Effects of Ovarian Hormone Replacement on the Ovariectomized (OVX) Female Rat Response to Ketamine

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ABSTRACT

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Ketamine has been shown to acutely and rapidly ameliorate depressive symptoms and suicidality. Given that women suffer from depression at two-times the rate of men, the current work first reviews the literature at the intersection between two subjects: the neurobiological mechanisms of ketamine's antidepressant effects, and how ovarian hormones might interact with these phenomena. The neuroinflammatory hypothesis of depression is emphasized. Next, we investigated how the ovarian hormones 17β-estradiol and progesterone interact with ketamine's effects on ovariectomized female Wistar rat behavior in the forced swim test. A secondary experiment analyzed the effects of ketamine on males, also in the forced swim test. No significant interaction or main effects were found among the females, whereas ketamine significantly reduced immobility among the males. Locomotor activity recordings confirmed that ketamine's effects in the forced swim test were not due to overall locomotor suppression.

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Abbreviations

5-HT	Serotonin
AKT	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CA1-3	*Cornu Ammonis* layers 1, 2, and 3 of the hippocampus
CD	Cluster of differentiation
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450 enzymes,
DG	Dentate gyrus region of the hippocampus
E2	17β-estradiol
EB	Estradiol benzoate
ECT	Electro-convulsive therapy
eEF2	Eukaryotic elongation factor 2
ER	Estrogen receptors
FDA	Federal drug administration
FST	Forced swim test
GABA	λ-aminobutyric acid
GPER1	G-protein-coupled estrogen receptor 1
HNK	Hydroxynorketamine
HPC	Hippocampus
HRT	Hormone replacement therapy
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
KYNA	Kynurenic acid

LPS	Lypopolysaccharides
MADRS	Montgomery–Åsberg depression rating scale
MAPK	Mitogen-associated protein kinase
MAPR	Membrane-associated progesterone receptor
MDD	Major depressive disorder
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-Aspartate
NO	Nitric oxide
OVX	Ovariectomy
P	Progesterone
PFC	Prefrontal cortex
PGRMC1	Progesterone receptor membrane component 1
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PR	Progesterone receptors
PMDD	Premenstrual dysphoric disorder
PSD	Post-synaptic density protein
QUIN	Quinolinic acid
SSRI	Selective-serotonin reuptake inhibitors
SPT	Sucrose preference test
TBI	Traumatic brain injury
TLR	Toll-like receptors
TRD	Treatment-resistant depression
TREM2	Triggering receptor expressed on myeloid cells 2
TNF	Tumor necrosis factor
TrkB	Tropomyosin receptor kinase B
TSST	Trier social stress test

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Estrogen & depression

An analysis of a World Health Organization research program, known as The Global Burden of Disease, shows that depression is one of the three top causes of non-fatal health loss globally (James et al., 2018) Sex differences in mood disorder prevalence rates begin to appear during adolescence (Buchanan, Eccles, & Becker, 1992; Seeman, 1997). There are many theories as to why this discrepancy between the sexes occurs. One line of evidence suggests it is, at least in part, due to shifts in ovarian hormone levels. Estrogens are a family of ovarian hormones of which there are three subtypes: estrone, estradiol, and estriol. The most potent estrogen, in terms of its affinity for estrogen receptors in both rats and humans, is 17β-estradiol (E2; Heldring, et al., 2007). The ovaries synthesize and release E2 in a cyclical manner, peaking during ovulation. In both sexes, the adrenal glands also synthesize and release E2; neurons and glia in the central nervous system (CNS) can also produce E2 via aromatization from testosterone (McCarthy, 2008). Like E2, progesterone (P) is produced in the ovaries, specifically by the corpus luteum, a temporary gland formed during the ovulatory phase of the human menstrual cycle and after the proestrus phase of the rat estrus cycle. P is also produced by the placenta, once it's formed during gestation, the adrenal glands, and again by cells in the CNS where it's formed by steroidogenesis from cholesterol in both males and females (González-Orozco & Camacho-Arroyo, 2019; McCarthy, 2008).

Douma and colleagues (2005) suggest that estrogen withdrawal, continued deficit, or changes throughout the menstrual cycle are correlated with mood-related distress (Yim, Stapleton, Guardino, Hahn-Holbrook, & Schetter, 2015). This idea is also supported by multiple research paradigms demonstrating a link between post-partum depression and E2 (Ahokas, Kaukoranta, Wahlbeck, & Aito, 2001; Bloch, Schmidt, Danaceau, Murphy, Nieman, &

Rubinow, 2000; Moses-Kolko, Berga, Kalro, Sit, & Wisner, 2009). Premenstrual dysphoric disorder (PMDD) further illustrates how ovarian hormones and depression are correlated (Fava et al., 1992; Pearlstein, 1995).

PMDD affects 3-8% of premenopausal women and can repeat monthly. Symptoms, such as depressive mood, arise in the luteal phase when P and estrogens are on the rise (Rapkin & Lewis, 2013). Importantly, the hormonal profiles of women with PMDD are indistinguishable from those without (Hantsoo & Epperson, 2015). This suggests that PMDD sufferers are perhaps more reactive to ovarian hormones at a neurobiological level. Other evidence demonstrating a hormone-depression link is that of menopause and hormone replacement therapy (HRT).

In the past, there have been a substantial proportion of postmenopausal women making use of HRT at one point in their lives, with some estimates being as high as 38% (Keating, Cleary, Rossi, Zaslavsky, & Ayanian, 1999). Today, due to efforts demonstrating associations between HRT and various negative health outcomes, postmenopausal women will typically receive HRT between the ages of 50-59 (or <10y post-menopause) and for shorter time periods (Chlebowski et al., 2010; Lobo, Pickar, Stevenson, Mack, & Hodis, 2016; Manson et al., 2013; Santen et al., 2010). In such cases, HRT is typically comprised of estrogens. Progestins, such as P, can also be administered either alone or in tandem with estrogens (Keating et al., 1999; Nelson, Humphrey, Nygren, Teutsch, & Allan, 2002). The effects of HRT on depressed mood during menopause are promising. One meta-analysis of 26 studies reveals that estrogens have a considerable effect size (d = .69) towards decreasing depressed mood, whereas progesterone solely or in tandem with estrogens was associated with less potent effects (Zweifel & O'Brien, 1997). Considering all the information, it is clear that ovarian hormones may worsen or improve depressive symptoms, but the direction of the relation changes due to various factors.

Understanding the basic theoretical framework behind the etiology of depression is therefore of critical importance.

The neurobiology of depression

One of the more prolific, but dated, theories behind the etiology of depression is known as the monoamine hypothesis of depression. To put it simply, proponents of this theory suggest that major depressive disorder (MDD) is caused by depleted levels of the monoamine neurotransmitters dopamine, norepinephrine, and serotonin (Hirschfeld, 2000; Nutt, 2008). While there are paradigms which support this theory, for example monoamine depletion diets (Ruhé, Mason, & Schene, 2007), there are multiple research contexts in which it is not supported (Nestler et al., 2002). For example, antidepressants induce a rapid increase in monoaminergic transmission, yet therapeutic effects usually take two to six weeks to begin (Wong & Licinio, 2001). Supporters of this theory will point to the fact that classical antidepressant drugs, such as monoamine-oxidase inhibitors, tricyclic antidepressants, and selective-serotonin-reuptake inhibitors, act on monoamine systems. However, plenty of evidence suggests antidepressants are simply ineffective. In general, antidepressant drug efficacy rates are low, with only one third of patients experiencing a therapeutic effect on their first attempt. Any given individual may be required to try several different types of antidepressants before therapeutic effects are achieved (Trivedi et al., 2006; Ioannidis, 2008). This observation is so common, the diagnosis of treatment-resistant depression (TRD) has been developed, although defining TRD has been a source of controversy (Fava 2003; Thase & Rush, 1997) In the context of clinical trials, Ioannidis (2008) has suggested that drug-placebo differences are generally small, but biased by the minority of severely depressed individuals who experience robust therapeutic effects. Shockingly, nearly all publications of randomized placebo-controlled clinical trials using, and

supporting the subsequent FDA approval of, antidepressant drugs have been found to have inflated their effect sizes, with these inflations ranging from 11 to 69% (Turner, Matthews, Linardatos, Tell, & Rosenthal, 2008). What's more, placebo effects in antidepressant clinical trials are extremely robust (Kirsch, 2014). In other words, the difference between antidepressant and placebo effects are actually quite small, due to the fact that placebo effects are quite large.

More recent research on depression suggests that neurodegeneration and atrophy is of paramount importance to its etiology, a theory known as the neurotrophic hypothesis of depression. Brain imaging research has consistently revealed that individuals suffering from MDD and anxiety-related disorders have lower hippocampal and prefrontal cortical volumes (Gurvits et al., 1996; Shah, Ebmeier, Glabus, & Goodwin, 1998; Sheline, Wang, Gado, Csernansky, & Vannier, 1996). Stress, a strong environmental component in developing a mood disorder, can, under some circumstances, cause neuronal apoptosis, dendritic atrophy, and decreases in trophic factors in the hippocampus (HPC; Duman & Monteggia, 2006; McEwen, 1999; Supolsky, Krey, & McEwen 1985). Santarelli and colleagues (2003) showed that induction of hippocampal neurogenesis is necessary for antidepressants to produce behavioral effects in a mouse model of depression. Furthermore, the less conventional electroconvulsive therapy (ECT), dubbed the most effective intervention for those with TRD (Abbott et al., 2013), causes increases in trophic factors in the HPC of rats (Nibuya, Morinobu, & Duman, 1995).

Of all the trophic factors, brain-derived neurotrophic factor (BDNF) is the most well-studied in the context of antidepressants, and research supports the claim that BDNF plays an important role in MDD. Post-mortem research shows that the HPC of humans with MDD had lowered levels of BDNF, while those who were taking antidepressants at the time of their death had increased levels (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001). Researchers have

observed similar effects in both the HPC and prefrontal cortex (PFC) of suicide victims in comparison to controls (Dwivedi et al., 2003; Karege, Vaudan, Schwald, Perroud, & La Harpe, 2005). Indeed, many have suggested that BDNF levels, in both serum (Aydemir et al., 2006; Gervasoni et al., 2005; Karege et al., 2002; Shimizu et al., 2003) and plasma (Kim et al., 2007; Lee, Kim, Park, & Kim, 2007) are a viable biomarker for MDD and even prescription adherence**.** This is backed up by the many studies showing that BDNF levels are increased when individuals (Aydemir, Deveci, & Taneli, 2005; Gervasoni et al., 2005; Gonul et al., 2005; Huang, Lee, Liu, & 2008; Yoshimura et al., 2007), or rodents (Nibuya, Morinobu, & Duman, 1995; Nibuya, Nestler, & Duman, 1996;) are administered antidepressants. When BDNF itself is administered, either centrally to the HPC (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002; Siuciak, Lewis, Wiegand, & Lindsay,1997), or peripherally (Schmidt & Duman, 2010), antidepressant-like effects are observed in rodents. Work by Shirayama and colleagues (2002) showed that these effects last for as long as 10 days after the infusion, long after the protein has degraded, suggesting that BDNF triggers mechanisms which sustain its effects on plasticity. Even less-conventional antidepressant interventions, like exercise (Oliff, Berchtold, Isackson, & Cotman, 1998) and ECT (Nibuya, Morinobu, & Duman, 1995) have been shown to increase central BDNF levels in rodents.

