and stressor exposure in the “middle” portion (i.e., bregma – 1.82 to – 2.46). Specifically, repeated ketamine injections significantly increased the number of DCX+ neurons found in the “middle” region of the hippocampal DG ($F_{1, 28} = 4.53, p < 0.05$; Figure 3.3B; for representative images, please see Figure A.1 in Appendix A) relative to the control groups. In contrast, the stressor regimen significantly reduced the number of “middle” hippocampal DCX+ neurons ($F_{1, 28} = 5.33, p < 0.05$; Figure 3.3B). Finally, in the “late/caudal” (i.e. bregma – 2.46 to – 2.92) hippocampal region, the stressor had no effect and the ANOVA for ketamine injection missed significance. However, based on our *a priori* hypothesis, we conducted follow up comparisons and found that the non-stressed ketamine-treated mice had significantly elevated DCX+ counts, compared to the remaining groups ($p < 0.05$; Figure 3.3C).

3.4.3.2 Corticosterone analysis

There were no significant differences in plasma corticosterone levels among the groups at either two or twenty-four hours after the last ketamine injection (Figures 3.4A and 3.4B, respectively).

3.4.3.3 Monoamine detection

Within the hypothalamus, levels of the 5-HT metabolite, 5-HIAA, were reduced by stressor treatment ($F_{1,28} = 4.32, p < 0.05$) while they were increased by ketamine treatment ($F_{1,28} = 8.11, p < 0.05$; data not shown). In contrast, there were no significant differences in hypothalamic 5-HT, NE, or MHPG.

Levels of 5-HIAA within the PFC were found to be significantly elevated by ketamine treatment ($F_{1,28} = 5.75, p < 0.05$; Figure 3.5A). None of the other assayed neurotransmitters (5-HT, NE, MHPG) were significantly affected by the ketamine or stressor treatments within this region.

Finally, repeated ketamine treatment also elevated levels of hippocampal MHPG ($F_{1,28} = 5.64, p < 0.05$; Figure 3.5B). Yet, neither NE or 5-HT (or 5-HIAA) were significantly influenced by any of the treatments.

3.4.3.4 Open field/novel object exploration/social preference-avoidance

During an open field test, there were no effects of either ketamine treatment or chronic stress on the total distance travelled in the open field arena ($p > 0.05$; Figure 3.6A). However, mice that received the ketamine treatment displayed a reduction in the number of entries into the center square of the open field arena ($F_{1,34} = 5.67; p < 0.05$; Figure 3.6B).

When mice were introduced back into the open field arena with a novel object (an empty wire mesh cage) present, a significant main effect of stress was found for the duration of time spent in a predetermined perimeter (the interaction zone) around the cage. Specifically, the chronically stressed mice spent less time in the interaction zone of the object than those in the control group ($F_{1,36} = 4.22; p < 0.05$; Figure 3.6C). Ketamine treatment had no effect ($p > 0.05$).

In a measure of social avoidance behavior, when mice were exposed to an unfamiliar conspecific mouse in the wire mesh cage, neither the ketamine treatment nor the chronic stress condition had any effects on the time spent in the interaction zone surrounding the mouse ($p > 0.05$; Figure 3.6D).

3.4.3.5 *Elevated plus maze*

Performance in the elevated plus maze was assessed as an additional measure of anxiety-like behavior and locomotor activity. There were no effects of ketamine treatment or CUS exposure on the time spent in the open arms, or on the number of entries into the open arms ($p > 0.05$; Figure 3.7A and 3.7B respectively). There was, however, a main effect of stress for the number of entries into the closed arms ($F_{1,36} = 4.35$; Figure 3.7C). Specifically, the CUS treatment increased the number of entries into the closed arms ($p < 0.05$), and ketamine did not modify this effect.

3.4.3.6 *Two-trial Y-maze test*

During the second trial of a two-trial Y-maze task, the number of arm entries into and time spent in each of the three arms was assessed. There were no effects of either ketamine or stressor exposure on the number of entries into the novel arm as a percentage of total arm entries, nor were there any effects of either treatment on time spent in the novel arm as a percentage of total arm time ($p > 0.05$; Figures 3.8A and 3.8B respectively).

3.4.3.7 Sucrose preference test

A significant stress x ketamine interaction was apparent for sucrose preference ($F_{1,37} = 4.01$; $p < 0.05$; Figure 3.9). Surprisingly, the stressor regimen and ketamine injections significantly reduced sucrose preference below that of controls at both weeks 4 and 5 of the experiment ($p < 0.05$). However, there appeared to be some degree of “rebound” at week 5, with ketamine + stress increasing sucrose preference above that of the previous week ($p < 0.05$). In fact, there was an overall significant increase in sucrose preference at week 5 compared to week 4 ($F_{3,37} = 5.13$; $p < 0.05$).

3.5 Discussion

Many current pharmacological treatments for depression are seriously limited in terms of both effect latency and general efficacy (Trivedi et al., 2006). Ketamine, a non-competitive NMDA receptor antagonist, has been found to have fast-acting antidepressant effects (Berman et al., 2000; Zarate et al., 2006) even in patients who are resistant to traditional antidepressants (Krystal, 2007). Ongoing research aims to understand the mechanisms underlying its rapid results and clearly distinct biological properties.

We presently found that while a single ketamine injection altered FST performance one hour after treatment, repeated administration of ketamine provoked long-lasting antidepressant-like effects that were still evident 8 days following the third of three ketamine injections. While these findings confirm that ketamine can produce antidepressant effects in a FST (Garcia et al., 2008; Li et al., 2010; Parise et al., 2013; Akinfiresoye and Tizabi 2013), they also further suggest that the efficacy of ketamine

might be augmented over time through cumulative dosing, which of course has important clinical ramifications.

It was surprising that ketamine did not reverse the chronic stressor induced reductions in sucrose consumption and actually itself reduced sucrose preference, which of course makes interpretation of this test difficult. It is possible that the drug induced some dissociative or other unexpected effects (although these should be minimal at the present relatively low dose) that could interfere with sucrose consumption. It is important to underscore that one recent study found that ketamine did not affect anhedonia, as measured using intracranial self-stimulation (Donahue et al., 2014). Similarly, ketamine did not influence the consumption of sugar pellets in chronically stressed rats (Rezin et al., 2009). Yet, others found that ketamine did have anti-anhedonic effects in rodents exposed to a number of different stressors (Garcia et al., 2009; Dwyer and Duman, 2013; Lally et al., 2014, 2015). Although it is unclear as to the source of the discrepancies in these ketamine behavioral findings, the dosing schedule and species used (mice vs rats) are obvious points of consideration.

In our investigation, ketamine did not modify the impact of CUS on elevated plus maze and novel object tests, suggesting that ketamine was not acting as an anxiolytic agent. In fact, ketamine itself (in the absence of the CUS) reduced the number of entries into the center of the open field arena, indicating a potential anxiogenic-like effect of the drug. In support of this interpretation, it has been well documented that anxiogenesis is one of the unwanted side effects of ketamine treatment (Short et al., 2017; Aust 2019). It is also reported that the anxiogenic effects of ketamine are transient, resolving within hours of administration (Bobo et al., 2016). It is therefore interesting to note that in this particular

instance, the open field test was done a full 24 hours after the last ketamine injection, suggesting that at least in our paradigm these effects were less transient. It would be interesting to determine how long the anxiogenic-like effects of ketamine persist, and also to attempt to decipher the underlying mechanism. Our data do not support a role of heightened HPA axis activity in this effect given that ketamine did not alter levels of corticosterone at either 2 or 24 hours after the last treatment, nor do central monoamines appear to be involved.

From a safety perspective, it is important to consider the potential long-term effects of repeated exposure to ketamine. Indeed, long-term exposure to ketamine has been associated with psychotic symptoms (Dillon et al., 2003) and tolerance may also develop over months (Liebrenz et al., 2007). However, these studies assessed recreational ketamine abusers and involved relatively high doses greatly exceeding those used in an antidepressant capacity. That being said, even at depression-relevant doses, ketamine has been reported to cause cognitive deficits in a number of tests (Neill et al., 2010; Rajagopal et al., 2014). In our current repeated injection study, ketamine did not produce any observable adverse effects in terms of cognitive performance in a two-trial Y-maze task, and ketamine-treated mice appeared perfectly normal in their locomotor skills. As well, the repeated ketamine injections actually increased hippocampal neurogenesis indicating positive effects on neuroplasticity.

Accumulating evidence has indicated that ketamine might exert its effects by influencing neuroplasticity (Li et al., 2011; Duman et al., 2012). Accordingly, we presently found that three injections of ketamine administered over the span of two weeks increased adult hippocampal neurogenesis. Interestingly, however, this effect was restricted to the

“middle and later” portions of the hippocampal DG. Similarly, the chronic stress reduced hippocampal neurogenesis, but only in the “middle” portion of this brain region. These data indicate the possible importance of the internal hippocampal anatomy in stressor and antidepressant sensitivity.

Ketamine’s neuroplastic effects might come about through the mTOR pathway, which typically integrates signals from various endogenous growth factors to promote rapid synaptogenesis and neurogenesis (Li et al., 2010; Zhou et al., 2014). Although not evaluated in the current study, we previously found that the mTOR inhibitor, rapamycin, prevented the antidepressant-like and neurogenic effects of erythropoietin (Osborn et al., 2013). Likewise, rapamycin was also reported to ameliorate many of the central actions of ketamine (Li et al., 2010). Whatever the case, it is important to note that numerous studies have shown a relationship between hippocampal neurogenesis and antidepressant efficacy (Malberg et al., 2000; Van Bokhoven et al., 2011) and that ablation of neurogenesis diminished the behavioral effects of a variety of antidepressants (Santerelli et al., 2003; Surget et al., 2008).

Finally, it is important to note that we only assessed neurogenesis after the repeated (three injection) ketamine treatment and such effects might not be apparent after a single dose. Interestingly, however, a recently published study reported that a single ketamine injection increased the proportion of functionally mature dentate gyrus neurons in adult rats (Soumier et al., 2016), raising the possibility that its neuroplastic effects could have immediate clinical consequences. However, given that the clinical effects of ketamine dissipate within several days to a couple of weeks, it would be useful for a future study to

conduct parallel time courses to address whether the neurogenesis changes are really linked to clinical efficacy in the long run.

3.6 Conclusions

In summary, ketamine provoked long-lasting antidepressant-like behavioural effects in the FST, as well as increased hippocampal neurogenesis. It is interesting that ketamine increased neurogenesis in the later but not the earlier portions of the dorsal DG, since the more caudal-ventral portions of the hippocampus are more related to anxiety and emotional processes, as opposed to the heavier memory role ascribed to more anterior-dorsal portions (Fuster-Matazano et al., 2011). Furthermore, the fact that ketamine itself reduced sucrose preference (much like our chronic stress regimen did), raises the possibility that the drug had unexpected “side” effects that could influence the motivation or proclivity for sucrose preference. Overall, our data supports the contention that ketamine can have beneficial antidepressant effects with repeated treatment but that its clinical effects do not stem from the obvious modulation of stressor-sensitive neurochemical pathways but could be linked to the modulation of neuroplastic processes.

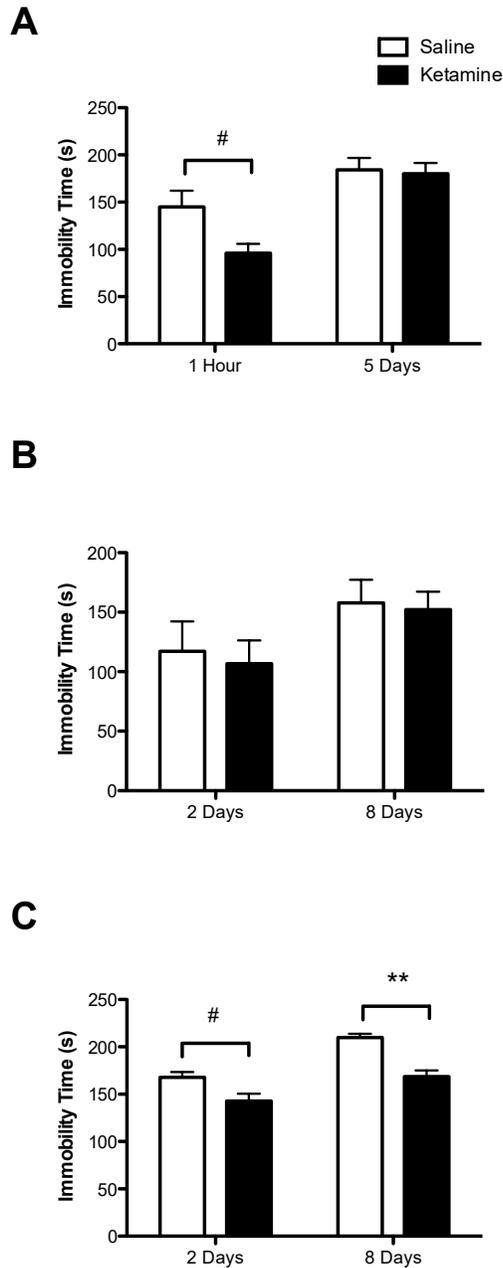


Figure 3.1. Effects of single and repeated ketamine treatment (10 mg/kg) on immobility time in the forced swim test. A. A single injection of ketamine reduced immobility time one hour but not 5 days after the injection. **B.** A single injection of ketamine had no effect on immobility time 2 or 8 days after the injection. **C.** Repeated ketamine treatment (three injections over 2 weeks) reduced immobility time both 2 days and 8 days after the final injection. Data expressed as means \pm SEM. # $p < 0.05$, ketamine-treated mice compared to saline-treated controls at given time point; ** $p < 0.001$, ketamine-treated mice compared to saline-treated controls at given time point.