Ketamine: A novel antidepressant

The first administration of ketamine to humans occurred soon after its synthesis at Jackson prison on August 3rd, 1964 (Domino, 2010). Decades later, it was discovered to have rapid antidepressant effects among individuals with MDD (Bergman et al., 2000). Historically, ketamine was largely used as an animal anesthetic and sedative, and indeed still is today. Additionally, sub-anesthetic doses of ketamine have robust analgesic effects while having little

impact on the respiratory system in comparison to opioids, like morphine or fentanyl (Vadivelu et al., 2016). Ketamine is thus commonly used in hospitals to this end. When Berman and colleagues (2000) first reported ketamine's antidepressant effects among individuals with MDD, it was largely ignored. It was another five years before the study was replicated (Zarate et al., 2006), but when it was, the original study went on to become cited thousands of times. Perhaps none could believe such an immediate, and robust, antidepressant effect was possible. Since Zarate et al (2006) replicated Berman's work, ketamine's antidepressant effects have been well established. One clinical trial currently recruiting participants at the National Institute of Mental Health has been running since 2017, under the supervision of Dr. Zarate (ClinicalTrials.gov Identifier: NCT03065335; National Institute of Mental Health, 2017). What's more, the medication Spravato (S-ketamine) by Johnson & Johnson was approved by the FDA as a breakthrough treatment in March 2019, being indicated for TRD only.

The neurobiological mechanism responsible for ketamine's antidepressant effects are not yet fully understood, but several theories have been postulated. The glutamate burst hypothesis (a.k.a. the disinhibition hypothesis; Zanos & Gould, 2018) posits that ketamine effectively reverses the synaptic and dendritic spine atrophy, notably in the PFC and HPC, known to be associated with stress and depression. Gerhard, Wohleb & Duman suggest this occurs in five steps – see Figure 1. First, ketamine blocks N-methyl-D-aspartate (NMDA) receptors on inhibitory λ-aminobutyric acid (GABA) interneurons. This disinhibition occurs preferentially on GABA interneurons due to their higher frequency of firing compared to pyramidal neurons. It occurs even more so on those NMDA receptors with a GluN2D subunit (Glasgow, Wilcox, & Johnson, 2018), which are highly concentrated among interneurons (Monyer, et al., 1994; Perszyk et al., 2016). Faster firing allows for NMDA receptors to be freer of their Mg+ ion

blockade, granting ketamine greater access to the NMDA pore to inhibit it's opening (Zanos & Gould, 2018). This is supported in humans, by evidence showing ketamine increases overall PFC activity in healthy individuals (Breier et al., 1997) and in rats, by evidence that MK-801 specifically increases pyramidal neuron-firing in the PFC (Homayoun., & Moghaddam, 2007). MK-801 is an NMDA antagonist which binds to the same site as ketamine. Second, by blocking the NMDA channels on GABA interneurons, ketamine reduces tonic firing of these interneurons, subsequently resulting in a disinhibition of glutamate neurons, and a burst in glutamate release. Anesthetic doses of ketamine do not cause an increase in glutamatergic transmission (Moghaddam, Adams, Verma, & Daly, 1997; Chowdhury et al., 2012). Third, as a result of the pre-synaptic glutamate burst, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are activated and upregulated on the post-synaptic PFC pyramidal neuron, resulting in greater depolarization of the post-synaptic membrane. Nearby post-synaptic voltage gated calcium channels open and an influx of calcium occurs. Fourth, BDNF is released by the post-synaptic neuron and binds to tropomyosin receptor kinase B (TrkB) receptors, also on the post-synaptic neuron, beginning several intracellular second messenger cascades. TrkB receptors auto-phosphorylate, allowing them to impact the cell for extended periods of time. This could explain how centrally administered BDNF produces prolonged antidepressant effects (Shirayama et al., 2002). Lastly, mammalian target of rapamycin complex 1 (mTORC1) signaling proteins are rapidly phosphorylated, increasing spine density in the PFC via proteins like post-synaptic density protein (PSD) 95, and synapsin. The importance of the mTORC1 signaling cascade is illustrated by paradigms which block mTORC1 via rapamycin, resulting in ketamine no longer producing antidepressant-like effects in animal models (Gerhard, Wohleb, & Duman, 2016; Koike, Iijima, & Chaki, 2011).

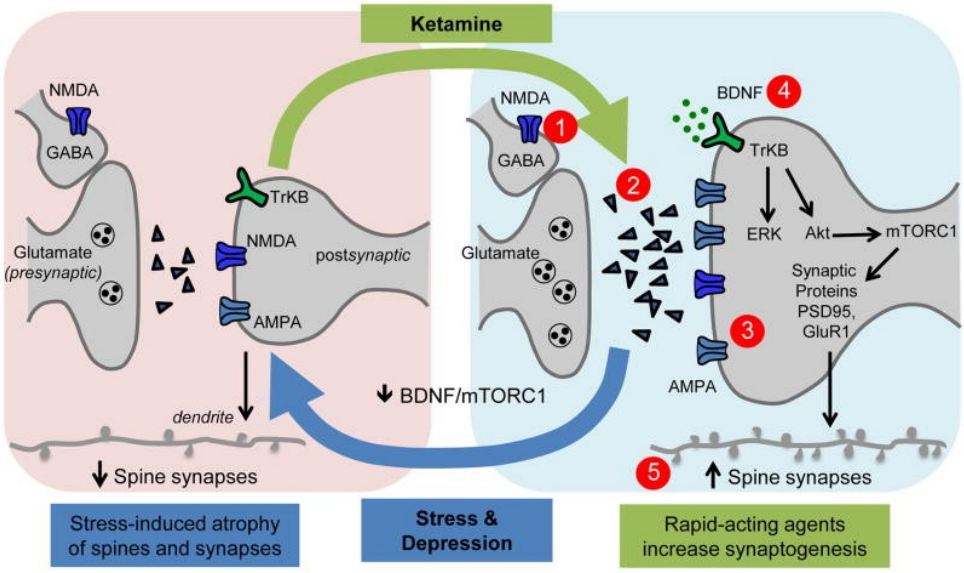


Figure 1 from Gerhard, Wohleb & Duman (2016). A hypothesized mechanism of ketamine's antidepressant effects.

A second hypothesis of ketamine's mechanism of action, assembled by several research groups, holds that a glutamate burst is not necessary (Autry et al, 2011; Kavalali & Monteggia, 2012; Nosyreva et al, 2013). Instead, ketamine's NMDA antagonism blocks spontaneous miniature excitatory post-synaptic currents at rest, which are mediated by NMDA activation. Moreover, it is suggested that it is the deactivation of eukaryotic elongation factor 2 (eEF2) kinase (a.k.a. CaMKIII), not the activation of mTORC1, at the root of this effect. Deactivation of eEF2 kinase, via ketamine administration, was shown to induce BDNF and dendritic protein translation, including AMPA subunits, in mouse models. This paradigm suggests that NMDA receptor activity at rest allows for eEF2 kinase to chronically phosphorylate eEF2, which then suppresses translation. It is suggested that acutely blocking NMDA, via ketamine, stops eEF2 phosphorylation via eEF2 kinase deactivation, which produces antidepressant-like effects (Autry et al, 2011; Kavalali & Monteggia, 2012; Nosyreva et al, 2013).

A third hypothesis, built upon the research of Dr. Panos Zanos, posits that NMDA antagonism, and subsequent second-messenger cascades, is not central to ketamine's antidepressant effects. This is because ketamine's R enantiomer produces stronger antidepressant-like effects in rodent models than the S enantiomer, despite R-ketamine having an almost four-fold lower affinity (Ki = 2.57) for the NMDA receptor than S-ketamine (Ki = .69; Moaddel et al., 2013). Research supporting this hypothesis shows that ketamine's antidepressant effects actually occur via its metabolites, specifically 2R and 6R hydroxynorketamine (2R-6R HNK), acting on AMPA receptors. Administering deuterated ketamine, which is not metabolized into other compounds due to a change in its molecular structure, produces no antidepressant-like effects in rodent models. Furthermore, administering 2R-6R HNK with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione (NBQX), an AMPA receptor antagonist, causes no antidepressant-like effects, whereas, without NBQX, the 2R-6R HNK metabolites produces the most potent effects compared to other metabolites (Zanos et al., 2016). Proponents of this theory, therefore, suggest ketamine's mechanism of action lies with its ability to activate AMPA receptors (Maeng & Zarate, 2007; Yang et al., 2015; Zanos et al., 2016).

Ketamine in females

Despite the many hypotheses of ketamine's antidepressant effects, much of the data has been derived from studies on males. As one might expect, there is a growing body of evidence which suggests that these theories do not hold true for females. In support of the glutamate burst hypothesis in males, social isolation stress (SIS) leads to a decrease in sucrose preference, a measure of anhedonia, as well as decreases in medial PFC (mPFC) spine density, PSD-95, and synapsin. Three hours after a single ketamine infusion all of these effects were reversed. In females, SIS also leads to a decrease in mPFC spine density, PSD-95 and synapsin, yet no

changes in sucrose preference and none of these effects were reversed by ketamine (Sarkar & Kabbaj, 2016). Furthermore, ketamine induces hippocampal glutamate in males but not females, but induces aspartate in the mPFC in females but not males (Franceschelli, Sens, Herchick, Thelen, & Pitychoutis, 2015). All this evidence is contrary to the glutamate burst hypothesis and suggests that the glutamatergic system in females reacts to ketamine differently than in males. Discounting another hypothesis of ketamine's mechanism of action in males, Carrier & Kabbaj (2013) have shown that ketamine does not induce decreases in eEF2 in the HPC and PFC of female rats, whereas this decrease has been shown to occur consistently in males (Autry et al, 2011; Carrier & Kabbaj, 2013; Kavalali & Monteggia, 2012; Nosyreva et al, 2013). One theory, that is supported by evidence in both males and females, is that which places chief importance on ketamine's metabolites. However, while both 2R and 6R-HNK are more abundant in females than males, so too are 2S and 6S-HNK (Zarate et al., 2012; Zanos et al., 2016). This evidence may explain the sex differences in terms of sensitivity to ketamine's antidepressant-like effects in rodents, but it only muddies the waters in terms of how ketamine may be exerting sex-specific effects. Making the story even less clear, the few meta-analyses conducted among clinically treated individuals have revealed no sex differences in ketamine's antidepressant effects (Romeo, Choucha, Fossati & Rotge, 2015), or a slightly higher sensitivity among males, but only at 7 days post-infusion (Coyle & Laws, 2015). Although more research must be done to replicate these findings, this suggests that there exists no difference between the sexes among humans in terms of response to ketamine. Nevertheless, a much more recent and comprehensive theory on the etiology of depression may be the missing link in explaining ketamine's effects in the female brain.