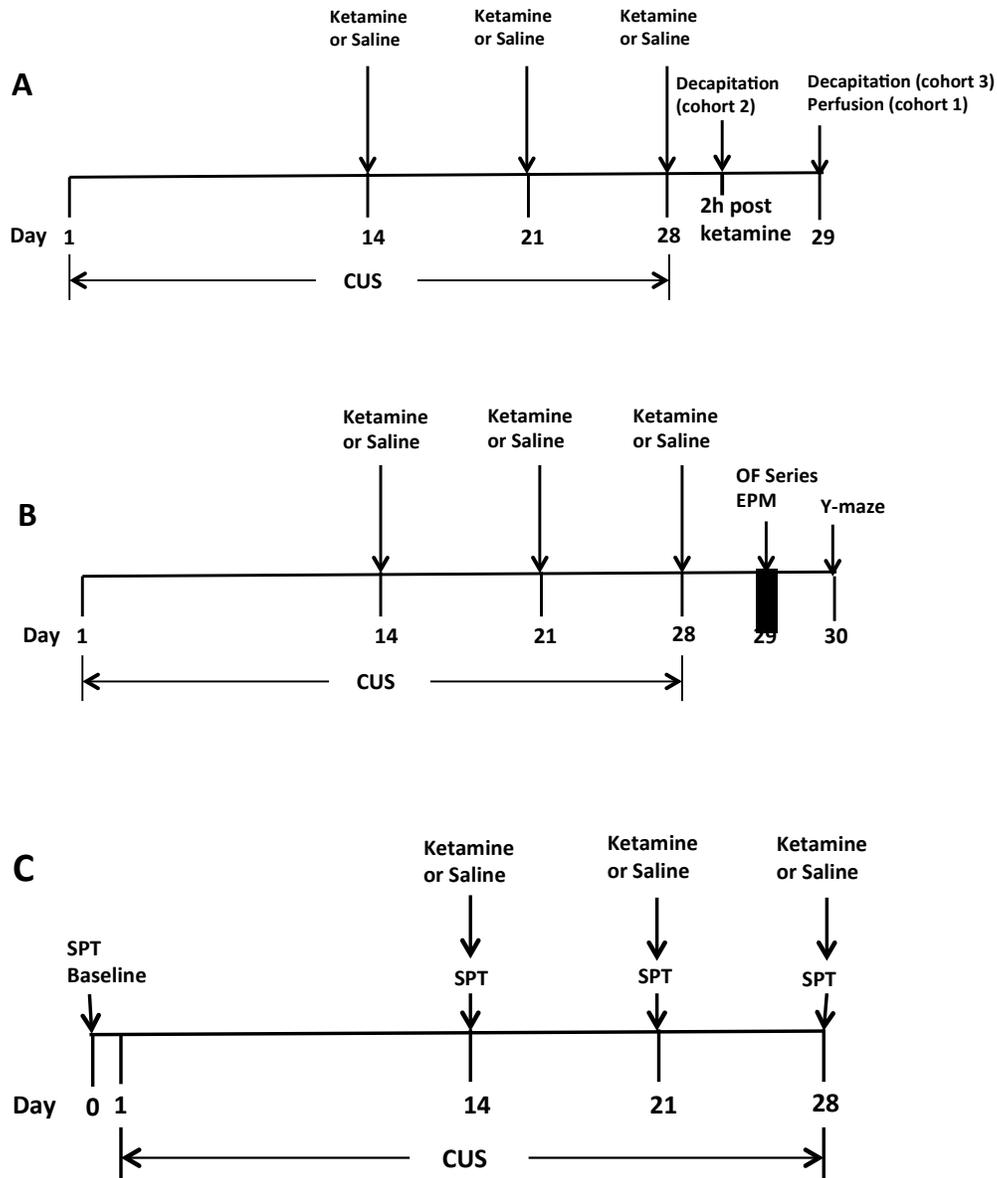


Figure 3.2 Timeline for Experiment 2.2. Mice were exposed to CUS for 28 days and were given ketamine injections (10 mg/kg, i.p.) on days 14, 21, and 28. **A.** Mice in cohort 1 were perfused 24 hours after the last ketamine injection and their brains were collected to assess hippocampal neurogenesis. Mice in cohorts 2 and 3 were rapidly decapitated 2 and 24 hours after the last ketamine injection on days 28 and 29, respectively, and their trunk blood and brain tissue were collected for analysis. **B.** Mice in cohort 4 were subjected to behavioral testing on days 29 and 30. OF series = open field test, novel object exploration, social preference-avoidance; EPM = elevated plus maze; Y-maze = two-trial Y-maze. **C.** Mice in cohort 5 underwent sucrose preference testing on days 14, 21, and 28, after the ketamine injections. SPT = sucrose preference test.

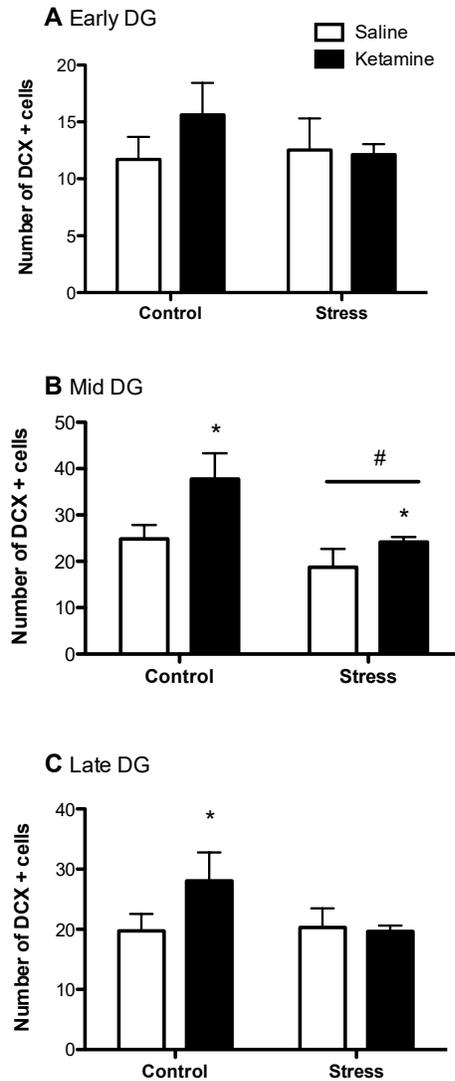


Figure 3.3. Effects of repeated ketamine treatment (10 mg/kg) and CUS on neurogenesis, as indicated by DCX staining in the DG. A. There was no effect of stress or ketamine on DCX staining in the operationally defined “early” (bregma – 1.22 to – 1.82) portion of the hippocampal DG. **B.** Repeated ketamine treatment increased DCX staining in the “middle” (bregma 1.82 to 2.46) portion of the hippocampal DG. Additionally, the CUS reduced DCX staining in this region, and this effect was reversed by ketamine treatment. * $p < 0.05$, relative to saline-treated mice; # $p < 0.05$, relative to non-stressed controls. **C.** Repeated ketamine treatment also increased DCX staining in the “later” (bregma 2.46 to 2.92) portions of the hippocampal DG. * $p < 0.05$, compared to all other groups. Data expressed as means \pm SEM.

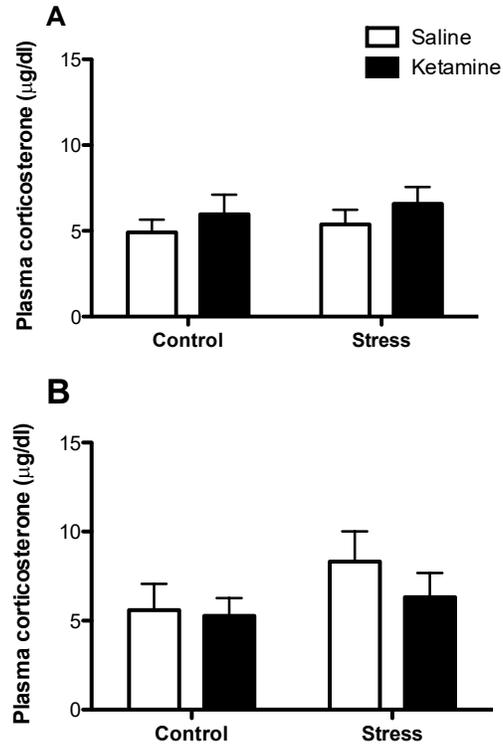


Figure 3.4. Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on plasma corticosterone. Plasma corticosterone levels were not affected by repeated ketamine treatment or CUS two hours (A) or 24 hours (B) after the final of three weekly ketamine injections. Data expressed as means \pm SEM.

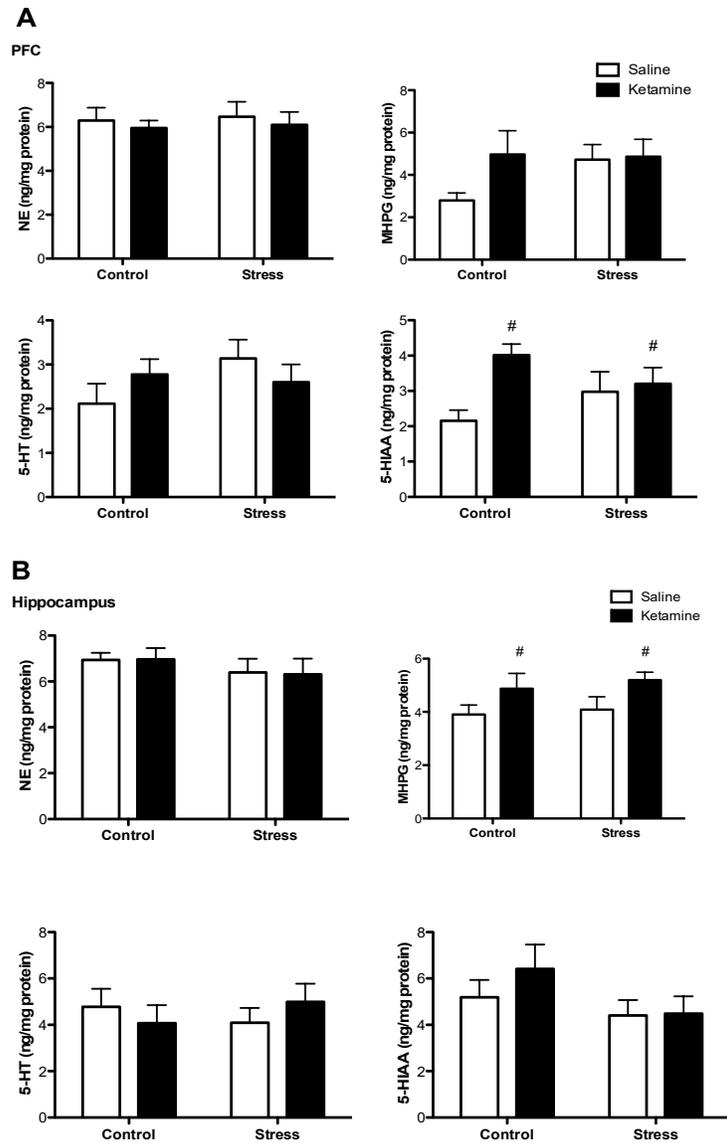


Figure 3.5. Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on monoamine levels. **A.** Ketamine treatment increased levels of the 5-HT metabolite, 5-HIAA, within the PFC. **B.** Hippocampal levels of the NE metabolite, MHPG, were increased by ketamine treatment. Data expressed as means ± SEM. # $p < 0.05$, relative to saline-treated mice.

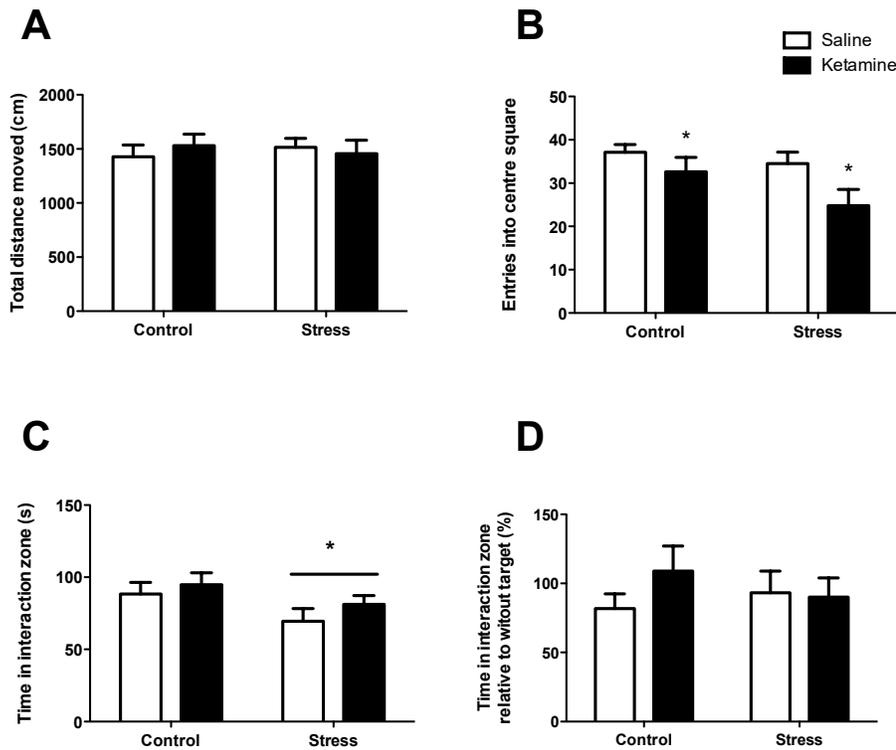


Figure 3.6. Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on open field performance, novel object exploration, and social preference-avoidance. A. There were no effects of either ketamine treatment or CUS on total distance travelled in the open field test. **B.** Ketamine treatment reduced the number of entries into the center square of the open field arena. * $p < 0.05$, relative to saline-treated mice. **C.** CUS reduced the duration of time spent in the interaction zone of a novel object (empty cage). * $p < 0.05$, relative to non-stressed controls. **D.** There were no effects of either ketamine treatment or CUS on the duration of time spent in the interaction zone of the cage when a novel mouse was placed in the cage. Data expressed as means \pm SEM.