Microglia, neuroinflammation, and ketamine

The neuroinflammatory hypothesis of depression, also known as the cytokine hypothesis, is comprehensive in the sense that it ties together both the monoamine and neurotrophic theories of depression. To put it simply, this theory posits that depression is caused by inflammatory processes (Maes et al., 2009; Miller, Maletic, & Raison, 2009; Raison & Miller 2016; Schiepers, Wichers, & Maes, 2005). Derived from yolk-sac progenitors during development (Gomez Perdiguero et al., 2015) microglia, a type of macrophage, are the brain's immune cells and make up approximately 10% of cells in the central nervous system (Salter & Stevens, 2017). Microglia are responsible for many processes, such as inducing apoptosis in nearby neurons and synaptic pruning, both of which are necessary for healthy brain development and maintaining homeostasis (Matcovitch-Natan et al., 2016; Paolicelli et al., 2011; Salter & Stevens, 2017; Schafer et al., 2012). Given their crucial role, microglia respond to changes in their immediate environment quickly as they are constantly surveilling and sampling it (Bernier et al.,2019). In the field of immunology, a macrophage engaging in an adaptive inflammatory response to pathogens in their environment can be referred to as an acute phase response (Lucas, Rothwell, & Gibson, 2006). In the case of microglia, this can be induced by viral (Olson & Miller, 2004; Chen, Zhong, & Li, 2019) or bacterial infection (Michel et al., 2005; Pascual, Ben Achour, Rostaing, Triller, & Bessis, 2012), traumatic brain injury (Davalos et al., 2005), and even alcohol consumption (Fernandez-Lizarbe, Pascual, & Guerri, 2009). A microglial response to threats such as these could entail the release of reactive nitrogen and oxygen species to cause oxidative stress and/or apoptosis in infected neurons (Miller & Raison, 2016). Microglia also release small proteins known as cytokines, which can be pro-inflammatory, such as interleukin (IL) - 1, 2, 6 and 18 (Gentleman et al., 2004; Salter & Stevens, 2017; Taib et al., 2017), tumor necrosis factor (TNF)

α (Ma, Zhang, & Baloch, 2016) and interferon (IFN) γ (Liu, Ho, & Mak, 2012), or anti-inflammatory, such as IL-4, 10 and 13, (Kumar, Alvarez-Croda, Stoica, Faden, & Loane, 2016; Lively & Schlichter, 2018; Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). Furthermore, microglia change shape when they become more active. This change in shape makes measuring microglia structure, via confocal microscopy and three-dimensional surface rendering, a convenient way of measuring microglial activation (Hong et al., 2015; Schafer et al., 2012). One important, and recently discovered, cue that microglia use to extend processes towards a target neuron is adenosine triphosphate (ATP; Dissing-Olesen et al., 2014). It was found that NMDA receptor activation mediates this ATP release. These effects were repeatable and reversible, and observed *in vivo* via two-photon imaging, indicating that these extensions were independent of neuronal death. However, it is hypothesized that this mechanism of microglial surveillance has evolved because ATP is also released during apoptosis, thus acting as a "find me" signal for all immune cells (Chekeni et al., 2010; Dissing-Olesen et al., 2014; Elliott et al., 2009). Importantly, microglia often engage in a biological process known as phagocytosis, whereby they extend a cup-shaped process to engulf a given target (Fu, Shen, Xu, Luo, & Tang, 2014). In fact, the term "phagoptosis", also known as primary phagocytosis, is now used to refer to phagocytosis-induced apoptosis of viable cells (Brown & Neher, 2012). Microglial phagocytosis is crucially involved in a plethora of neurodegenerative diseases, as it is the process responsible for synaptic pruning and the removal of protein aggregates. Phagocytosis is triggered by multiple extracellular chemotactic signals and pathogens binding to multiple families of receptors. A synapse, for example, is phagocytosed via releasing the complement component 3 and 1q (C3 & C1q) "eat me" proteins, which are recognized by complement receptor 3 (CR3) on microglia (Salter & Stevens, 2017; Schafer et al., 2012; Stevens et al., 2007; Veerhuis, Nielsen, & Tenner,

2011). On the other hand, certain types of bacteria are recognized by toll-like receptors (TLR), and apoptotic debris are recognized by triggering receptor expressed on myeloid cells 2 (TREM2; Fu, Shen, Xu, Luo, & Tang, 2014).

The idea that microglia-mediated neuroinflammation contributes to the etiology of depression is not an exceptionally new one (Herbert & Cohen, 1993; Raison, Capuron, & Miller, 2006). Phenomena such as sickness behavior, an adaptive behavioral strategy where an organism's motivational state is reorganized to optimize coping with illness, illustrates the connection between brain and body (Dantzer, 2009). While sickness behavior clearly demonstrates how depressive behavior is elicited by immune challenges, the neuroinflammatory hypothesis of depression posits that stress is the first step in leading to depression (Miller & Raison, 2016). A striking body of evidence shows how stress induces inflammation both peripherally and centrally. Researchers have shown that the Trier social stress test (TSST) elicits increases in circulating IL-6 (Bierhaus et al., 2003) and Il-1β (Aschbacher et al., 2012). Aschbacher and colleagues' work (2012) showed that peripheral immunoreactivity to stress even predicts future depressive symptoms in a cohort of post-menopausal women. Studies of older adults have also found that peripheral pro-inflammatory IL-1 family cytokines were positively correlated with future depressive symptoms (Milaneschi et al., 2009; van den Biggelaar et al., 2007). Van den Biggerlaar and colleagues' (2007) work demonstrated that *ex vivo* whole blood cytokine production in response to LPS administration also predicted future depressive symptoms. Administration of LPS to cell cultures is a common laboratory technique to induce immune cell reactivity because LPS is found in the outer membrane of gram-negative bacteria (Osborn, Gander, Parisi, & Carson, 1972). Stress-induced increases in pro-inflammatory cytokines, which are increased in depressed populations (Capuron and Miller, 2004; Howren et

al, 2009; Maes et al, 2009; Miller, Maletic, & Raison, 2009; Mossner et al, 2007; Raison et al, 2006; Raison & Miller 2016; Schiepers, Wichers, & Maes, 2005), affect the CNS through several mechanisms which are hypothesized to then cause depression.

Centrally administered LPS, which causes microglia to release pro-inflammatory cytokines, has been shown to reduce rat hippocampal neurogenesis (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003). In fact, the number of activated microglia, indicated by CD68 labelling, was inversely correlated with new neurons, indicated by BrdUrd labelling. Ekdahl's research showed that new hippocampal neurons simply do not survive around activated microglia, and conversely, inhibiting microglial activation, via minocycline, gives rise to increases in new neurons. The damaging effects of activated microglia to their neighboring neurons are likely mediated by cytokines such as IL-1β and IL-6 (Gebicke-Haerter, 2001; Hanisch, 2002). Monje, Toda, & Palmer's (2003) work, using indomethacin as an inflammatory blockade, also confirmed that microglial activation is negatively associated with neurogenesis. This work ties together both the neuroinflammatory and neurodegenerative hypotheses of depression.

Common to both the monoamine and neuroinflammatory hypotheses of depression is the kynurenine pathway, which consists of the following reactions: Tryptophan, an essential amino acid and precursor for the monoamine neurotransmitter serotonin (5-HT; Fernstrom, 1983; Schaecter & Wurtman, 1990), is catabolized into kynurenine by the enzyme, and rate-limiting factor (Soliman, Mediavilla-Varela, & Antonia, 2010), indoleamine 2,3-dioxygenase (IDO; Guillemin, Smith, Smythe, Armati, & Brew, 2003). Kynurenine is then converted into either quinolinic acid (QUIN), a neurotoxic NMDA-receptor agonist, or kynurenic acid (KYNA) an NMDA, AMPA, and kainate receptor antagonist, which has been shown to be protective against

excitotoxicity (Bay-Richter et al., 2015; Zunszain et al., 2012). The kynurenine pathway is controlled by the immune system, with the synthesis of certain downstream metabolites, like QUIN, occurring within microglia (Schwarcz, Bruno, Muchowski, & Wu, 2012; Suzuki et al., 2019). When this pathway is more actively engaged, for example due to pro-inflammatory cytokines which induce IDO (Guillemin, 2012; Kindler et al., 2019; O'Connor et al., 2009), less tryptophan is available for 5-HT synthesis (Dantzer, O'Connor, Lawson, & Kelley, 2011; Halaris et al., 2015; Maes, Leonard, Myint, Kubera, & Verkerk, 2011; Myint, 2012; Myint, Schwarz, & Müller, 2012). This directly relates the neuroinflammatory and monoamine hypotheses of depression, the latter of which argues that depression is due to lowered levels of 5-HT. Indeed, this process resulting in 5-HT depletion has been implicated in the etiology of depression for decades (Lapin, 1973).

Recently, the kynurenine pathway was suggested as a mechanism through which ketamine might affect neurodegeneration via microglia-mediated neuroinflammation (Miller, 2013). Several studies now have shown that LPS induces QUIN in microglia (Bahrami, Firouzi, Hashemi-Monfared, Zahednasab, & Harirchian, 2018; Garrison et al., 2018; Rodrigues et al., 2018). Moreover, higher levels of neurotoxic QUIN was found in the cerebrospinal fluid of suicide victims (Erhardt et al., 2013). Furthermore, QUIN was increased, specifically in microglia, in the post-mortem brains of severely depressed individuals (Steiner et al., 2011).

Establishing ketamine's relevance to this paradigm-shift in our understanding of the neurobiology of depression, Walker and colleagues' (2013) showed that ketamine reverses LPS-induced depressive-like behavior in rodents. What's more, this effect occurred when ketamine was given 10 hours after LPS administration, giving the inflammatory and kynurenine pathways enough time to be activated. Pre-treatment with ketamine blocked LPS-induced depressive-like

behavior from developing. Interestingly, ketamine had no impact on LPS-induced inflammatory activation in the form of plasma cytokine levels. The researchers hypothesized that ketamine, an NMDA antagonist, was instead blocking QUIN's ability to act as an NMDA-receptor agonist. As mentioned previously, a requirement for ketamine's antidepressant effects to take hold is the upregulation of AMPA-mediated glutamatergic neurotransmission, likely due to ketamine's metabolites (Maeng & Zarate, 2007; Yang et al., 2015; Zanos et al., 2016). Their hypothesis was confirmed via administering NBQX 15 minutes before ketamine, restoring depressive-like behavior (Miller, 2013; Walker et al., 2013).

Verdonck and colleagues (2019) were the first to show that microglia are a direct target of ketamine and, specifically, the production of quinolinic acid within microglia. In a mouse model of LPS-induced depression ketamine induced changes in microglia resulting in a neuroprotective phenotype. They observed that ketamine reversed the LPS-induced increase in QUIN in brain parenchyma (as in the whole brain, not specific to any particular area), whereas ketamine had no effect on QUIN in control animals. Translating this work to a clinical perspective, the researchers also tested kynurenine pathway metabolite concentration in 15 individuals with treatment resistant depression in response to ketamine. The standard dose of 0.5mg/kg ketamine was infused over 40 minutes, with the frequency of administration determined by the patient's psychiatrist. Blood samples were taken before and after each infusion for analysis. They found that the KYNA:QUIN ratio before the first ketamine infusion to be a significant predictor of final Montgomery–Åsberg Depression Rating Scale (MADRS) score. This effect was driven primarily by QUIN plasma levels. QUIN concentrations before each ketamine infusion were the best predictor of ketamine efficacy and were the only significant predictor of relative change (i.e. before and after ketamine) in MADRS scores. These findings by

Verdonck et al (2019) confirm the hypothesis of Walker et al (2013) that ketamine directly affects microglial QUIN levels, in both mice and humans, resulting in protection from neuroinflammatory processes, and that the degree to which ketamine impacts QUIN levels correlates with relief of depressive symptoms in humans.

Further linking ketamine to inflammatory processes, researchers have shown that ketamine reverses the effects of LPS, in the form of pro-inflammatory cytokine release, in microglia *in vitro* (Chang et al., 2009). This makes sense, given that others have shown that NMDA and LPS have very similar effects on microglia. Both produce a release in pro-inflammatory cytokines and the adoption of a more amoeboid-like shape, indicating a more active state (Kaindl et al., 2012). NMDA receptors have been implicated in cytokine-induced neurotoxicity in previous research as well. When Chao, Hu, Ehrlich & Peterson (1995) administered both IL-β and TNF-α to *in vitro* fetal brain cells, they noticed a marked increase in neuronal injury. When these cytokines were administered in conjunction with MK-801, the increase in neuronal injury was ablated. Recently, researchers showed that ketamine reduced pro-inflammatory cytokines, microglia phagocytic markers in the rat HPC, as well as and depressive-like behavior in a rat model (Chen et al., 2017).