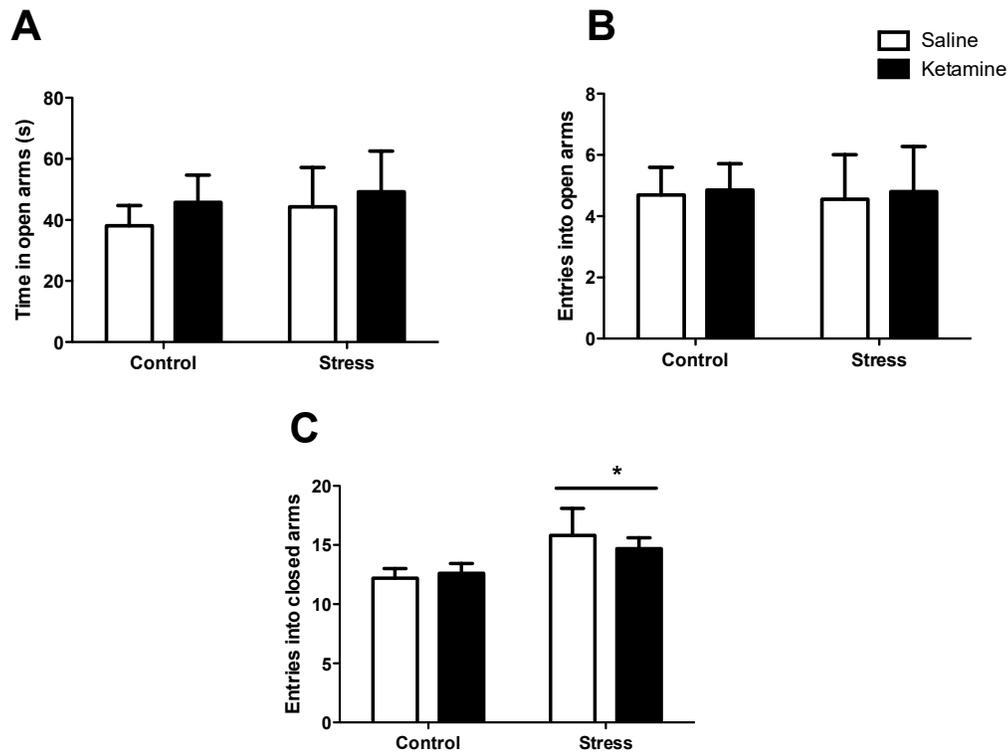


Figure 3.7. Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on performance in the elevated plus maze. A. There were no effects of either ketamine or stressor treatment on time in open arms. **B.** There were no effects of either ketamine or stressor treatment on entries into open arms. **C.** CUS increased the number of entries into the closed arms. Data expressed as means \pm SEM. * $p < 0.05$, relative to non-stressed controls.

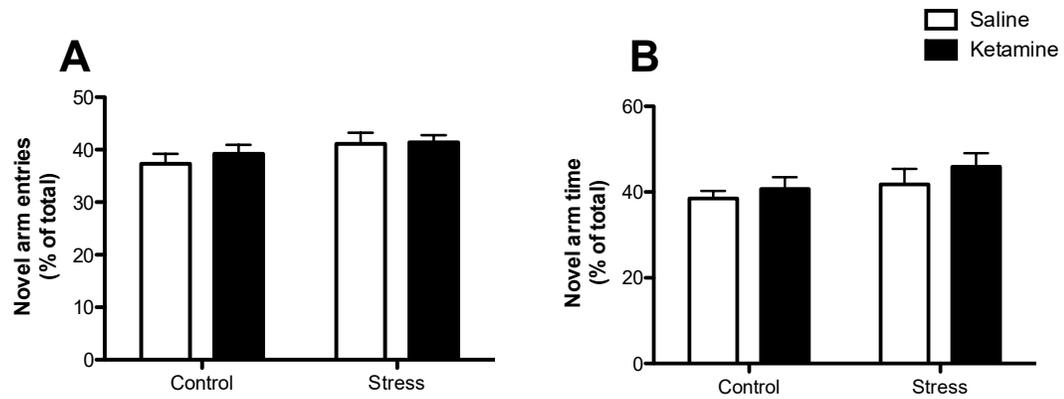


Figure 3.8. Effects of repeated ketamine treatment (10 mg/kg, i.p.) and CUS on performance in a two-trial Y-maze. A. There were no effects of either ketamine treatment or CUS exposure on the number of novel arm entries as a percentage of total arm entries. **B.** There were no effects of either ketamine treatment or CUS exposure on the amount of time spent in the novel arm as a percentage of total arm time. Data expressed as means \pm SEM.

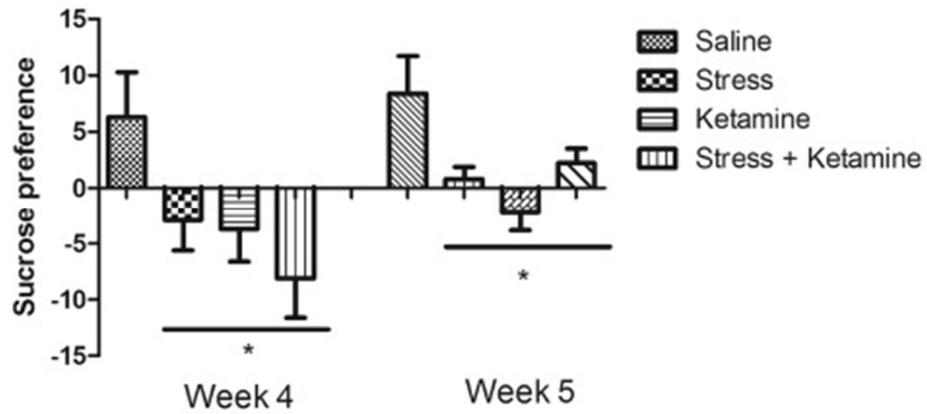


Figure 3.9. CUS and repeated ketamine (10 mg/kg) treatment time-dependently altered sucrose preference. In particular, ketamine and the CUS regimen reduced preference at weeks 4 and 5 relative to vehicle treatment (and adjusted for baseline preference). Data expressed as means \pm SEM. * $p < 0.05$, relative to non-stressed, saline-treated controls.

Chapter 4. Endogenous JNK affects stress relevant processes, and its phosphorylation state can be influenced by acute ketamine treatment.

4.1 Abstract

Ketamine has recently been found to possess potent and rapid antidepressant properties, yet the mechanisms underlying these effects are not clearly understood. We hypothesized that ketamine may be imparting its effects through interactions with the JNK pathway. Accordingly, we presently found that ketamine (10 mg/kg) increased levels of p-JNK in the PFC, but not in the hippocampus. The JNK antagonist, SP600125 (but not the p38 antagonist, SB203580), increased plasma corticosterone levels under resting conditions and in the context of an acute stressor. SP600125 also reduced open field exploration, but prevented the stressor-induced increase in open arm exploration in an elevated plus maze. Finally, SP600125 promoted noradrenergic activity in the central amygdala and locus coeruleus under resting conditions but prevented the stressor-induced noradrenergic effects within the hypothalamic PVN. These data suggest that inhibiting endogenous JNK can have stressor-like corticoid, behavioral and central monoamine effects under basal conditions, and provisionally suggest that antidepressants like ketamine could be modulating JNK activity.

4.2 Introduction

Current pharmacological treatments for depression, namely monoaminergic drugs such as SSRIs, are significantly limited, taking weeks to months to provide symptomatic relief, and they are only effective in 60-70% of patients (Thase et al, 2005; Trivedi et al., 2006). In what is considered one of the largest breakthroughs in depression treatment,

ketamine, an NMDA receptor antagonist and dissociative anesthetic, has emerged as a potent and rapid antidepressant agent (Duman et al., 2012; Duman et al., 2016). Indeed, a single sub-anesthetic infusion of ketamine provokes antidepressant effects within hours, and has been shown to be effective in historically treatment resistant patients (Berman et al., 2000; Zarate et al., 2006). Unfortunately, ketamine has its own limitations, such as dissociative side effects and potential for abuse (Murrough et al., 2013b; Schatzberg, 2014). Moreover, the safety of long-term ketamine use has not been firmly established (Moda-Sava et al., 2019). Consequently, research is currently focused on trying to understand the mechanisms underlying its antidepressant effects in order to design novel antidepressants that do not have the limitations associated with ketamine.

The MAPK protein, JNK, has recently gained attention for its suspected role in depression pathology (Jovicic et al., 2015; Hollos et al., 2018), and there is reason to believe that ketamine might be interacting with the JNK pathway. JNK is a stress-activated kinase that is involved in cytokine regulation, cell death, and cell proliferation and survival, and its overactivation can lead to neuronal degeneration and functional impairment (Nishima et al., 1997; Davis 2000). Postmortem PFC tissue from depressed patients displayed increased levels of active JNK, as does the PFC from animals exposed to both acute and chronic stress (Galeotti and Ghelardini, 2012; Martin-Hernandez et al., 2018; Rosa et al., 2018). JNK has also been associated with impaired neuroplasticity, and in fact both *JNK1* knockout mice and those treated with a JNK inhibitor displayed increased hippocampal neurogenesis, which was accompanied by reduced depressive-like behaviors (Mohammed et al., 2018).

Interestingly, JNK has been found to negatively regulate the GR, whose activation is associated with depression-related processes including neurogenesis, inhibition of inflammation, and HPA axis regulation (Munck, 2005; Davies et al., 2008; Kadmiel and Cidlowski, 2013). Likewise, ketamine has recently been reported to increase GR expression and activity in the hippocampus (Wang et al., 2019). Moreover, in the study by Wang et al. (2019), ketamine's antidepressant-like effects were dependent upon its modulation of the GR.

Surprisingly, little is known about the role of JNK in the response to psychogenic stressors, which is of significant interest given that stressor exposure is the primary environmental challenge believed to contribute to depression (Hayley et al., 2005; Pittenger and Duman, 2008). Indeed, repeated or particularly severe exposure to psychogenic or neurogenic stressors induces enduring pathological hormonal and neurotransmitter changes that culminate in behavioral pathology (Anisman, 2009; McEwen et al., 2012). The current work therefore has two principal goals: first, to investigate the role of JNK in the hormonal, neurochemical, and behavioral response to an acute stressor, and second, to determine if ketamine administration can affect JNK activity. Together these findings could provide information that may guide future research directions.

4.3 Methods

4.3.1 Animals

Male CD1 mice were purchased from Charles River Laboratories (Laprairie, Quebec, Canada) at 8-10 weeks of age. Mice were singly housed in standard polypropylene cages (27 × 21 × 14 cm), and maintained on a 12-hour light/dark cycle (light phase: 0700–

1900 h). Water and Ralston Purina mouse chow (St. Louis, MO, USA) were provided *ad libitum*, and room temperature was maintained at 21 °C. Animals were acclimatized to the laboratory for a period of one week before experimental procedures were commenced. All experiments were approved by the Carleton University Committee for Animal Care and adhered to the guidelines outlined by the Canadian Council for the Use and Care of Animals in Research.

4.3.2 Experiment 3.1a: Investigating the role of JNK in the behavioral and neurochemical response to acute stress

In experiment 3.1, we tested our hypothesis that the JNK pathway is involved in the neurochemical and behavioral responses to an acute stressor. Mice were treated with the JNK inhibitor, SP600125, prior to experiencing an acute stressor; behavior, corticosterone, and central monoamines were examined. To this end, mice were divided into four treatment groups as follows: 1. vehicle + stress, 2. vehicle + no stress, 3. JNK antagonist + no stress, 4. JNK antagonist + stress. Two cohorts were used. Cohort one was subjected to behavioral testing (open field test and elevated plus maze). Cohort two underwent rapid decapitation and their trunk blood and brain tissue were collected for corticosterone and central monoamine analysis.

4.3.2.1 Treatments

Mice were given an i.p. injection of either the JNK antagonist, SP600125 (30 mg/kg), or vehicle [dimethyl sulfoxide (DMSO) diluted with saline to a ratio of 1:4]. Fifteen minutes later mice in the stress group had their bedding wetted with 250 ml of

water. After 10 minutes of wet bedding, mice were immediately placed into a “restraint bag” for 15 minutes. The restraint apparatus consisted of a conical shaped plastic bag with an opening at the end to allow breathing. The animals were placed snugly in the plastic bag with their tails taped down to prevent excessive movement. Mice therefore had a total stress exposure of 25 minutes. Five minutes after being removed from the restraint bag, mice were either rapidly decapitated and their trunk blood and brain tissue kept for analysis, or subjected to behavioral testing (see Figure 4.1). End points were therefore 45 minutes from the time of vehicle or drug injection. (N = 32 per cohort; n = 8).

4.3.2.2 Open field test

Five minutes after the termination of the stressor exposure, mice were placed into a clear, Plexiglas open field arena (40 × 40 cm), which was illuminated by ambient fluorescent ceiling lights. Mice were placed in one corner of the arena and allowed to freely explore the space for 5 minutes. Activity was recorded by a video camera that was mounted directly above the open-field apparatus. The video camera was connected to tracking software (EthoVision, Noldus, Netherlands), which scored latency to enter the center, time spent and number of entries into the center square, middle region, and outer square.

4.3.2.3 Elevated plus maze

Immediately after the open field exposure, mice were subjected to an elevated plus maze test for 5 minutes in duration. Specifically, mice were placed into the center of the Plexiglas apparatus (30 cm high), which was composed of four arms (6 × 30 cm), two of which were enclosed by walls and two of which were open. Time spent and number of

entries into each of the four arms was recorded by an experimenter who was blind to the animal groups. To be scored as an entry into an arm, all four paws had to be placed into that arm.

4.3.2.4 Plasma corticosterone analyses

Forty-five minutes after the injection, mice were rapidly decapitated and trunk blood was collected. The trunk blood was collected in tubes containing 10 µg of EDTA. The blood was kept on ice before being centrifuged at 3600 rpm for 8 minutes, and 50 µL of plasma was then collected for the determination of corticosterone levels. Plasma was immediately frozen at -80 °C until analyzed. Corticosterone levels were measured by a commercial RIA kit (ICN Biomedicals, CA, USA). Inter-assay variability was avoided by assaying all samples (in duplicate) within a single run.

4.3.2.5 Monoamine detection

Forty-five minutes after the injection, mice were rapidly decapitated and brain tissue was collected. Brain regions were removed from coronal brain sections that were obtained using a chilled microdissecting block that contained slots (0.5 mm apart) for single-edged razor blades. The PFC, central amygdala, hippocampus, PVN, and locus coeruleus were collected. The tissue was immediately placed in a homogenizing buffer containing 14.17 g monochloroacetic acid, 0.0186 g EDTA, 5.0 mL methanol and 500 mL H₂O, and stored at -80 °C until analyzed. HPLC was performed on collected brain samples to determine levels of central monoamines and metabolites. Brain tissue was sonicated in the homogenizing buffer that they were stored in. Tissue was then centrifuged at (15 000

× g for 20 min), and the supernatants were passed through a radial compression column (C18 reverse phase, 8 mm × 10 cm) connected to a three-cell coulometric electrochemical detector (ESA model 5100, A). Each liter of the mobile phase used for the separation comprised 0.1 g disodium EDTA, 1.3 g heptane sulphonic acid, 35 mL acetonitrile and 6.5 mL triethylamine. The mobile phase was subsequently filtered (0.22 mm filter paper) and degassed, after which phosphoric acid was used to adjust the pH to 2.5. Determination of the area and height of the peaks was carried out with the aid of a Hewlett-Packard integrator. The protein concentration in each sample was determined using a BCA protein analysis kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkman, PC800 colorimeter).

4.3.3 Experiment 3.1b: Inhibition of p38 prior to an acute stress

A separate cohort of 48 mice was used to determine if any effect of JNK inhibition on corticosterone levels was specific to this kinase, or whether the alternate MAPK, p38, might also have such consequences. Indeed, p38 has recently been implicated in depressive-like behavioral and neurochemical pathologies (Bruchas et al., 2011). To this end, the treatment groups were as follows: 1. vehicle + stress, 2. vehicle + no stress, 3. JNK antagonist + no stress, 4. JNK antagonist + stress, 5. p38 antagonist + no stress, 6. p38 antagonist + stress.

4.3.3.1 Treatments

The timeline for Experiment 3.1b was identical to that used in Experiment 3.1a. Mice were given an i.p. injection of either SP600125 (30 mg/kg; JNK antagonist), SB203580 (1 mg/kg; p38 antagonist) or vehicle (DMSO diluted with saline to a ratio of 1:4). Fifteen minutes later, the mice in the stress group had their bedding wetted with 250 ml of water, followed 10 minutes later by 15 minutes of restraint bag stress, as described in Experiment 3.1a. Mice therefore had a total stress exposure of 25 minutes. Five minutes after being removed from the restraint bag, mice were rapidly decapitated and their trunk blood and brain tissue kept for analysis. End points were therefore 45 minutes from the time of vehicle or drug injection. (N = 48; n = 8 per group)

4.3.3.2 Plasma corticosterone analysis

Plasma corticosterone was analyzed exactly as described in Experiment 3.1a

4.3.3.3 Western blot analysis

It was of interest to assess whether the JNK antagonist or the p38 antagonist affected hippocampal levels of phosphorylated GR. Indeed, hippocampal GR receptors have an important role in the regulation of HPA responses and, as already mentioned, JNK is believed to affect GR phosphorylation status. To this, end western blot analyses were conducted on whole tissue samples (collected as described in section 4.3.2.5) using antibodies directed at the Ser234 phosphorylation site in mice.

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Samples were diluted with lysis buffer containing a protease inhibitor yielding whole cell lysate concentrations of 10 µg of protein in 10 µl and 10 µl loading buffer (5% glycerol, 5% β-mercaptoethanol, 3% SDS and 0.05% bromophenol blue). The 20 µl sample was heated in boiling water for 5 minutes to denature the proteins. Proteins were separated by electrophoresis (120 V) on 8.5% sodium dodecyl sulphate-polyacrylamide gels and transferred overnight at 4°C (180 mA) onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour in a solution of non-fat dry milk (5% w/v) dissolved in tris-buffered saline with tween (TBS-T) buffer (10 mM Tris-base (pH 8.0), 150 mM sodium chloride, 0.5% Tween-20). Membranes were then incubated with a monoclonal mouse anti-pGR primary antibody (1:1000; Abcam, Cambridge, MA, USA) diluted in 3% BSA in TBS-T for 1 hour at room temperature. After incubation with the primary antibody, membranes were washed at room temperature in TBS-T three times for a total of 30 minutes. Membranes were then incubated in a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 5% milk in TBS-T at room temperature for one hour. After three final washes in TBS-T for a total of 30 minutes, pGR was visualized with a chemiluminescent substrate (Perkin Elmer, Waltham, MA, USA; 5 minutes) and exposed on a Kodak film. Band density was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized against β-actin.

4.3.4 Experiment 3.2: Ketamine's effects on JNK in the context of stressor exposure

The aim of Experiment 3.2 was to test our hypothesis that the JNK pathway may play a role in ketamine's effects. To this end, mice were divided into four treatment groups as follows: 1. saline + no stress, 2. saline + stress, 3. ketamine + no stress, 4. ketamine + stress.

4.3.4.1 Treatments

Mice were given an i.p. injection of either saline or ketamine (10 mg/kg). Thirty minutes later, mice in the stress group were subjected to 30 minutes of acute restraint stress, while the no-stress group remained undisturbed in their home cages. The restraint stress consisted of placing mice into a conical shaped plastic bag with an opening at the end to allow for breathing, and their tails were taped down to prevent excessive movement. Immediately after the stressor ended, mice were returned to their home cages and provided with new nestlets for behavioral observation. Thirty minutes later, mice were rapidly decapitated with their brain tissue collected for western blot analysis (see Figure 4.7; N = 32; n = 8 per group).

4.3.4.2 Nestlet test

Immediately after stressor exposure mice were given a new nestlet and their behavior was recorded for 30 minutes. The total number of interactions with the nestlet, and the total amount of time spent interacting with the nestlet, were both rated by an experimenter who was blind to the treatment groups.

4.3.4.3 *Western blot analysis*

Mice were rapidly decapitated 5 minutes after the nestlet test, and their brains were collected for western blot analysis of pGR (Ser 234), GR, pJNK, and JNK. Brains were sliced into coronal sections using a chilled microdissecting block containing slots (0.5mm apart) for single-edged razor blades. The PFC and hippocampus were dissected from the sections using razor blades. All tissue was collected bilaterally and immediately placed on dry ice. The tissue was stored at -80°C until processing. On the day of extraction, tissue was homogenized in 10X its volume of radioimmunoprecipitation assay (RIPA)-like buffer (0.1% SDS, 1 mM sodium ortho-vanadate in 10 mM Tris) with a protease inhibitor cocktail (Roche's Complete Mini EDTA-free). Samples were centrifuged at 6000 rpm for 10 minutes, and the supernatant was collected in fresh tubes. To determine protein concentration, Pierce's BCA Protein Assay Kit was used. The protein samples were then mixed with 5X loading buffer (5% glycerol, 5% β -mercaptoethanol, 3% SDS and 0.05% bromophenol blue), heated in a heating block (set to 105°C) for 5 minutes, and subsequently stored at -20 °C.

Samples (20 ug) were separated by electrophoresis (160 V) on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred for 60 minutes at room temperature (100 V) onto Millipore's Immobilon-FL PVDF fluorescent compatible membrane. To determine total protein, membranes were incubated in REVERT[®] (LI-COR Biotechnology) for 5 minutes, washed 2 \times 2 minutes (in 6.7% glacial acetic acid and 30% methanol, in water) and imaged on a LI-COR Odyssey imaging system at 700 nm for 2 minutes. Membranes were then blocked for 1 hour in 0.5% fish gelatin (Sigma cat # 7041) in TBS, and incubated overnight at room temperature with one of the following primary antibodies (in 0.05% fish

gelatin in TBS with 0.1% tween): monoclonal rabbit anti-pJNK (1:1000; Cell Signaling cat #4668), polyclonal rabbit anti-JNK (1:1000; Cell Signaling cat #9252), monoclonal rabbit anti-pGR (1:1000; Cell Signaling cat #97285; reacts with GR phosphorylated at Ser 234 in mice, which is the phosphorylation site targeted by JNK), and monoclonal rabbit anti-GR (1:1000; Cell Signaling cat #3660). Membranes were washed 4 × 5 minutes in TBS and then incubated with anti-rabbit 800 infrared conjugate (1:20000; LI-COR Biotechnology) in 0.5% fish gelatin blocker with 0.2% tween and 0.01% SDS at room temperature for 1 hour. Membranes were then washed 4 × 5 minutes in TBS-T, 2 × 5 minutes in TBS, and read on the LI-COR Odyssey Fc system at 800 nm for 6 minutes.

4.3.5 Statistical analysis

Data were analyzed by 2 by 2 ANOVAs. For ANOVAs exhibiting a significant interaction, post hoc analyses using Fisher's PLSD were conducted to determine the exact nature of the relationship. Data were evaluated using a StatView (version 6.0) statistical software package available from the SAS Institute, Inc.

4.4 Results

4.4.1 Experiment 3.1a Investigating the role of JNK in the behavioral and neurochemical response to acute stress

4.4.1.1 Open field test

Although no significant interaction was apparent, a main effect for the JNK antagonist was evident for total distance moved in the open field, ($F_{1,27}=5.42$; Figure 4.2). Specifically, the inhibitor significantly reduced total distance moved compared to vehicle-treated controls in both the stressed and non-stressed mice ($p < 0.05$). However, the JNK antagonist did not significantly influence the number of entries into the inner square compared to vehicle-treated controls in both stressed and non-stressed mice ($p=0.07$; data not shown).

4.4.1.2 Elevated plus maze

Again no significant interaction was apparent for the JNK antagonist and acute stressor. However, there was a main effect for the stressor treatment with regard to the number of entries into the open arms of the elevated plus maze ($F_{1,26}=5.13$; Figure 4.3). The follow up comparisons indicated that there was a significant increase in number of open arm entries evident in the acutely stressed mice ($p < 0.05$) and although again no significant interaction was apparent, this effect was restricted to the vehicle-treated animals. Indeed, it is clear from Figure 4.3 that SP600125 treatment appeared to prevent the stressor induced elevation in open arm entries. In contrast, the stressor and JNK inhibitor did not significantly affect the number of entries into the closed arm of the elevated plus maze ($p > 0.05$; data not shown). The total number of arm entries was also unaffected by either treatment ($p > 0.05$; data not shown).

4.4.1.3 Corticosterone analysis

The effects of the JNK antagonist on corticosterone levels of mice subjected to an acute stressor were assessed. A significant JNK inhibitor \times stressor interaction was apparent ($F_{1,25}=6.024, p < 0.05$). As shown in Figure 4.4, post hoc analyses revealed that both the acute stressor and JNK antagonist individually increased corticosterone levels. Moreover, the corticosterone elevation was further enhanced in mice that received both treatments, such that hormonal levels significantly exceed that of the individually treated animals ($p < 0.05$).

4.4.1.4 Monoamine detection

Levels of NE and its metabolite, MHPG, were assessed in the central amygdala, PVN region of the hypothalamus, locus coeruleus, as well as the hippocampus and PFC. There were no significant monoamine variations within the PFC or hippocampus as a function of the stressor or JNK inhibitor treatments; hence, these data are not shown. Treatment with the JNK antagonist however did provoke significant differences in NE levels within the central amygdala ($F_{1,25}=5.00; p < 0.05$; Figure 4.5, top panel). Specifically, the antagonist reduced NE levels, relative to vehicle-treated controls ($p < 0.05$), and this effect was observed in both the stressed and non-stressed animals. However, there were no significant differences with regards to MHPG accumulation in the amygdala (Figure 4.5, top panel).

Within the PVN, the effect of the JNK antagonist on NE levels varied as a function of stress treatment ($F_{1,24}=5.41; p < 0.05$; Figure 4.5, middle panel). Indeed, post hoc comparisons revealed that the stressor treatment increased PVN NE levels compared to non-stressed vehicle controls ($p < 0.05$); and that this effect was attenuated by the JNK

antagonist ($p < 0.05$). Again, there were no significant differences in MHPG levels in the PVN following the various treatments (Figure 4.5, middle panel). However, the JNK antagonist did significantly increase MHPG levels within the locus coeruleus of both stressed and non-stressed mice ($F_{1,24} = 5.12$; $p < 0.05$; Figure 4.5, bottom panel), in the absence of any significant variations of NE levels.

4.4.2 Experiment 3.1b Inhibition of p38 prior to an acute stress

4.4.2.1 Corticosterone analysis

In a separate cohort of animals, the effects of JNK and p38 inhibition on corticosterone levels were examined in mice exposed to an acute stressor. The JNK inhibitor and p38 inhibitor animals were analyzed separately. For the JNK inhibitor, a significant JNK inhibitor x stressor interaction was once again observed ($F_{1,28} = 6.37$; Figure 4.6, top panel). Further post hoc analysis revealed that both the JNK inhibitor and the stressor significantly increased corticosterone levels ($p < 0.05$), but there was no further effect of stress on the corticosterone levels of the JNK inhibitor-treated animals. For the p38 inhibitor test, there was a significant main effect of stress on corticosterone levels, ($F_{1, 28} = 7.63$; Figure 4.6, bottom), wherein the stressor increased levels of corticosterone in both vehicle and p38 inhibitor-treated animals ($p < 0.05$). However, the p38 antagonist had no significant effect on corticosterone levels.

4.4.2.2 Western blot analysis

There were no significant differences between mice treated with vehicle and those treated with the JNK antagonist, SP600125, or the p38 antagonist, SP203580, in either of the control and stress conditions, on hippocampal levels of phosphorylated GR ($p > 0.05$).

4.4.3 Experiment 3.2: Ketamine's effects on JNK in the context of stressor exposure.

4.4.3.1 Nestlet test

Mice were assessed to determine if pre-treatment with ketamine (10 mg/kg) modified any stress-induced changes in nest building behavior. There were no effects of either ketamine or acute stress on the number of interactions with the nestlet ($p > 0.05$; Figure 4.8, top panel). A main effect of stress was found for the duration of time spent interacting with the nestlet ($F_{1,27} = 4.22$; Figure 4.8, bottom panel) whereby the acute stressor reduced interaction time in both vehicle and ketamine-treated groups ($p < 0.05$). Ketamine treatment, however, did not have any effects on interaction time ($p > 0.05$).

4.4.3.2 Western blot analysis

p-JNK and JNK

Within the PFC, there was a significant main effect of ketamine treatment on the levels of p-JNK ($F_{1,28} = 4.57$; data not shown) wherein ketamine treatment increased p-JNK levels ($p < 0.05$). Ketamine treatment also increased the levels of p-JNK relative to total JNK in this region ($F_{1,27} = 4.85$, $p < 0.05$; Figure 4.9, top panel). There was no effect of either ketamine treatment or stress on levels of total JNK.

Within the hippocampus, there were no effects of ketamine treatment or acute stress on levels of p-JNK, total JNK, or the ratio of p-JNK to total JNK ($p > 0.05$; Figure 4.9, bottom panel). For representative immunoblots, please see Figure A.2.

p-GR and GR

Within the PFC, there were no effects of ketamine treatment or acute stress on levels of p-GR, total GR, or p-GR relative to total GR ($p > 0.05$; Figure 4.10, top panel). Likewise, levels of p-GR, total GR, and p-GR relative to total GR remained unchanged by all treatment conditions in the hippocampus ($p > 0.05$; Figure 4.10, bottom panel). For representative immunoblots, please see Figure A.3.