Much of the evidence presented thus far, points to microglia playing a pivotal role in ketamine's antidepressant effects. Ketamine's NMDA-antagonism make it capable of stopping neuronal ATP release, stopping microglia from potentially damaging nearby neurons, as previously discussed (Dissing-Olesen, 2014). Ketamine reduces the neurotoxic effects of QUIN being produced within microglia (Verdonck et al., 2019). Ketamine also reduces the microglial response to otherwise activation-inducing molecules like LPS (Chang et al., 2009) and stops microglial production of pro-inflammatory cytokines (Chen et al., 2017). Unfortunately, the vast

majority of this research has been conducted using the male sex. Similarly, the theoretical bases which guide such experiments has also, historically, used the male sex. An important consequence of this, beyond the inability of generalizing these findings to over 50 percent of the population, is the lack of understanding how ketamine might interact with ovarian hormones. Fortunately, extensive research has been conducted investigating the role of estrogens on microglia.

Ovarian hormones and neuroinflammation

The classical mechanism through which lipid-soluble hormones like E2 act on neurons and glia is via nuclear estrogen receptors (ERs). Hormones like E2 diffuse across the cell membrane passively and bind to nuclear receptors in the cytoplasm. These ligand-bound receptors dimerize with other ligand-bound receptors, forming a hetero or homodimer (Cowley et al., 1997; Pace et al., 1997; Mangesldorf et al., 1995), and translocate to the nucleus where they act as transcription factors. Nuclear estrogen receptors were thought to only function this way, but more recently membrane-bound estrogen receptors have been discovered, such as G-protein-coupled estrogen receptor 1 (GPER1), and act more rapidly (Maggiolini & Picard, 2010). Estrogens, as a whole, have been found to be neuroprotective towards multiple pathologies, most notably those in which microglia are implicated, such as Alzheimer's disease (Fillit et al., 1986; Henderson, Paganini-Hill, Emanuel, Dunn, & Buckwalter, 1994; Kawas et al., 1997, Paganini-Hill, & Henderson, 1994; Tan et al., 1996) and multiple sclerosis (Confavreux, Hutchinson, Hours, Cortinovis-Tourniaire, & Moreau, 1998; Gold & Voskuhl, 2009; Laffont, Garnier, Lélu, & Guéry, 2015; Voskuhl et al., 2016).

One way that E2 impacts microglia is via the nuclear estrogen receptor (ER) α. Researchers have found that systemic administration of E2 reduces LPS-induced microglia

activity in a dose-dependent manner by decreasing the expression of proteins associated with phagocytosis, by inhibiting morphological changes, and by inhibiting cell migration. ER knockout mouse models demonstrated that ERα is responsible for these effects. For example, microglia activity was unaffected by the absence or presence E2 administration in ERα-null mice (Vegeto et al., 2003). Previous research by Bruce-Keller and colleagues (2000) showed that, in a dose-dependent manner, E2 attenuates microglia phagocytosis, and the release of superoxide, a neurotoxic free radical. These effects were mediated by the phosphorylation of mitogen-activated protein kinase (MAPK). Additionally, Vegeto, Pollio, Ciana, & Maggi (2000) also found that E2 reduces the buildup of free-radicals, specifically, nitrous oxide, in microglia.

Another way that E2 can impact microglia is via GPER1. E2 binding to GPER1, located predominantly on the endoplasmic reticulum, but also the Golgi apparatus and nuclear membrane, results in the mobilization of intracellular calcium, and the production of nuclear phosphatidylinositol 3,4,5-trisphosphate (PIP_3; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). PIP_3 is an effector of multiple downstream signaling proteins, particularly the protein kinase AKT which plays a crucial role in several cellular processes, such as cell survival (Downward, 2004; Pap & Cooper, 1998; Song, Ouyang, & Bao, 2005) and proliferation (Kuo et al., 2008; Lawlor & Alessi, 2001). Zhao and colleagues (2016) were the first to show that GPER1 mediates E2's anti-inflammatory effects on microglia in a rat model of cerebral ischemia. Both E2, or the GPER1 agonist, G1, alone were able to attenuate LPS-induced increases in pro-inflammatory TNF-α and IL-1β. Co-administration of the GPER1 antagonist, G15, with E2 reversed these anti-inflammatory effects, and E2 administration to GPER1-knockdown rats had reduced anti-inflammatory effects. As little is known about the effects of estrogens on microglia, even less is known about the effects of progesterone.

P acts on neurons and glia through classical genomic mechanisms by binding to nuclear progesterone receptor (PR), of which there are two isoforms (PRA, PRB), and via nonclassical, a.k.a. nongenomic, membrane-bound progesterone receptors (mPRs; Ellmann et al., 2009). P has been investigated in the context of traumatic brain injury (TBI) and stroke for its neuroprotective effects for over a decade. Interestingly, there has been several conflicting lines of research in the field of TBI, with some showing P as being neuroprotective following TBI (Pettus, Wright, Stein, & Hoffman, 2005; Roof, Duvdevani, Heyburn & Stein, 1996; Roof, Hoffman, & Stein, 1997; Shahrokhi et al., 2010; Stein, 2008; Wright et al., 2007), and more recent work showing it has no neuroprotective effects (Allitt et al., 2016). Moreover, a large scale meta-analysis (n = 2400) showed that P given after TBI in humans produced no amelioration (Lin et al., 2015). Fortunately, evidence from the field of hypoxic ischemia is clearer, with several studies finding that P exerts neuroprotective effects in the face of an ischemic challenge (Cervantes et al., 2002; Chen, Chopp, & Yi, 1999; Dang, Mitkari, Kipp, & Beyer, 2011; Gibson et al., 2005; Gibson, Coomber, & Murphy, 2011; González-Vidal, et al., 1998; Ishrat, Sayeed, Atif, 7 Stein, 2009; Kumon et al., 2000; Moralí et al., 2005). It is also noteworthy to mention that post-menopausal women are at a drastically higher risk of stroke than pre-menopausal women, and it is thought that this is due to the protective effects of ovarian hormones (Wenger, Speroff, & Packard, 1993). Whether or not this effect occurs due to estrogens, progesterone, or both, has been a hotly debated subject. Dong and colleagues (2018) showed that P following ischemia significantly reduced neuronal death and improved learning and memory in male mice only, with female mice showing no improvement. Tameh and colleagues (2018) found that a mechanism responsible for this is P's ability to upregulate certain NMDA receptor subunits, specifically NR1, NR2A, and

NR3B, which mediate neuronal survival signaling cascades (Liu et al., 2007; Nakanishi et al., 2009). By upregulating these subunits, P serves to inhibit NMDA-mediated apoptosis.

Work by Bali, Morgan, and Finch (2013) revealed that P exerts its effects on microglia by binding to progesterone receptor membrane component 1 (PGRMC1, a.k.a. 25-Dx, a.k.a. ventral midline antigen or VEMA). PGRMC1 belongs to neither the classical, nor the membrane-bound progesterone receptor subfamilies, but rather the membrane-associated progesterone receptor (MAPR) family (Piel et al., 2016). PGRMC1 is the P-binding protein in a single-transmembrane protein complex (Gellersen, Fernandes, & Brosens, 2009; Theis & Theiss, 2019; Thomas, 2008) and has been found on the membranes of the Golgi apparatus, endoplasmic reticulum, and mitochondria of CNS cells (Sakamoto et al., 2004; Xu et al., 2011). Both E2 and P upregulate PGRMC1 expression in the ovariectomized rat HPC (Bali et al., 2012). Activating PGRMC1 with P reinstates neuronal activity (Labombarda et al., 2003), enhances spinogenesis (Wessel et al., 2014), and enhances neuronal migration and myelination from Schwann cells in the spinal column, where PGRMC1 has been found in the cell membrane (Castelnovo, Magnaghi, & Thomas, 2019; Theis & Theiss, 2019). What's more, P has been shown to induce BDNF expression, an effect which has been shown to be mediated by both the classical PR in cortical slice explants (Jodhka et al., 2009), and by PGRMC1 in cultured glial cells (Su, Cunningham, Rybalchenko, & Singh, 2012). This suggests that ketamine, E2, and P, each on their own, would increase BDNF expression. However, this is contradictory to seminal research which showed that P can antagonize E2's synaptogenesis-inducing effects at certain time points (Woolley & McEwen, 1993

). Prior to the discovery that PGRMC1 is crucial for microglial activation (Bali, Morgan, & Finch, 2013), work by the same authors showed that P antagonizes E2-mediated neurite

outgrowth *in vitro,* but only when microglial cells were also present. P had no effect on neurite outgrowth with only neurons and astrocyte cocultures (Wong et al., 2009). This line of research culminated in several additional important findings. Firstly, that activating PGRMC1 induces microglial activation to the same degree as stimulation from LPS, as indicated by CD11b protein expression. Second, that when microglia are activated via P-binding to PGRMC1 they inhibit new neurite outgrowth, they inhibit neurites from growing further. Third, that P binding to PGRMC1 inhibits BDNF release from astrocytes, further hindering neuritogenesis. Fourth, that PGRMC1 knockdown stopped LPS and injury (*in vitro* scratch-wounding) induced microglia activation (Bali, Morgan, & Finch, 2013; Bali, Arimoto, Morgan, & Finch, 2013). Together, these findings suggest that PGRMC1 is critical for microglial activation, and that E2 and P may be acting to antagonize one another, with E2 causing quiescence in microglia, but P activating them.

This is of course counter-intuitive when considering P's seeming neuroprotective effects in the context of ischemia. Looking to P's broader effects on inflammation could, perhaps, clear these muddied waters.

P also has multiple anti-inflammatory effects in LPS-stimulated microglia *in vitro*, as indicated by Lei and colleagues' work (2014). LPS upregulated the pro-inflammatory cytokine TNF-α, inducible nitric oxide synthase (iNOS), an enzyme precursor for the free radical nitric oxide (NO), and cyclooxygenase-2 (COX-2), an enzyme precursor for prostaglandin. All of which are upregulated during inflammation. P attenuated these LPS-induced increases in a dose-dependent manner. Additionally, P decreased the LPS-induced phosphorylation of several other kinases such as p38, c-Jun N-terminal kinase, and extracellular regulated kinase MAPKs. Importantly, P decreased the LPS-induced activation of the protein complex nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a transcription factor which controls the expression of various target genes, especially those involved in immune and inflammatory responses (Lei et al., 2014). NF-κB and pro-inflammatory cytokines have a bidirectional connection: NF-κB is activated by pro-inflammatory cytokines, and NF-κB directly promotes pro-inflammatory cytokine production by binding to cytokine promoter regions in the genome (Oeckinghaus & Ghosh, 2009). These pleiotropic effects of P, along with the PGRMC1 activational mechanism, all specific to microglia, demonstrate the relevance of understanding how P, E2 and ketamine might interact with one another. Of course, much more comprehensive research still needs to be done to understand the complexity of these phenomena.