4.5 Discussion

Stressor related disorders such as depression are associated with a wide array of biological changes, including monoamine alterations, deficiencies in neurogenesis and trophic factor signaling, as well as HPA axis abnormalities (Duman et al., 2001; Hayley et al., 2005; Bland et al., 2006; Anisman et al., 2009). A better understanding of the molecular pathways contributing to the negative effects of stress upon CNS functioning is essential for developing novel treatment strategies. In the present paper, we report data suggesting a tonic inhibitory role for the MAPK, JNK, in regulating central monoamine utilization, corticosterone release and behavioral activity. At the same time, however, the role of endogenous JNK appears to change in the context of acute stressors, wherein JNK inhibition attenuates certain features of the challenge. Importantly, inhibition of the alternate MAPK, p38, had no significant impact upon corticosterone alone or in the context

of the stressor, suggesting a selective role for JNK in this regard. With respect to our second goal, we found evidence that ketamine influences JNK activity, at least in the PFC, suggesting that JNK modulation could play a role in ketamine's antidepressant effects.

JNK is increasingly being recognized as playing a role in pathological processes associated with depression including neuroplasticity, inflammation, and HPA axis regulation (Jovicic et al., 2015; Hollos et al., 2018; Mohammed et al., 2018). We currently report that the JNK antagonist, SP600125, greatly increased circulating corticosterone levels. The fact that the JNK antagonist elevated corticosterone levels in both stressed and non-stressed mice suggests that JNK may be regulating basal HPA tone. In this regard, JNKs are known to be involved in the regulation and activity of GRs in the hippocampus, a region intimately involved with HPA axis regulation through negative feedback mechanisms (Davies et al., 2008). Moreover, in addition to JNK inhibiting GR expression, the converse also appears to hold true, wherein JNK activity is down-regulated by glucocorticoid binding to GR (Bruna et al., 2003). Thus, it is possible that bi-directional JNK-GR interactions could normally play a role in modulating basal HPA tone and possibly even reactivity to stressors.

Given that JNK has been reported to phosphorylate GR (Meller et al., 2003), one would expect JNK inhibition to result in decreased levels of phosphorylated GR. However, we presently did not find any differences in levels of phosphorylated GR within the hippocampus at 45 minutes post JNK inhibition. It is possible that such changes might have been present at other times. Indeed, a previous report indicated that the JNK antagonist provoked c-Jun phosphorylation after 15 min and this effect was absent by 1 hour (Bevilaqua et al., 2003). Alternatively, another possibility is that we missed subtle changes

in phosphorylation that might have been apparent in separate nuclear or cytoplasmic fractions. Indeed, when phosphorylated by JNK, GR is inhibited and remains in the cytoplasm, whereas, when activated, GR translocates to the nucleus where it acts as a transcription factor (Davies et al., 2008). We currently used whole hippocampal tissue punches (thereby combining all cellular fractions together), which might have reduced the signal to noise ratio.

Although a peripheral site of action cannot be presently ruled out, it is likely that the JNK inhibitor acted centrally since previous studies indicate that the drug had inhibitory effects in the brain within one hour following intraperitoneal injection (Wang et al., 2004; Yatsushige et al., 2007). Moreover, JNK3 could be the target underlying the present findings since this is the isoform that is enriched within the brain (Gupta et al., 1996). Although SP600125 inhibits all three JNK isoforms (Mehan, 2011), the fact that no significant difference was found in circulating corticosterone levels in JNK1-deficient mice (Unger et al., 2010), is consistent with a central JNK3 mechanism controlling HPA responsivity. Yet, one should not discount the possibility that JNK could modulate corticosterone release by acting directly on the adrenal glands since one previous finding did indicate that adrenal JNK expression was induced in response to ACTH binding (Watanabe et al., 1997).

The behavioral findings generally support the notion that JNK inhibition can influence exploration within the context of a stressful environment. Indeed, mice systemically injected with the JNK antagonist displayed decreased exploration in an open-field arena. Our open field findings could reflect a number of processes being affected by JNK inhibition, including those related to anxiety, locomotion or general malaise. The fact

that the JNK antagonist did not significantly reduce the number of entries into the inner zone of the arena argues against processes linked to anxiety being affected. In contrast, the significant reduction in total distance travelled (and velocity; data not shown) raises the possibility that JNK inhibition was somehow impacting locomotor capability or promoting malaise. Whatever the case, it is unlikely due to any direct neuronal damage given the short timeline of the study and since it has been reported that systemic SP600125 administration actually increased recovery of motor function after spinal cord injury (Yoshimura et al., 2011), suggesting that the inhibitor does not cause deficits in motor performance. Alternatively, reduced exploration might have been driven by some degree of malaise; however, gross qualitative observation of the animals did not reveal any obvious signs of sickness such as hunched posture, piloerection or ptosis. This is not to say that some “sub-clinical” sickness wasn’t apparent; indeed, we previously found that the corticoid changes elicited by immunological stressors (e.g. LPS) were accompanied by malaise (Hayley et al., 2005). The previous finding that SP600125 inhibited LPS induced prostaglandin production (de Oliveira et al., 2008), coupled with the evidence that hypothalamic and peripheral prostaglandins are important for sickness behaviors (Johnson et al., 1993), suggests that such mechanisms were not involved in the present behavioral findings.

In contrast to open field changes, the JNK antagonist alone did not significantly affect elevated plus maze performance, but it did reverse the stressor induced increased entry into the open arms. Increased entries into open arms in the elevated plus maze has been reported by many to be a measure of impulsivity (Szumlinski et al., 2001; Niimi et al., 2011). Hence, the increased entries into the open arms may have been a reflection of impulsivity in the group that was exposed to an acute stressor, and JNK inhibition may

have interfered with processes important in this regard. At this juncture, we are uncertain as to the exact mechanisms or processes that underlie or contribute to the complex, task-dependent behavioral consequences of JNK inhibition.

In addition to affecting HPA output, the JNK antagonist altered noradrenergic activity in stressor-sensitive brain regions, including the central amygdala, PVN and locus coeruleus. The fact that the JNK inhibitor, SP600125, influenced NE activity within the amygdala and locus coeruleus independent of any stressor effects, suggests that (like its HPA actions) endogenous JNK is potentially having a tonic regulatory influence over neurochemical activity in these brain regions. It is particularly interesting to note that (in contrast of the hippocampus) the amygdala has long been thought to have a primarily excitatory role upon hypothalamic CRH neurons, thereby facilitating corticosteroid release (Tanaka et al., 1983; Feldman et al., 1994). Similarly, locus coeruleus NE projections are known to impart excitatory effects upon limbic brain regions resulting in enhanced vigilance (Abercrombie and Jacobs, 1988; Chan-Palay, 1993). Hence, endogenous JNK could normally be acting to restrain such responses.

It is curious to note that the pattern of stressor + SP600125 induced hypothalamic changes paralleled the elevated plus maze changes but was in opposition to the corticoid effects. This divergent pattern of effects appeared to largely stem from the fact that the stressor alone appeared to preferentially affect hypothalamic NE and elevated plus maze behavior. Given that mice treated with the JNK antagonist did not differ from control on these measures suggests that inhibiting endogenous JNK can overcome stressor effects. Yet, in the absence of the stressor, the JNK antagonist itself clearly induced stressor-like effects (at least, with regards to corticosterone and open field exploration). These data

indicate that hypothalamic amine responses associated with JNK inhibition were clearly distinguishable from those of the amygdala and locus coeruleus; possibly, reflecting a divergent role for JNK in affecting neuroendocrine vs. anxiety/vigilance-relevant processes.

Findings from Experiment 3.2 appear to support our hypothesis that ketamine can modulate JNK activity in the absence of an immune challenge. Indeed, a single injection of ketamine provoked increased levels of p-JNK/JNK within the PFC without modifying levels of JNK protein. There is already some evidence that ketamine can modulate JNK activity in the context of immunostimulation and neuropathic pain. For example, in 2008, Wu et al. reported that ketamine suppressed the LPS-induced increase in JNK activity and subsequent AP1 activation, and ketamine was later reported to inhibit spinal nerve ligation-induced JNK activation (Mei et al., 2011). However, to the best of our knowledge, this is the first time ketamine has been reported to modulate JNK activity in the absence of direct immune stimulation. Since GR activation is associated with reductions in active (or phosphorylated) JNK, we are not entirely sure why we saw an increase in our experiment. That being said, we can speculate that one possibility may be through reduced NF- κ B signaling. Ketamine has been found to inhibit NF- κ B, and it has also been reported that inhibition of NF- κ B can lead to increased JNK activation (Nakano et al., 2004; Sun et al., 2004).

Ketamine was recently reported to increase activity of the GR (Wang et al., 2019) and therefore it was surprising that we did not find any effects of ketamine on levels of GR or pGR in the PFC or Hippocampus. Furthermore, since it has been established that JNK phosphorylates (and thereby deactivates) the GR, we would expect that the increased pJNK

in the PFC of ketamine-treated mice would have been associated with increases in pGR as well. As mentioned earlier, it is possible that had we separated cytosolic and nuclear fractions we may have been better able to detect small changes in this protein. Furthermore, although we did not find similar effects in the hippocampus, it is possible that had we looked at a different time point they may have been observed.

Finally, ketamine did not inhibit the stress-induced reduction in interaction time with the nestlet. This is not particularly surprising since findings from Chapters 2 and 3 both highlight the lack of ability of ketamine to modulate stress-induced deficits in exploratory behavior. This finding further supports the contention that ketamine's antidepressant effects are not related to acute stress reactivity.

4.6 Conclusions

To the best of our knowledge, this is the first report of behavioral, hormonal and neurochemical changes in response to systemic JNK antagonism alone and in the context of a psychologically relevant stressor. Although in stressful situations JNK might contribute to certain behavioral features and monoamine changes, its most robust effects appear to be evident in the absence of a stressor. Our results are consistent with a normal role for endogenous JNK in keeping HPA functioning in check under basal conditions and in the modulation of behavioral and monoamine activity. These data certainly speak to the need for further assessment of the potential importance of JNK and other MAPKs in the long-term consequences of stressors. It is also important to consider the possibility that selective JNK or MAPK targeting drugs could offer new therapeutic agents for treating

stressor disorders. Indeed, repeated stressors could sensitize HPA and monoamine activity resulting in psychiatric illness and we speculate that such increased sensitivity would be reflected in an exaggerated basal state that could stem from disruptions in endogenous JNK functioning. Finally, it appears that ketamine is able to modulate JNK activity in the absence of direct immune stimulation, at least within the PFC. Future studies that look to further classify the relationship between the JNK pathway and the antidepressant effects of ketamine are warranted.

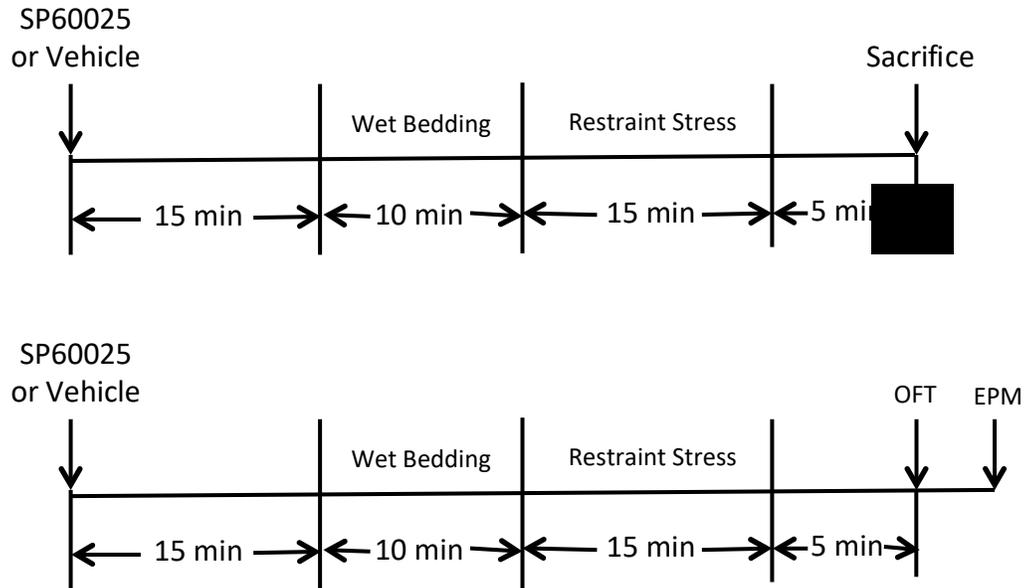


Figure 4.1. Timeline for Experiment 3.1a. Mice were injected with the JNK antagonist, SP60025 (30 mg/kg, i.p.), or vehicle (DMSO diluted with saline to a ratio of 1:4) 15 minutes prior to stressor exposure (10 minutes of wet bedding followed by 15 minutes of restraint stress). Mice in cohort 1 (top) were sacrificed and blood and brain tissue was collected for analysis. Mice in cohort 2 (bottom) were subjected to behavioral testing. OF = open field test; EPM = elevated plus maze.

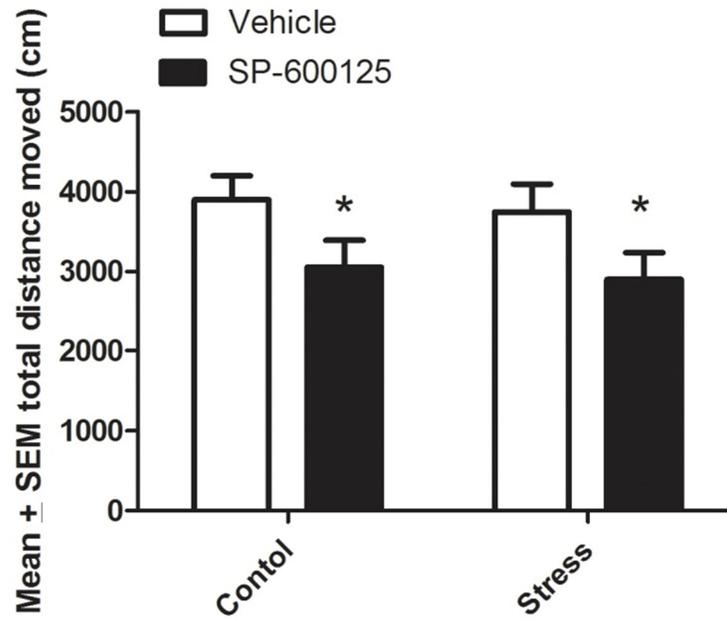


Figure 4.2. Effects of JNK antagonism and acute stress in the open field test. Mice that received the JNK antagonist, SP600125, displayed a significant reduction of total distance moved in the open field compared to vehicle-treated controls. * $p < 0.05$, relative to vehicle-treated mice. Data expressed as means \pm SEM.