Ketamine binds to ERα

It has been shown that estrogens act to mediate enhanced sensitivity to ketamine in depression models using rodents (Carrier & Kabbaj, 2013; Franceschelli et al., 2015; Sarkar & Kabbaj, 2016). Furthermore, both female mice (Zanos et al., 2016) and humans (Zarate et al., 2012) metabolize ketamine to produce higher levels of the crucially important ketamine metabolites 2R-6R/2S-6S HNK than males. It is thought that this occurs due to women having higher levels of the CYP2A6 and CYP2B6 enzymes, responsible for ketamine's metabolism (Ho et al., 2018). E2 and P are capable of inducing these enzymes
(Choi, Koh, Jeong, 2013), with E2 doing so via ERα (Higashi et al., 2007). ERα is therefore critical in understanding how ketamine and ovarian hormones might interact. In most tissues, including the CNS, E2 binding to ERs precipitates an upregulation of PRs (Graham & Clarke, 1997). Thanks to work by Alves et al., (2000) it is known that this occurs via E2 binding to ERα receptors in the hippocampus of wild-type mice (Vasudevan & Pfaff, 2008). Both E2 and P lead

to the induction of PGRMC1 in female OVXd rat hippocampal neurons in the CA1, CA3, and DG (Bali et al., 2012), although it is unclear which ER/PR-binding mechanisms cause this.

Evidence that ketamine binds to ERα, was provided by Ho and colleagues (2018). Radioligand binding assays, coupled with surface plasmon resonance, demonstrated that E2, ketamine, and 2R-6R/2S-6S HNK bind to ERα receptors in cultured astrocytes. They also found that the same compounds act in an additive manner to induce AMPA receptor subunits, again *in vitro*. This is thought to be crucial for ketamine's antidepressant effects, as previously discussed, and this effect was ablated by ERα knockdown. Given that all three compounds also lead to ERα trafficking to the nucleus, it follows that the authors hypothesized a potential positive feedback loop. Namely, when ERα binds to estrogen-response elements, CYP2A6, CYP2B6, and AMPA subunit transcription is induced, leading to more ketamine metabolism and more AMPA receptors (Ho et al., 2018). Research has yet to be conducted to determine how PRs might be impacted by these processes but it is likely that, via ERα binding, ketamine and its metabolites cause PR upregulation. Virtually nothing is known of ketamine's ability to impact PGRMC1. How this might be manifested behaviorally is an even greater mystery.

Hippocampus as a candidate for ketamine-hormone interactions

Depression involves multiple brain regions. The HPC is perhaps the most well studied brain region in regards to MDD. A meta-analysis of MRI results yielded an average reduction of 8-10% in HPC volume among individuals with unipolar depression (Videbech & Ravnkilde, 2004). The HPC is also a site of continuous neurogenesis as documented in adult macaques (Kornack & Rakic, 1999). That is, while depression may decrease HPC volume, this change may not necessarily be permanent. Outside the context of antidepressant research, it is worth noting that HPC volume declines naturally with age, and that there exists an interactive effect of gender

and age on HPC volume decline. As we age, HPC volume declines linearly among men, whereas there is no such correlation among women (Pruessner, Collins, Pruessner, & Evans, 2001).

Morphological changes in the HPC such as neurogenesis and synaptogenesis are, in part, mediated by BDNF (Liu, Diorio, Day, Francis, & Meaney, 2000; Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001; Vaynman, Ying, & Gomez-Pinilla, 2004). What's more, researchers have shown that peripheral BDNF leads to both hippocampal neurogenesis among adult mice, as well as antidepressant-like effects in the forced swim test (FST; Schmidt & Duman, 2010). Ketamine is well documented to both increase hippocampal BDNF, and produce antidepressant-like effects in the FST in male rodents (Garcia et al., 2008; Reus et al., 2011; Yang, Hu, Zhou, Zhang, & Yang, 2013; Choi, Lee, Park, Kim, & Son, 2017). Furthermore, it is likely that the neuroprotective effects of E2 are partly due to BDNF upregulation (Sato et a., 2007;). Some speculate that the BDNF gene contains an estrogen response element such that E2 could upregulate BDNF directly (Scharfman & MacLusky, 2006). Others have shown that estrogen receptors are found on BDNF-expressing neurons (Sohrabji & Lewis, 2006; Miranda, Sohrabji & Toran-Allerand, 1993). It is perhaps through this mechanism that E2 mediates dendritic spine growth and synaptogenesis in the HPC, as previously discussed (Gould, Woolley, Frankfurt & McEwen, 1990; Woolley & McEwen, 1993).

What makes the HPC particularly interesting in terms of ketamine's effects is, as previously discussed, that the HPC-PFC circuits are likely the most crucial for ketamine's antidepressant effect. Within this circuit ketamine selectively disinhibits GABA interneurons, as per the glutamate burst hypothesis – see Figure 1. With regards to the neuroinflammatory hypothesis, the HPC has been the focus of several research groups who found that ketamine

generally suppresses glial inflammation in this area (Chen et al., 2017; Peters, Villasana, & Schnell, 2018; Tan et al., 2017).

Conclusion

The etiology of major depressive disorder is complex, and there is no single explanation for its manifestation. Likewise, it's possible that there are many valid explanations as to why women suffer from MDD more than men, but ovarian hormones are surely implicated. Despite the discovery of ketamine's antidepressant effects being relatively recent, there's plenty of evidence supporting the various hypotheses concerning its mechanism of action in the CNS. However, evidence specific to the female sex is lacking. That which has been gathered only demonstrates that well-researched theories do not generalize across the sexes. Considering the neuroinflammatory hypothesis of depression may help shed light on ketamine's mechanism of action among females. Indeed, a massive body of literature supports the relevance of this hypothesis, particularly the role of microglia, to novel antidepressants like ketamine. The literature provides more than sufficient evidence to warrant the idea that circulating E2 and P would interact with ketamine's effect on the CNS. In believing some, one might suspect that the three molecules would act additively to reduce depressive symptoms. Believing other lines of research, one might expect E2 to act additively with ketamine, but that P would antagonize this via acting on PGRMC1 on microglia. Evidence which indicates P has neuroinflammatory effects is contradicted by evidence which shows it has anti-inflammatory effects. *In vivo* experimentation looking specifically at the HPC is the next step in investigating these complex phenomena, as much of the literature uses *in vitro* models. Exploring this is paramount to the likely eventuality that ketamine, or a similar compound, will be administered to those seeking treatment for MDD on a larger scale.

The goal of this project was to elaborate on the interaction between ketamine and ovarian hormones. Although the link between E2 and depression isn't always easy to interpret, research clearly shows that exogenous E2 produces antidepressant-like effects in the FST(Rocha, Fleischer, Schaeffer, Rohrer, & Hickey, 2005; Rachman, Unnerstall, Pfaff, & Cohen, 1998), much like ketamine. The role of progesterone in MDD has been less well elucidated.

Primary Manipulations & Hypotheses

Research on FST behavior very similar to the current work was conducted by Carrier & Kabbaj (2013) using nearly identical hormonal manipulations. This research was used to formulate some of the current hypotheses. The current project's primary manipulations were ketamine dose (five groups: 0, 2.5, 5, 10, & 20mg/kg), and hormone (three groups: Low E2, high E2, & high E2 + P) resulting in a total of 15 groups. Rats were ovariectomized after two weeks of handling in order to control for ovarian hormone levels experimentally. Rats were tested in the forced swim test chamber before and after exposure to their respective drug-hormone conditions. We also tested the rat's locomotor activity before and after ketamine in order to ensure that any effects observed in the forced swim test were not due to the motor side-effects of ketamine. We hypothesized a dose-dependent response to ketamine such that rats would be less immobile in the forced swim test. As per the results of Carrier & Kabbaj (2013), the current work hypothesized that the effects of ketamine in the low E2 and high E2 groups would be similar, but that the addition of P in the high E2 + P group would make rats more responsive to ketamine, pushing levels of immobility down even further. A control experiment was conducted using two groups of males, saline and 10mg/kg ketamine, of males. In the same direction as the females, we hypothesized that ketamine would reduce immobility in the FST.

Method

Animals & Surgeries

145 female and 20 male Wistar rats were used for this experiment, the equivalent of ten per group. All were between two and three months of age and weighed approximately 250-270g. Of the 145, 51 rats were obtained via a colony housed in the animal care facility at Concordia University, Montreal, QC. The remainder were obtained via Charles River, Saint-Hyacinthe, QC. All were pair-housed under a 12-hour reversed light-dark cycle (dark 2000 to 800) in shoe-box cages (25.5 cm wide x 46.6 cm long x 21.6 cm high) and were provided with food and water *ad libitum*. All activities were carried out in agreement with the Canadian Council on Animal Care and were approved by the Concordia Animal Research Ethics Committee.

All females underwent OVX surgeries. Anesthesia was achieved via isoflurane gas (Richmond, ON, Canada). Ovaries were removed and E2 capsules were implanted subcutaneously via a unilateral lumbar incision. The antibiotic benzylpenicillin (0.1mL/rat, SC; Rafter 8 Products, Calgary, AB, Canada) was administered before OVX and the analgesic Anafen (0.4mL/kg, SC; Merial Canada Inc., Baie d'Urfé, QC, Canada) following OVX.

Drug & Hormone

A racemic mixture of R (–) and S (+) ketamine hydrochloride (Narketan, CDMV, Lavaltrie, QC, Canada) dissolved in 0.9% NaCl was administered via intraperitoneal (IP) injections 24 hours before the forced swim. The injection time was also exactly 48 hours before the locomotor tests. The five doses administered were 0, 2.5, 5.0, 10.0, and 20.0 mg/kg.

As previously mentioned, female rats were further separated into hormonal groups. All received E2 capsule implants made of Silastic tubing (1cm long, inner diameter 1.96mm). The capsules contained a 5% concentration of E2 in cholesterol and released a chronic low dose of

E2 (20 pg/mL serum concentration; Sigma-Aldrich, St. Louis, MO) from 8 to 24 days post-implant. This level of E2 is similar to that seen during the diestrus phase in normally cycling female rats (Almey, Hafez, Hanston, & Brake, 2013; Overpeck, Colson, Hohmann, Aplestine, Reilly, 1978). Those in the high E2 and high E2 + P groups received E2 injections in addition to the E2 capsules already implanted. The E2 (10µg/kg, SC; Sigma-Aldrich, St. Louis, MO) dissolved in sesame oil was administered immediately after ketamine. This dose has previously been shown in the Brake lab to produce levels of E2 at approximately 75-90pg/mL, thus mimicking levels observed during the proestrus phase (Almey et al., 2013). Rats in the high E2 + P group received a second injection of progesterone (500µg, SC; Sigma-Aldrich, St. Louis, MO) dissolved in sesame oil four hours before the forced swim test session. This dose has been shown to produce sexual receptivity in ovariectomized rats (Chesler & Juraska, 2000; Edwards, Whalen & Nadler, 1968). Male rats received sesame oil SC injections in place of the E2 and P injections, as well as saline, or 10mg/kg ketamine.

Competitive enzyme-linked immunosorbent assays (Enzo Life Sciences) were used to assess if our hormonal manipulations produced the expected hormone levels. Blood was collected the day after day three of testing – see Figure 2a. Blood was collected in ice cold vials and then immediately fractionated via centrifuge to separate the plasma, which was stored at -20˚C. The E2 assay (ADI-901-1740) reports cross-reactivity levels for E2 at 100%, for estrone at 17.8%, and for estriol 0.9%. The P assay (ADI-900-011) reports no cross-reactivity levels.

Apparatus

Forced Swim Test (FST) Chambers. Testing was conducted using a transparent glass cylinder 23.5cm in diameter filled with water at 25±1°C to approximately 40cm high such that the animal's tail could not touch the bottom. Plexi-glass cylindrical inserts were placed inside the top of the chambers in order to stop the rats from escaping. All sessions were recorded from a horizontal point of view via webcam (Logitech HD 1080p). Videos were scored with the behavioral recording software OdLog 2. As per Porsolt and colleagues (1977, 1978), immobility was defined as the animal floating, making only those movements required to keep its head above water. Rats were placed in the water for ten minutes during the initial habituation session before exposure to drug and hormone treatments, and for five minutes during a second test session 24 hours after the habituation session.