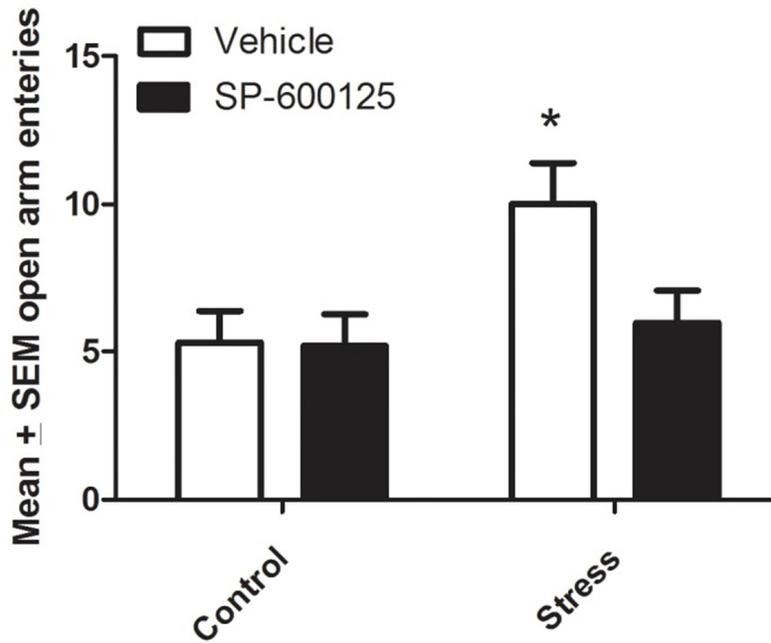


Figure 4.3. Effects of acute stress and JNK inhibition on performance in the elevated plus maze. Vehicle-treated mice that were subjected to an acute stressor displayed increased entries into the open arms of the plus maze, relative to the non-stressed or JNK antagonist (SP600125)/stress groups. * $p < 0.05$, relative to all other groups. Data expressed as means \pm SEM.

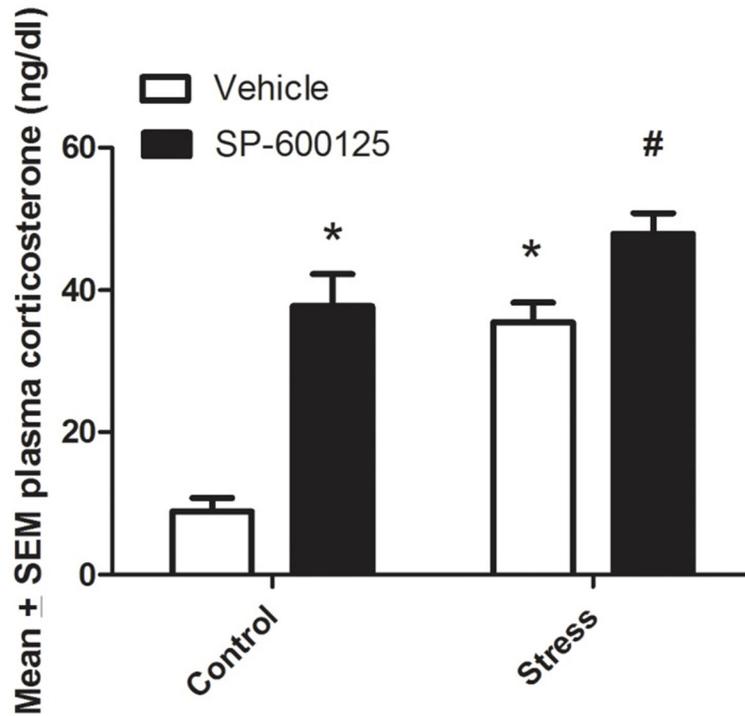


Figure 4.4. The effect of the JNK antagonist, SP600125, on corticosterone levels varied as a function of stressor treatment. The JNK antagonist and stressor individually increased corticosterone levels. However, the corticosterone rise was most pronounced in mice that received both the JNK antagonist plus the stressor. * $p < 0.05$, relative to vehicle control group; # $p < 0.05$, relative to vehicle stress group. Data expressed as means \pm SEM.

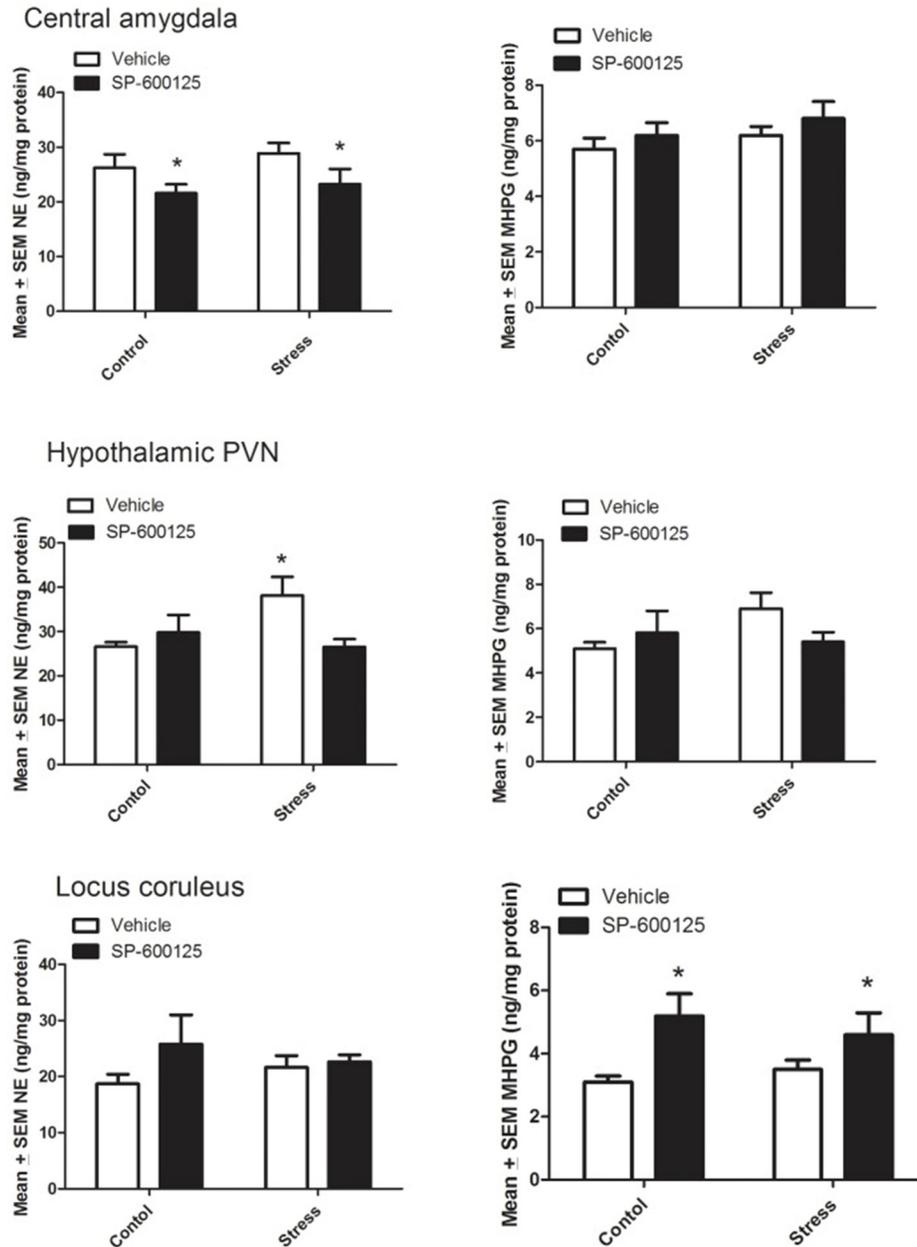


Figure 4.5. Effects of JNK antagonism and acute stress on levels of central monoamines. The JNK antagonist, SP600125, significantly reduced NE levels in the central amygdala of both non-stressed and stressed animals compared to vehicle-injected controls (top panel, left; $*p < 0.05$, relative to vehicle-treated mice). The stressor-induced elevation of hypothalamic NE levels was prevented by SP600125 treatment (middle panel, left; $*p < 0.05$, relative to non-stressed controls). The JNK antagonist significantly increased levels of the NE metabolite, MHPG, in locus coeruleus of both non-stressed and stressed mice (bottom panel, right; $*p < 0.05$, relative to vehicle-treated mice). Data expressed as means \pm SEM.

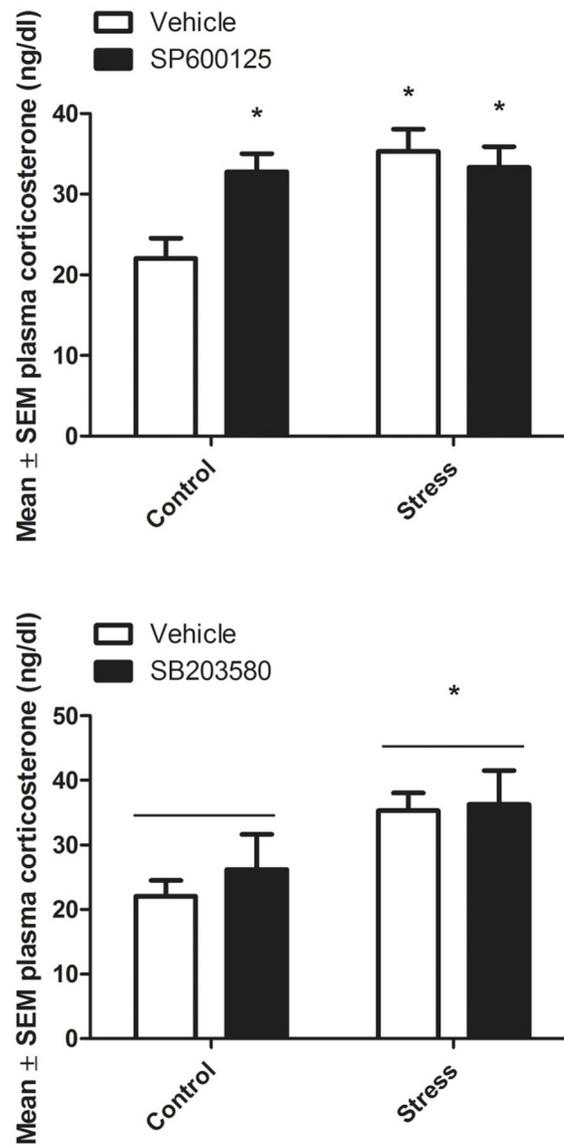


Figure 4.6. Effects of JNK and p38 inhibition on plasma corticosterone in the context of acute stress. The JNK antagonist, SP600125, and stressor exposure increased corticosterone levels compared to vehicle-treated controls, $*p < 0.05$, relative to vehicle-treated control group (top). In contrast, the p38 inhibitor, SB203580, did not affect corticosterone levels in any group (bottom). $*p < 0.05$, relative to non-stressed controls. Data expressed as means \pm SEM.

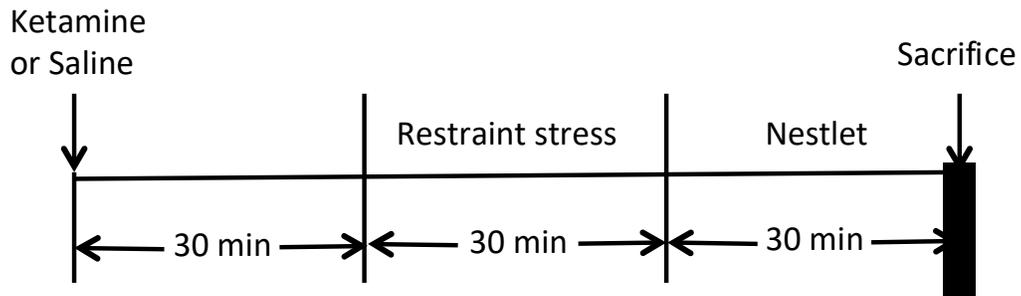


Figure 4.7 Timeline for Experiment 3.2. Mice were treated with ketamine (10 mg/kg, i.p.) or saline 30 minutes prior to an acute restraint stress. Mice were subsequently given a fresh nestlet and their nestlet interactions were recorded for 30 minutes. Mice were then sacrificed and their brain tissue was collected for western blot analysis.

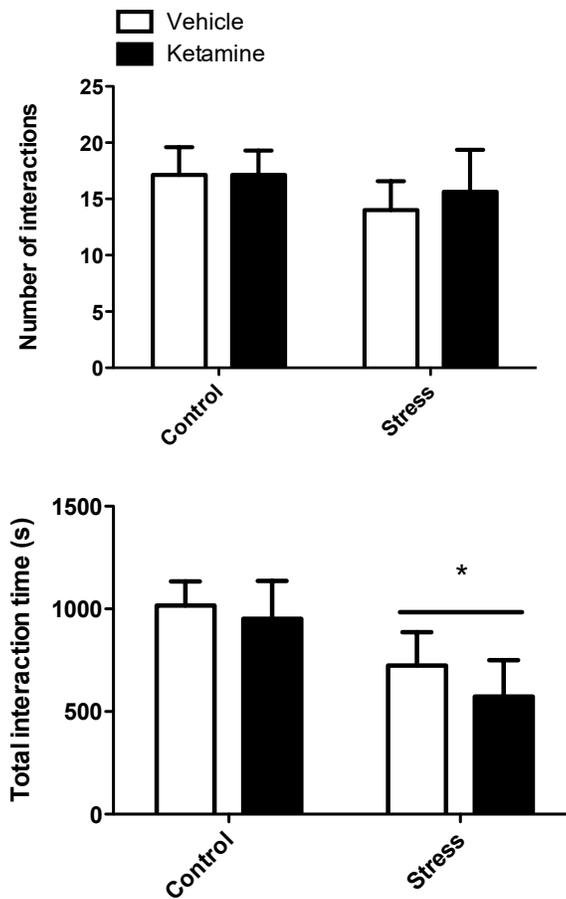


Figure 4.8. Effects of ketamine and acute restraint stress on nestlet behavior. There were no effects of either ketamine treatment (10 mg/kg; i.p.) or acute restraint stress on the number of interactions with the nesting material (top panel). In contrast, acute stress reduced the duration of time spent interacting with the nesting material in both vehicle and ketamine-treated mice (bottom panel). Data expressed as means \pm SEM. * $p < 0.05$, relative to non-stressed controls.