Locomotor activity chambers. Locomotor activity and animal position (center vs. edge) were recorded for ten minutes in a plexiglass cubic chamber (40.7cm long x 41cm wide x 39.2cm high) via an 8 x 8 infrared beam matrix. The chamber was surrounded by Styrofoam walls and cover such that the chamber is dark during recording. Rats were placed in the chambers for ten minutes during each session; once 20 minutes before the FST habituation session, and once 48 hours after ketamine and E2 administration – see Figure 3. Truscan software (Truscan Activity Monitoring System; Coulbourn Instruments, Allentown, PA, USA) provided the raw data in the form of distance travelled in the chamber.

Testing Protocol

After a handling period of two weeks, all female rats underwent OVX and E2 capsule implantation. Female rats were then randomly assigned to one of the 15 groups, whereas the males were separated into two groups. This was followed by a week-long recovery period, and

then once-daily injections of saline for two days to habituate to the receiving of intraperitoneal (IP) injections – see Figure 2a. Day one of testing consisted of three components. First, rats were tested in the locomotor chambers for a period of ten minutes. Second, approximately 20 minutes after the locomotor test, rats were tested in FST for a period of ten minutes, known as the habituation session. Third, rats were injected with ketamine or saline, and subsequently E2 or sesame oil. Injections were approximately two hours after the habituation session such that the FST test session the following day was exactly 24 hours after both injections. Day two of testing had two components. First, rats were injected with P or sesame oil. Second, exactly four hours after the P injection, rats were placed in the FST for five minutes, known as the test session. Day three of testing consisted of placing the rats in the locomotor test, again for ten minutes. This test was timed to occur exactly 48 hours after the ketamine and E2 injections of testing day one. The day after, all rats were euthanized – see Figure 2b. Male rats were subjected to the same testing schedule, without surgery, and were administered sesame oil in place of hormones.

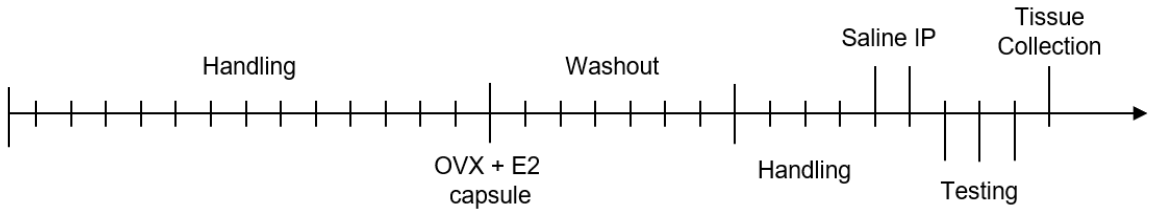


Figure 2a. Experimental timeline for all rats. Each tick mark represents one day. OVX indicates the time of ovariectomy.

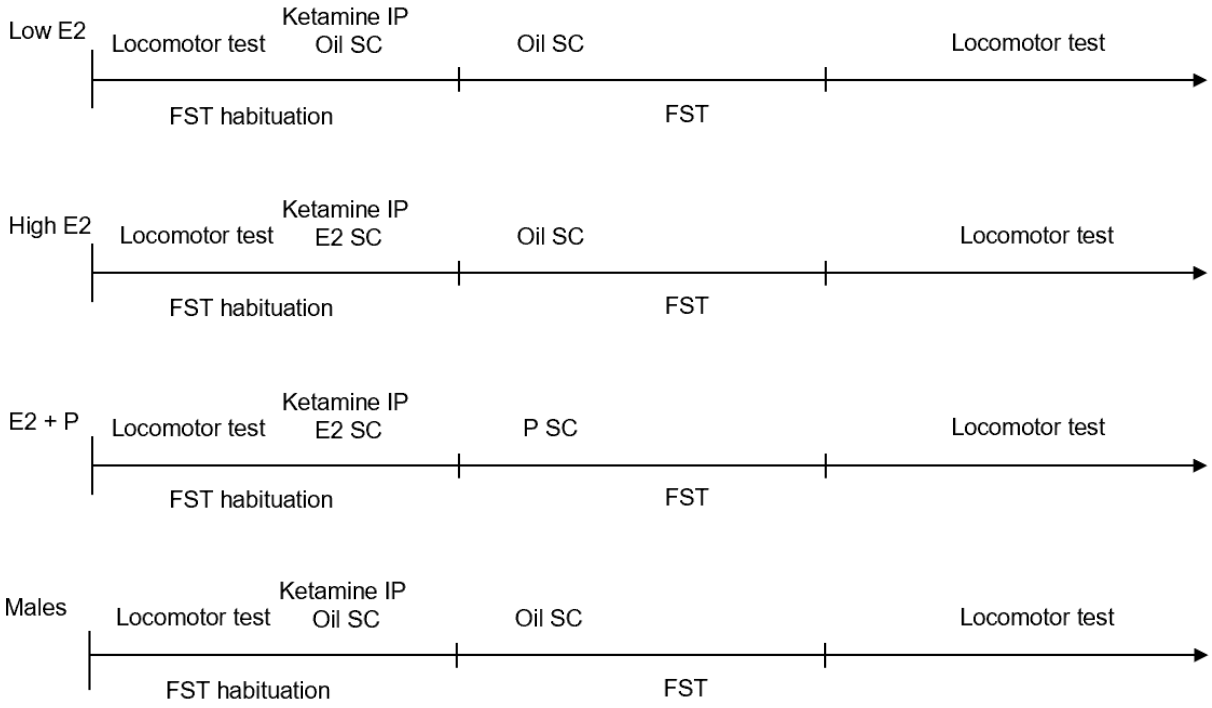


Figure 2b. Daily breakdown of testing protocol, separated by hormonal group for females. Each tick mark represents one day. IP = intraperitoneal injection. SC = subcutaneous injection. E2 = 17β-estradiol. P = progesterone.

Statistical Analysis

The design of the current research has two factors: hormone, and ketamine. The hormone factor had three levels and the ketamine factor had five levels, resulting in 15 female groups in total. The statistical analysis was therefore a two-way 3 x 5 ANOVA in terms of analyzing both the forced swim and locomotor test data. Of primary interest was an interaction between the two factors. However, a main effect of either factor would have been of interest as well due to the exploratory nature of this research. Male rats were separated into two groups (n = 10 each): saline and 10mg/kg. Results were analyzed via Welch's T-test which assumes unequal variance between the two groups.

A priori power analysis revealed that the design yielded a power of .779, based on an f effect size of 0.25. The f effect size is the default of the statistical software used to perform the power analysis and is analogous to partial eta-squared (η_p^2; Faul, Erdfelder, Lang, & Buchner, 2007; 2009; Lakens, 2013). The current work reports eta-squared (η^2) as an effect size for several reasons. In calculating various eta squared statistics in the same data set, one can compare them to each other because they have the same denominator (SS_{Total}), making these values easier to interpret (Kline, 2013). This is not the case for partial eta squared because the denominators change across the different factors. Eta-squared is also in the original metric of the dependent variables, making it directly comparable across studies employing similar paradigms (Lakens, 2013).

Results

The ANOVA for the females revealed no statistically significant interaction effect between the factors, as well as no main effects of both the hormone and ketamine factors – see Appendix A. This was the case for both the locomotor and forced swim test data – see Figures 3 through 6. In the framework of null-hypothesis significance testing, the experimental hypotheses are rejected based on the criteria of $p < .05$ – see Appendix A. Note that a lack of statistically significant differences in the locomotor test reveals that ketamine did not impact locomotor behavior – see Appendix C. Moreover, as can be seen in Figure 3, ketamine had the greatest impact in reducing immobility, in terms of percent-change from the saline dose, in the high E2 + P group. This effect became apparent when looking at the data broken down minute by minute – see Figure 5. Effect sizes also revealed there was a strong effect of hormone alone on immobility but in the opposite direction of these hypotheses, with the high E2 + P group being the most immobile in the control ketamine group ($\eta^2 = .25$) – see Appendix B.

A Welch's T-test revealed a significant decrease in immobility ($p = .0005$, $d = 1.089$), and a near-significant increase in climbing ($p = 0.0677$, $d = 0.846$), among the 10mg/kg ketamine-treated males compared to those given saline – see Figure 7**.**

As can be seen in Table 1, hormone levels were within acceptable ranges to properly represent the appropriate phases of the estrus cycle of an intact female rat. Note that blood was collected 3 days after E2 injection and 2 days after P injection, thus allowing for a significant amount of metabolism and clearance.

Table 1

Hormone levels (pg/mL)

	n	mean	SEM
E2 capsule only	10	18.69	3.27
E2 capsule + injection	21	39.72	5.59
P injection	8	364.19	49.82

Table 1. ELISA results in picograms per milliliter of blood plasma separated by hormonal treatment, *i.e.* animals in the E2 capsule + injection bar are from both the high E2 and the high E2 + P group.

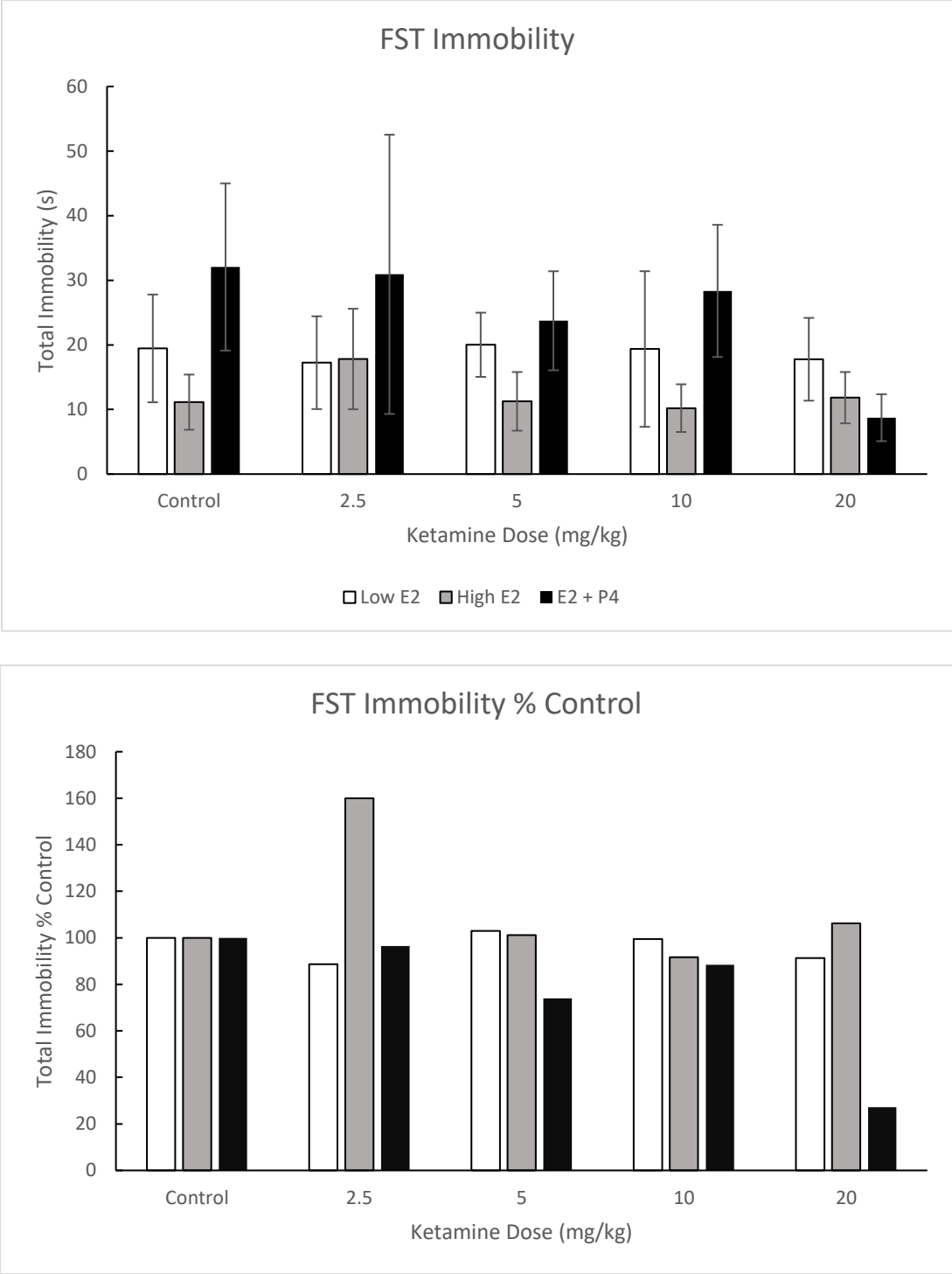


Figure 3. Total immobility in seconds (top) and as a percentage of the effect of hormone in the ketamine control group (bottom) during the five-minute forced swim test among the females (n = 9-10 per group).

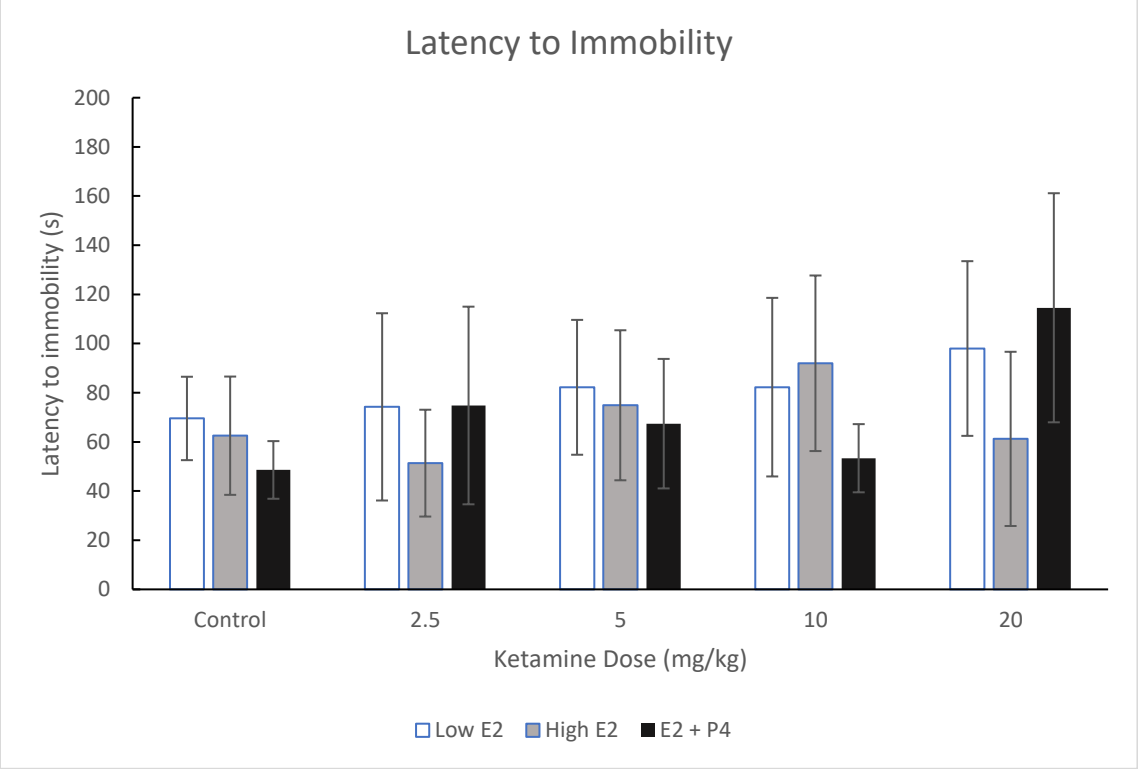
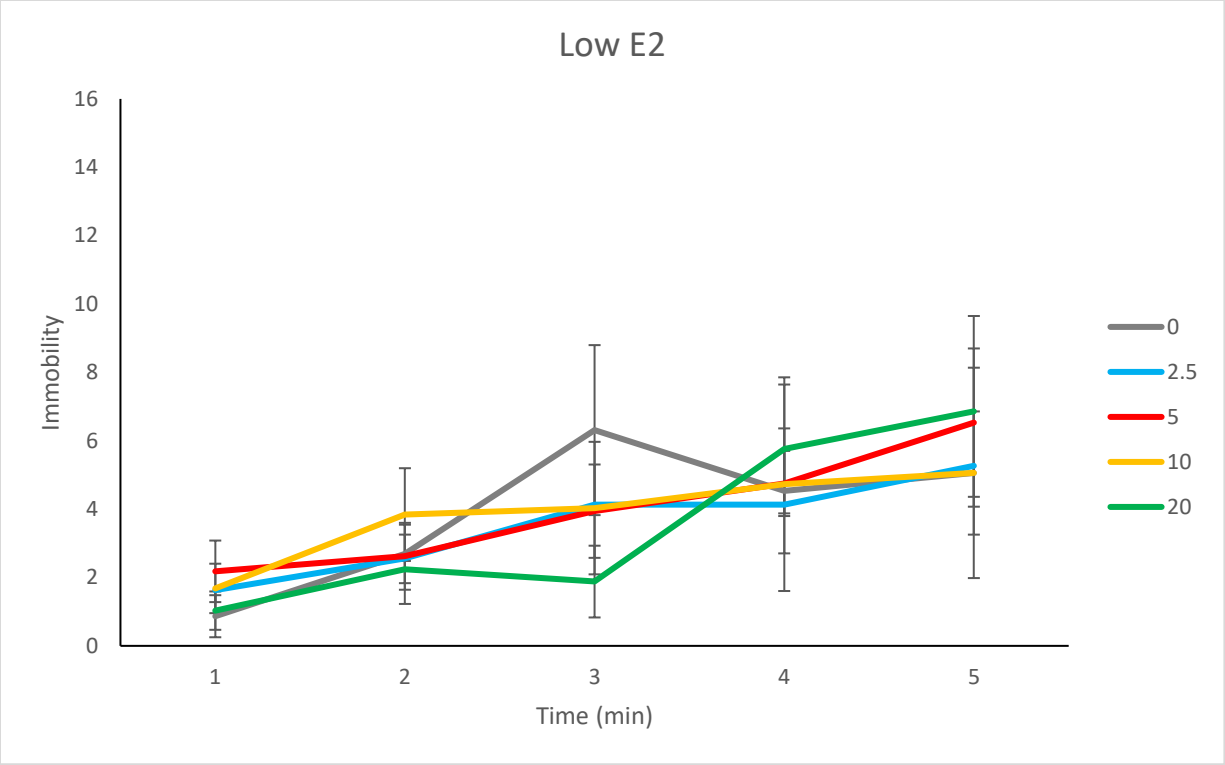
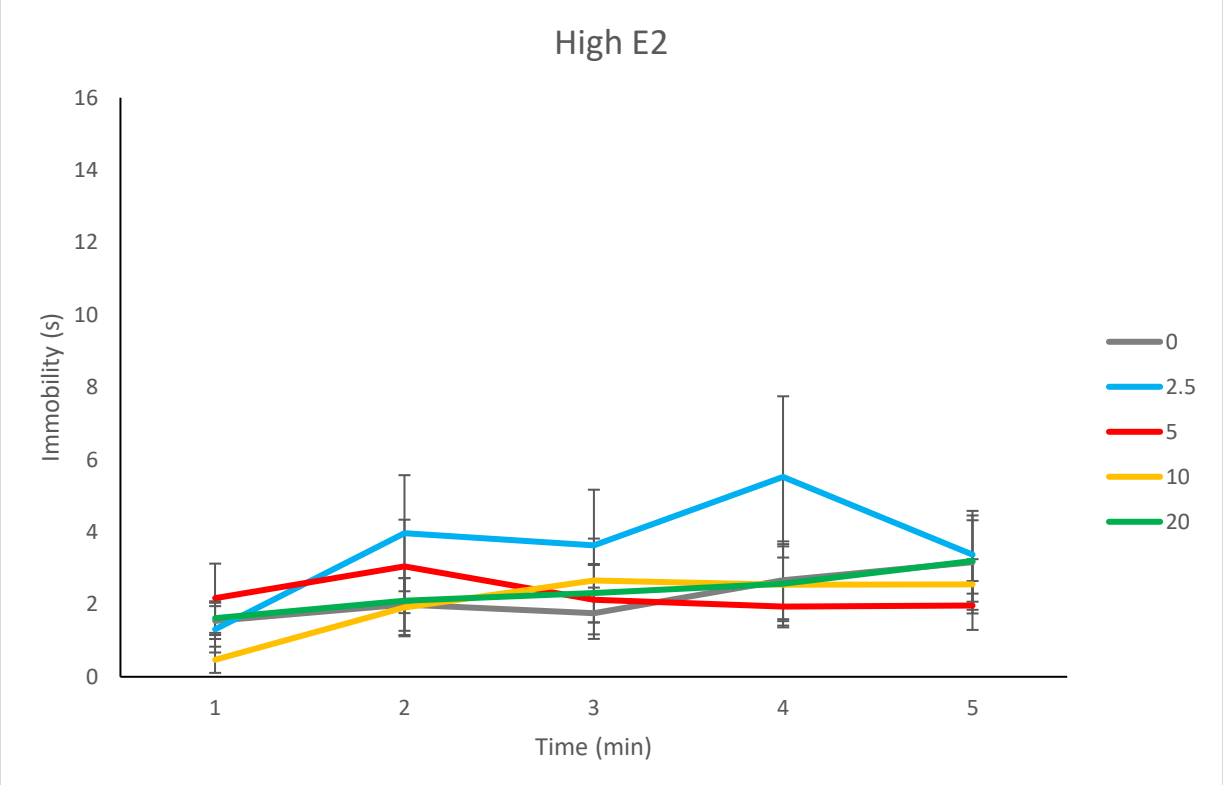


Figure 4. Latency to immobility measured from the moment the rats are placed into the forced swim chambers on day two of testing among the females (n = 9 – 10 per group).