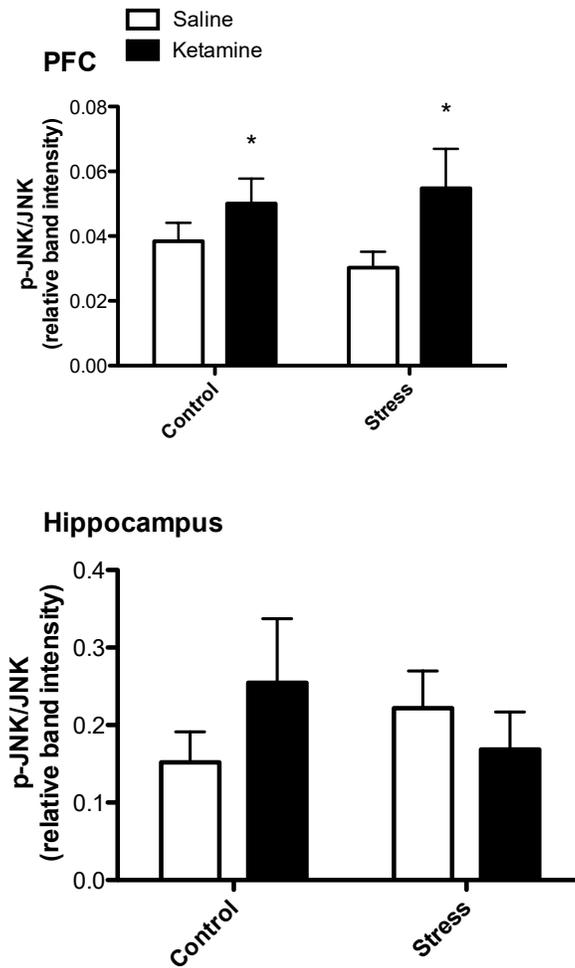


Figure 4.9. Effects of ketamine and restraint stress on JNK activity in the PFC and hippocampus. A single ketamine treatment (10 mg/kg; i.p.) increased levels of p-JNK (relative to total JNK) in the PFC (top panel), but had no significant effect on p-JNK levels (relative to total JNK) in the hippocampus (bottom panel). There was no effect of stress in either brain region. Data expressed as means \pm SEM. * $p < 0.05$, relative to saline-treated mice.

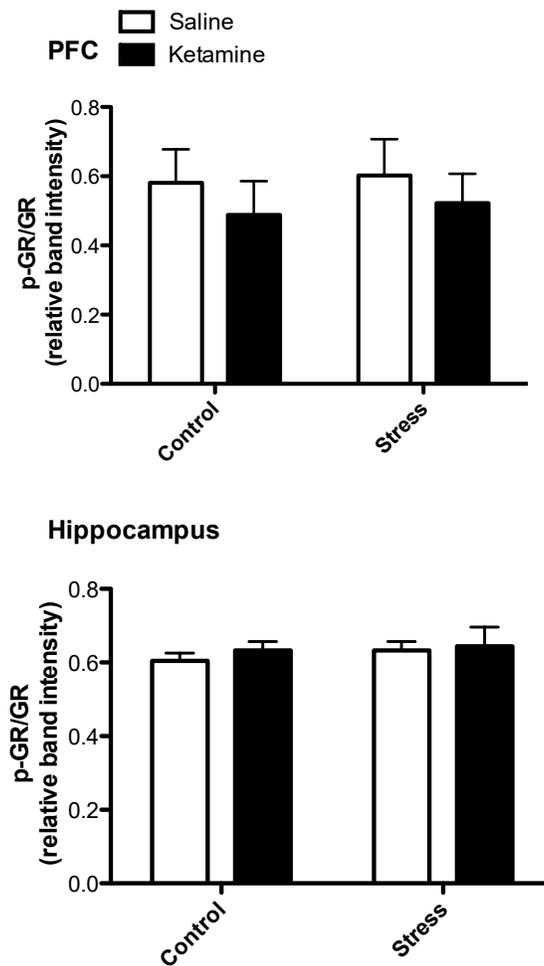


Figure 4.10. Effects of ketamine and restraint stress on GR activity in the PFC and hippocampus. There were no effects of either acute stress or a single ketamine treatment (10 mg/kg, i.p.) on levels of p-GR (relative to total GR) in the PFC (top panel) and hippocampus (bottom panel). Data expressed as means \pm SEM.

Chapter 5. General Discussion

In the present thesis, we were predominantly concerned with increasing our understanding of the mechanisms underlying ketamine's rapid antidepressant effects. Specifically, we wanted to test our hypothesis that ketamine would influence multiple processes that have been implicated in the pathophysiology of depression, including central monoamine activity, neuroplasticity and neurogenesis, inflammation, and HPA axis regulation. To this end, in Chapters 2 and 3, we tested our hypotheses that both acute and repeated ketamine treatment would modify the neurochemical, neuroplastic, hormonal, and behavioral consequences of acute and chronic stressors, respectively. We also began an investigation into the possible influence of the JNK pathway in ketamine's antidepressant effects in Chapter 4, by first exploring the outcome of JNK inhibition on neurochemistry and behavior, and then by testing our hypothesis that ketamine would act upon JNK and its stress-relevant downstream targets.

Although it is generally accepted that the monoamine hypothesis of depression is overly simplified, monoamines are still recognized as important for mood regulation (Yoon et al., 2017; Wang et al., 2018) and we cannot argue against the fact that for many patients, increasing the levels of certain monoamines leads to remission of depressive symptoms. Surprisingly, before our work began, very little was known about how ketamine affected central monoamine levels, especially within the context of stressor exposure. When considering the entirety of monoaminergic data from Chapters 2 and 3, we found that generally ketamine did not have a remarkable impact on the stress-induced changes to levels of NE, 5-HT or their respective metabolites, MHPG and 5-HIAA, in the PFC, hippocampus or hypothalamus. This indicates that ketamine was not altering the

neurochemical response to stressors. That being said, ketamine did affect serotonergic activity within the three brain regions at various points in Chapters 2 and 3, which is in line with a subsequent report that ketamine promotes 5-HT utilization in multiple brain regions (Thelen et al., 2016). It has also been reported that 5-HT is important for ketamine's antidepressant effects (Zhang et al., 2017).

In Chapter 2 we also found that ketamine had no effect on the stress-induced corticosterone elevations that were provoked by both the acute restraint stress and LPS. Moreover, ketamine did not alter corticosterone levels under any conditions whatsoever in Chapters 2 or 3. This important finding further supports our contention that ketamine is not affecting acute stress reactivity, and therefore its rapid antidepressant effects likely stem from other pathways. In this regard, we reported in Chapters 2 and 3 that ketamine blunted the LPS-induced elevations in two depression-relevant inflammatory cytokines (IL-1 β and TNF- α), and also increased hippocampal neurogenesis, both of which will be discussed shortly.

Anxiety is frequently comorbid with depression, and many antidepressants are also effective in treating anxiety symptoms. It was of interest to assess the ability of ketamine to modify stress-induced behavioral changes over a number of anxiety-sensitive tests. Surprisingly, ketamine failed to blunt any of the stress-induced behavioral changes across all Chapters and tests. Moreover, ketamine itself had anxiety-like effects in some tests, suggesting that ketamine does not act as an anxiolytic agent, and instead may promote anxiety-like behavior. Although there are reports of acute ketamine treatment having anxiolytic effects in rodents (Silvestre et al., 2002), which is what we expected to find, opposite effects have also been reported (Baber et al., 2001; da Silva et al., 2010).

Furthermore, reports from clinical trials actually list anxiety as one of the most common side effects of ketamine treatment for depression (Short et al., 2017; Aust 2019). The literature also suggests that the anxiogenic effects of ketamine are transient, lasting only a couple of hours (Bobo et al., 2016). It is therefore interesting to note that in our repeated ketamine injection study in Chapter 3, we found increased anxiety-like behavior among the ketamine-treated mice 24 hours after the last ketamine injection. Based on our findings, it appears that ketamine may have anxiogenic effects that persist beyond its duration of action. A study published in 2009 by Engin et al. found anxiolytic effects of ketamine at 50 mg/kg, but not at 10 mg/kg, which is the dosage used in our studies. It is therefore possible that ketamine may have an anxiogenic effect at lower doses, whereas higher doses yield anxiolytic effects.

When the research trials with ketamine began, patients typically received a single infusion of the drug (Berman et al., 2000; Zarate et al., 2006). Although the infusions were very effective at relieving depressive symptoms, inevitably patients would relapse, usually within a week (Berman et al., 2000; Zarate et al., 2006; Ibrahim et al., 2012). Naturally, repeated ketamine-dosing schedules began to be used in an effort to sustain remission, however the question of whether or not repeating treatment would prolong ketamine's effects, or even reduce them over time, remained for the most part unanswered.

In Chapter 3 we found that a single ketamine injection had antidepressant-like effects in the FST one hour, but not 48 hours, after treatment. Importantly, when we looked at FST performance after repeated ketamine treatment (three injections administered over a two-week period), we found that significant antidepressant-like effects were still detectable 2 days and 8 days after the last ketamine injection. This clearly indicates that

repeated ketamine treatment may have a more durable effect than a single treatment, and patients might still benefit from treatments that are spaced farther apart. Our findings support those from Murrough et al., (2013) who investigated the consequences of multiple (up to six) ketamine infusions in depressed patients, and suggested that repeated infusions might promote more lasting antidepressant effects than a single infusion, although this study lacked a group receiving only a single infusion for direct comparison.

Being able to prolong the interval before depressive symptoms return has great clinical value and is an active area of research (Costi et al., 2019; Hasler, 2019). At this point, the main use for repeated ketamine treatment would be to elicit relief from depressive symptoms until a less invasive strategy can be implicated, since not only are the infusions costly and inconvenient, but the safety of repeated ketamine has not been established (Hasler, 2019). In fact, there are researchers and clinicians directly involved in ketamine treatments who express concerns about the safety of long-term use (Murrough et al., 2013; Moda-Sava et al., 2019).

There is evidence that repeated administration of NMDA receptor antagonists, including ketamine, may have neurotoxic effects in rodents (Olney et al., 1989; Olney et al., 1991), and prolonged abuse of ketamine can lead to deleterious brain changes (Liao et al., 2010; Liao et al., 2011). Functionally, chronic ketamine use has been associated with decreased performance on memory tasks in humans (Morgan et al., 2010) and animals (Tan et al., 2011). It was therefore reassuring that we did not observe any cognitive deficits associated with repeated ketamine treatment in the two-trial Y-maze test in Chapter 3. This discrepancy could be partly due to the relatively low dose that we used in our studies. Additionally, we used only three ketamine treatments (less than 6, which is currently

standard protocol at the majority of ketamine clinics in North America), and therefore additional studies looking at longer-term exposure to antidepressant-relevant doses of ketamine are needed before definitive conclusions regarding any effects on cognition can be drawn.

It is known that patients with depression show signs of hippocampal atrophy and neuronal loss (Sheline et al., 1996; Czeh et al., 2001; Colla et al., 2006). This, coupled with the fact that traditional antidepressants increase neurogenesis, and neurogenesis is suspected to be essential for the behavioral effects of antidepressants, implies that neurogenesis may be an important factor in depression pathology and recovery (Taupin, 2006; Boldrini et al., 2012; Miller and Hen, 2015). Although evidence suggests that the antidepressant effects of ketamine are mediated by synaptic plasticity in the PFC, likely through activation of the mTOR pathway (Li et al., 2010; Li et al., 2011), the effects of ketamine on hippocampal neurogenesis have been less clear. Certainly at anesthetic doses, ketamine appears to be neurotoxic to the developing animal brain (Slikker et al., 2007; Shi et al., 2010; Liu et al., 2011; Paule et al., 2011) and ketamine was found to reduce postnatal neurogenesis in rodents (Huang et al., 2016). One study also reported that ketamine had no effect on neurogenesis (Tung et al., 2008). However, at the time of our current investigation, the majority of the literature surrounding ketamine and neurogenesis was based on anesthetic doses, given acutely, and often in developing animals. Therefore it was of particular interest to assess neurogenesis in our repeated, subanesthetic ketamine protocol.

In Chapter 3, we found that repeated ketamine treatment led to increased neurogenesis in the DG of the hippocampus. Our findings are reassuring that when given

at a subanesthetic dose, ketamine appears to promote rather than reduce neurogenesis. Since our results were published, there have been other reports of subanesthetic doses promoting neurogenesis (Ma et al., 2017; Michaelsson, 2018), although these were based on single injections. The complete story of ketamine's impact on neurogenesis is not straightforward, and it may be that while high doses of the drug can in fact be neurotoxic, low or subanesthetic doses may promote neurogenesis. Our work contributes to the growing body of evidence indicating that drug dosage is a key factor in how glutamate activity can foster both neurogenesis and neuronal toxicity (Winkelheide et al., 2009; Rubio-Casillas and Fernández-Guasti, 2016).

BDNF is a trophic factor that is strongly associated with hippocampal neurogenesis (Huang and Reichardt, 2001). Since ketamine has been found to increase levels of BDNF in animals, and it has been reported that ketamine's antidepressant effects are dependent upon its synthesis (Autry et al., 2011), we have reason to suspect that ketamine could increase neurogenesis through its regulation of BDNF. In this regard, ketamine might increase BDNF by de-suppressing its translation indirectly via the dephosphorylation and subsequent activation of eukaryotic elongation factor 2 (eEF2) (Garcia et al., 2008; Zunszain et al., 2013). Ketamine has also been shown to inhibit GSK-3, a protein kinase whose activity has been connected to impaired neurogenesis, and itself is a negative regulator of BDNF. Therefore it is reasonable to suspect that ketamine could also be increasing BDNF through its inhibition of GSK-3. Follow up studies should attempt to clarify if ketamine's antidepressant effects are dependent on neurogenesis, and if so, what role the above-mentioned proteins may play.

Given the fact that inflammatory processes have been implicated in depression pathology, it was of interest to assess the ability of ketamine to modify the impact of an immunological stressor (LPS) on sickness behavior, inflammatory cytokines, and neurochemistry. As seen in Chapter 2, ketamine had no effects on the sickness symptoms or elevated corticosterone levels caused by LPS. In contrast, ketamine dose-dependently blunted the LPS-induced increase in two proinflammatory cytokines, IL-1 β and TNF- α . The fact that ketamine did not have any effects on the LPS-induced increases of any other measured cytokines, namely IL-6, IL-10, IFN- γ and GM-CSF, indicates that ketamine may be acting quite selectively.

Notably, IL-1 β and TNF- α have been associated with NF- κ B signaling, whereas the others act mainly through the JAK-STAT signaling pathway (Mercurio and Manning, 1999; Kiu and Nicholson, 2012). Hence our findings are particularly interesting since NF- κ B has been shown to play an important role in depression-related pathology, including impaired neurogenesis and synaptic plasticity (Koo et al., 2010; Caviedes et al., 2017), and these particular processes have been linked to ketamine's antidepressant effects (Chapter 3 of this document; Li et al., 2010; Ma et al., 2017). There is evidence that ketamine inhibits LPS-induced NF- κ B activity (Taniguchi and Yamamoto, 2005) even at antidepressant-relevant doses (Yu et al., 2007). Therefore, a useful next step would be to determine to what extent, if any, ketamine's antidepressant effects are dependent upon NF- κ B signaling. This information could provide valuable insight for the investigation of new therapeutic targets.

Interestingly, in addition to negatively regulating neurogenesis, GSK-3 also promotes inflammation (Jope et al., 2017). Furthermore, aberrant GSK-3 regulation is

thought to underlie the pathology of mood disorders such as depression, and the inhibitory control of GSK-3 is an important feature of antidepressant treatments (Li and Jope, 2010; Jope et al., 2017). Given its implication in both inflammation and neuroplasticity, and the fact that in Chapters 2 and 3 we found that ketamine was able to modulate stress-induced changes in these two processes, we speculate that GSK-3 might play a critical role in ketamine's antidepressant effects. Indeed, since the publication of our ketamine studies, there has been some published support of this hypothesis (Zanos and Gould, 2018).

That the JNK signaling pathway might play a role in the pathophysiology of depression has been a relatively recent idea. Indeed, reports describing a connection between the two are now emerging, and it is becoming increasingly clear that JNK may have a place in antidepressant research (Holloos et al., 2018). In Chapter 4 we reported that JNK inhibition, but not p38 inhibition, caused marked elevation in plasma corticosterone, similar to the increase seen with acute psychogenic stressor exposure. Moreover, stressed mice given the JNK inhibitor showed an even further increase in corticosterone than either treatment alone. This finding was quite surprising, as we had hypothesized that the JNK inhibitor would blunt the corticosterone response to acute stress based on the understanding that JNK is activated in response to acute stress, and its inhibition has been shown to have antidepressant-like effects in the tail suspension test (Galeotti and Ghelardini 2012).

Since JNK regulates GR activity in the hippocampus, and the GR is an important regulator of HPA axis activity (Davies et al., 2008), we suspect that through its interactions with the GR, JNK may have a tonic inhibitory role on HPA axis activity under basal conditions. Although ketamine did not alter corticosterone levels in any of our tests, it is possible that ketamine administration could theoretically modulate the spike in

corticosterone caused by JNK inhibition through its own interaction with the GR. Indeed, ketamine has very recently been shown to improve depressive-like behaviors in chronically stressed mice, and that effect was associated with the rescue of both stress-induced GR reductions, and reductions in the nuclear translocation of the GR (Wang et al., 2019). In Chapter 4 we also found that ketamine increased the ratio of p-JNK/JNK within the PFC. Active (or phosphorylated) JNK phosphorylates and thereby deactivates the GR, preventing its translocation to the nucleus. Interestingly, the increased nuclear translocation of GR caused by ketamine treatment has also been associated with reductions in corticosterone (Wang et al., 2019), and therefore provides support for our hypothesis. This could have significant value for research into novel therapeutic targets. One caveat here is that the ketamine-induced increase in p-JNK was evident only in the PFC and not the hippocampus. However, this does not mean that hippocampal JNK activity was not affected, and it could simply have been undetected at the time-point and with the fractions analyzed.

In Chapter 4 we also found no changes in the levels of p-GR after JNK inhibition or stressor exposure. Since JNK normally phosphorylates GR, we expected to see a reduction in p-GR, and our findings do not exclude the possibility that we may have simply missed detecting any alterations at our particular end point. It is also possible that the effects of JNK inhibition on GR phosphorylation may have been picked up had we separated nuclear and cytoplasmic fractions, since p-GR typically resides in the cytosol whereas dephosphorylated GR translocates to the nucleus.

Conclusions

Taken together, the data presented here indicate that ketamine is generally not modulating the behavioral, neuroendocrine, or neurochemical response to acute or chronic stressors, and therefore its rapid antidepressant effects must stem from other avenues. Indeed, our data suggest that instead ketamine can modify neuroplastic and inflammatory processes, and furthermore it is able to blunt the chronic and systemic stressor-induced changes to both. Ketamine treatment also led to increased levels of p-JNK, which itself may be a tonic inhibitor of HPA axis activity, and it is therefore suspected that ketamine's antidepressant effects might involve its interaction with this pathway.

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Appendix A. Supplementary figures

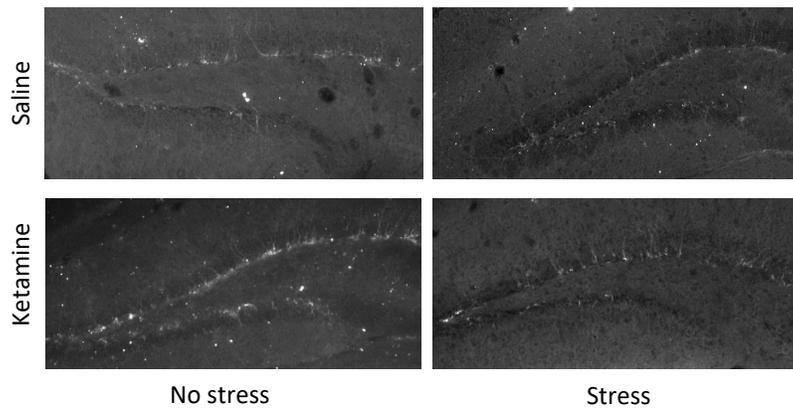


Figure A.1. Representative images of the effects of repeated ketamine treatment (10 mg/kg) and CUS on DCX + cells in the central portion (bregma $- 1.82$ to $- 2.46$) of the hippocampal DG.

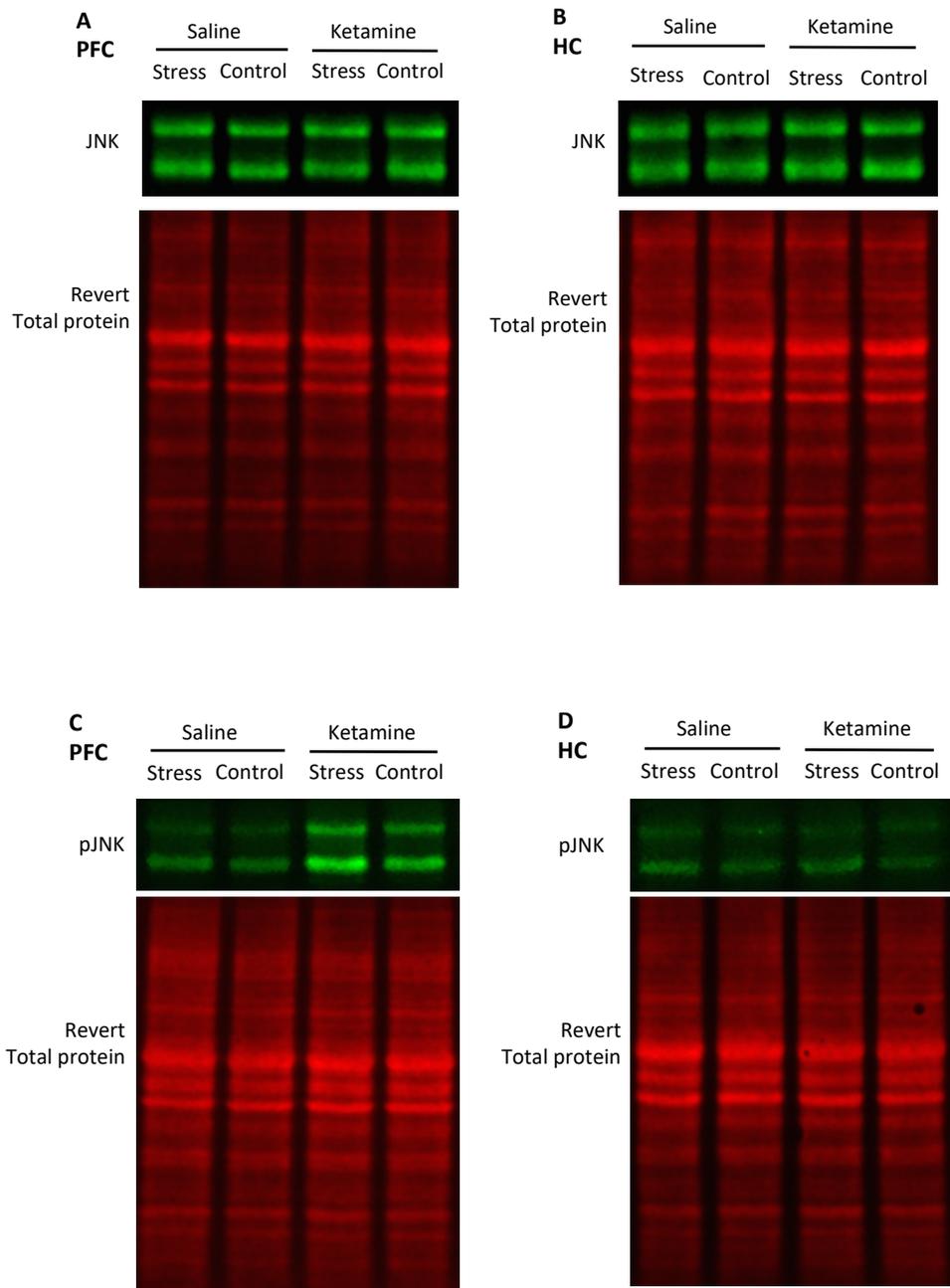


Figure A.2. Representative immunoblots for the effects of ketamine (10 mg/kg) and restraint stress on levels of JNK in the PFC (A) and hippocampus (B), and levels of pJNK in the PFC (C) and hippocampus (D). PFC = prefrontal cortex; HC = hippocampus.

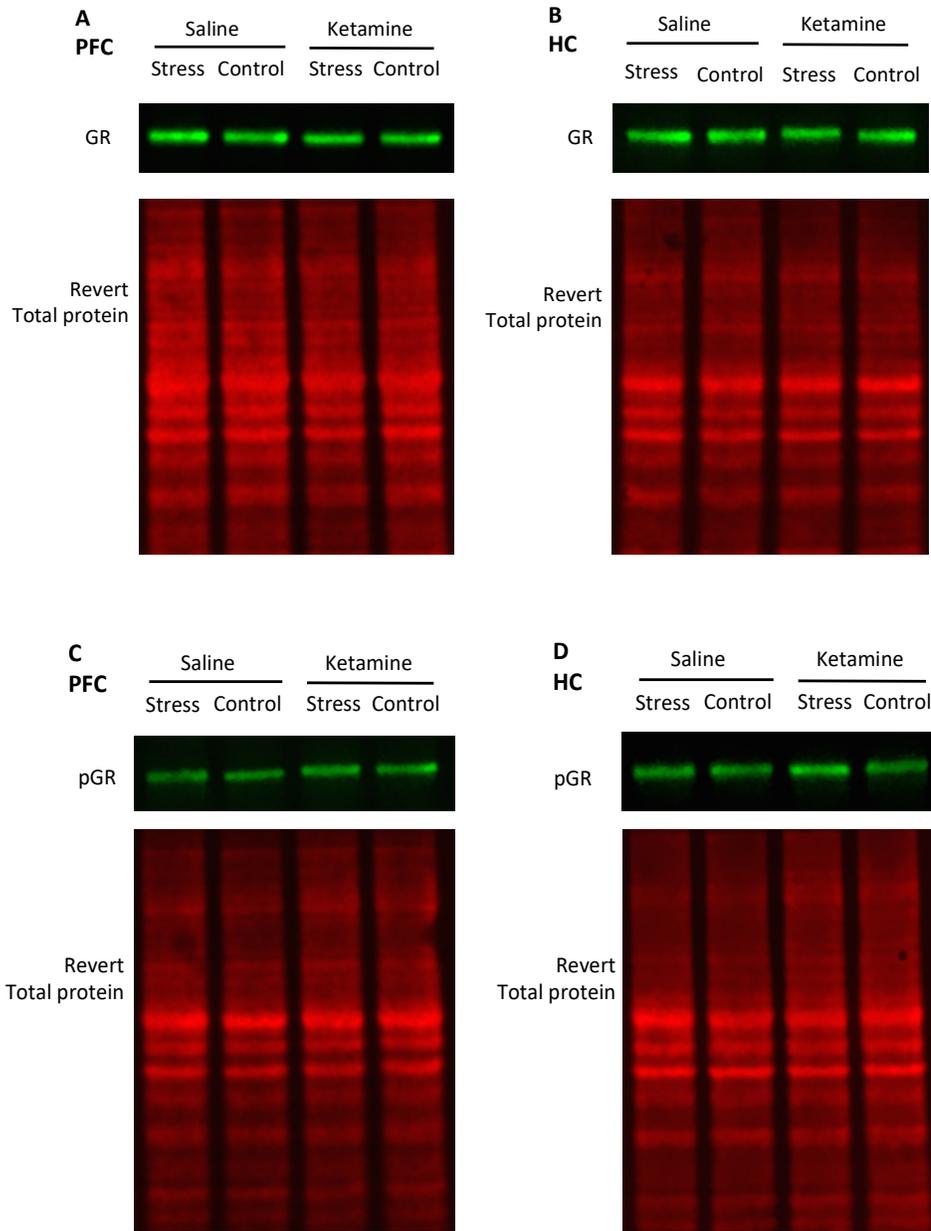


Figure A.3. Representative immunoblots for the effects of ketamine (10 mg/kg) and restraint stress on levels of GR in the PFC (A) and hippocampus (B), and levels of pGR in the PFC (C) and hippocampus (D). PFC = prefrontal cortex; HC = hippocampus.