Low E2
Immobility
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Time (min)
0
2.5
5
10
20


High E2
Immobility (s)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Time (min)
0
2.5
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20

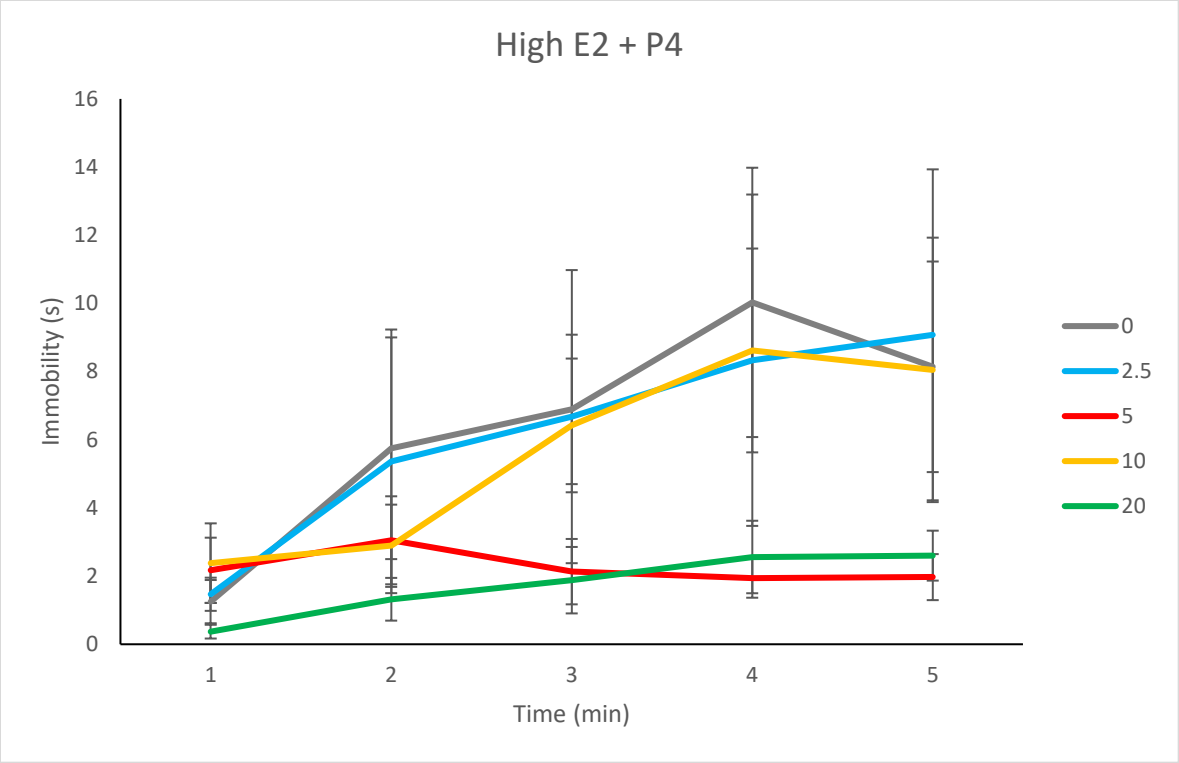

Figure 5. Cumulative immobility per minute in the FST, separated by hormonal condition (n = 9 – 10 per group). Rats in the high E2 + P condition responded to the 5 and 20mg/kg dose such that immobility remained low throughout the entirety of the test.

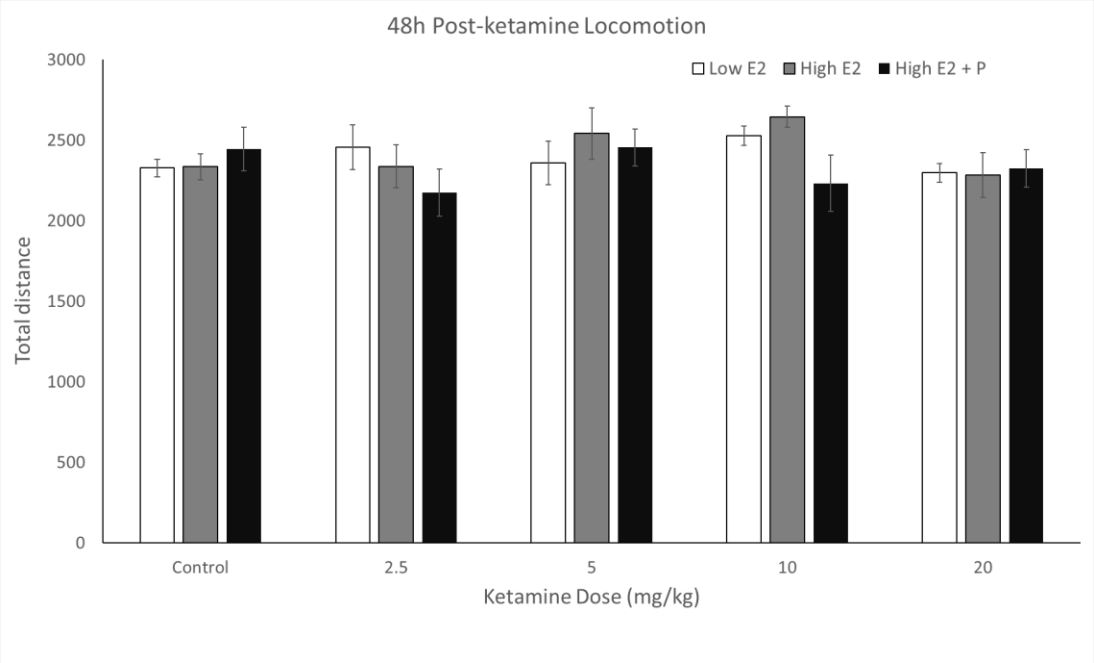

Figure 6. Locomotor activity 48 hours after ketamine and estrogen administration among the females (n = 6 – 9 per group).

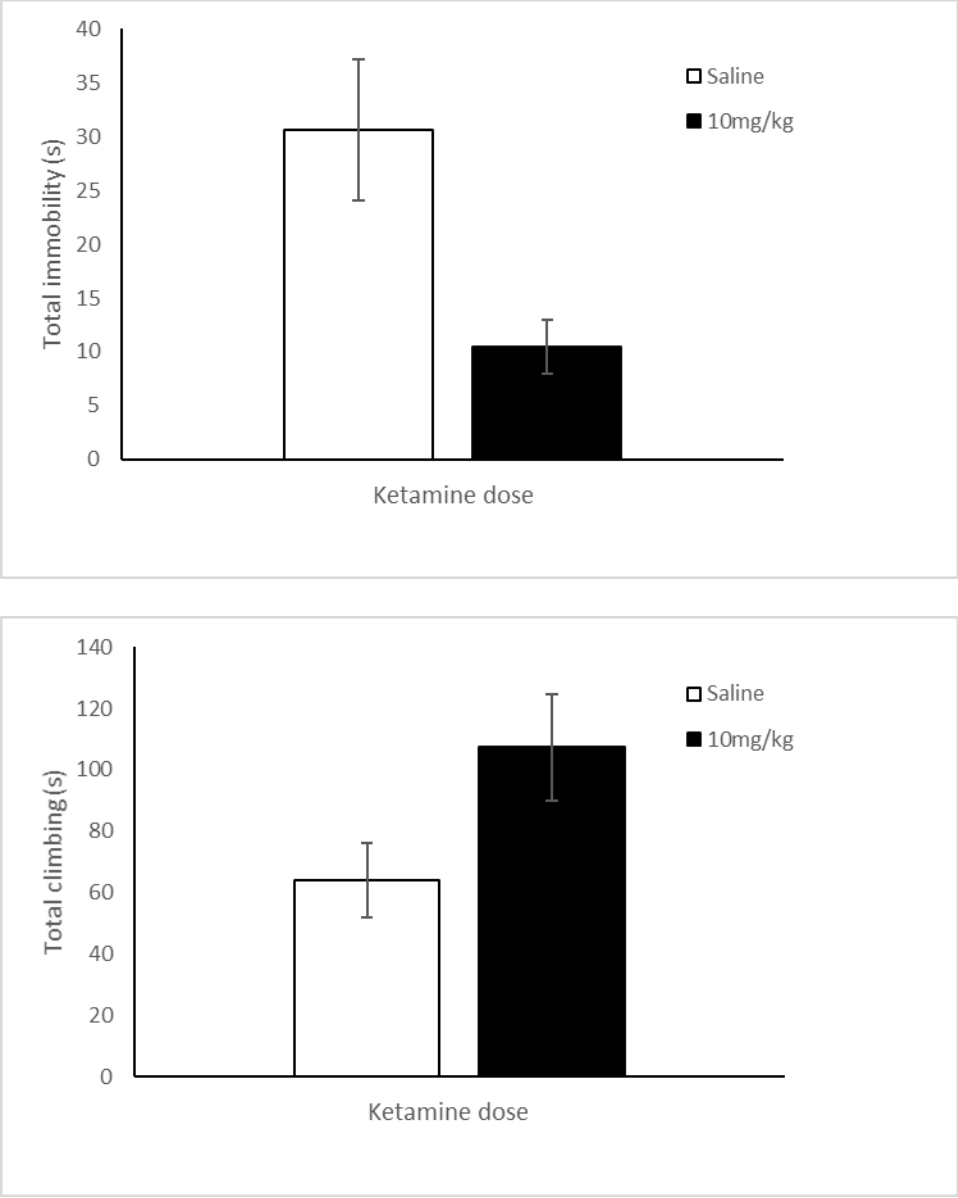

Figure 7. Total immobility in seconds (top) and total climbing in seconds (bottom) during the five-minute forced swim test among the males (n = 10 per group).

Discussion

The goal of this study was to analyze the behavioral effects of ketamine and ovarian hormones in the forced swim test (FST). The primary research hypotheses, concerning the female rats, were two-fold. First, that rats would respond to ketamine in a dose-dependent manner, becoming less immobile in the FST as ketamine dosage increased. Second, that, with the addition of P, in the high E2 + P group, rats would spend even less time immobile in the FST in response to ketamine than those in the other hormone conditions. ELISAs confirmed that the hormonal manipulations accurately represented the desired phase of the rat estrus cycle. The female's data revealed no statistically significant main effects nor an interaction effect. However, as can be seen in figure 3, rats in the high E2 + P condition responded to ketamine in a near dose-dependent manner where the 20mg/kg ketamine group spent the least time immobile across all 15 groups. This is in-line with previous work (Carrier & Kabbaj, 2013) who showed that OVXd female rats only responded to ketamine in the FST when both estrogen benzoate (EB) and P were on board, which will be discussed further.

Male rats responded to the 10mg/kg ketamine dose by spending significantly less time immobile then those that received saline. This shows that the FST, in our hands, was sensitive to the antidepressant-like effects of ketamine, as many have shown in the past (Garcia et al., 2008; Réus et al., 2011; Yang et al., 2013)

Concordant & Discordant Findings

Using ovariectomized Sprague-Dawley female rats, Carrier & Kabbaj (2013) examined the role of the ovarian hormones estrogen benzoate (EB) 24 hours and P four hours before the FST, much like the current work. Rats were injected with either saline or 2.5mg/kg of ketamine 30 minutes before the FST. Their findings showed no effect of this small dose of ketamine on

OVX rats treated with EB alone (2 or 10mg/kg), nor P alone (500ug). Only rats treated with 2mg/kg of EB in tandem with 500ug of P were responsive to this low dose of ketamine. Although this somewhat agrees with the current work's results, the design of Carrier & Kabbaj (2013) was such that ketamine was still onboard during the time of the FST, whereas in the current work it is not. The half-life of ketamine ranges from 2-3 hours (Clements & Nimmo, 1981; Grant, Nimmo & Clements 1981). It is possible that EB and P increased susceptibility to ketamine at a pharmacological level, producing antidepressant-like effects.

In a separate experiment, Carrier & Kabbaj (2013) found that intact female rats responded to ketamine as expected in the FST at all doses tested (2.5, 5, 10mg/kg) in comparison to saline. Theoretically, the current work should have replicated these findings, especially at the 10mg/kg dose which is what the literature has consistently found to produce positive results (Browne & Lucki, 2016; Burgdorf et al., 2013; Koike, Iijima, & Chaki, 2013; Reus et al., 2011). The fact that these results were not replicated in the current work could be due to a variety of factors such as the strain of the animal, the experimental timeline, or the type of estrogen administered. Browne & Lucki (2016) posit that Wistar rats are insensitive to ketamine at doses of 2.5 and 5.0mg/kg in the FST following chronic treatment. There is also a dearth of studies using females throughout the literature in general making it difficult to interpret our findings. Moreover, the FST has been shown to be inconsistent in the literature in terms of success rates and validity.

The forced swim test: consensus or controversy?

Due to the inclusion of hormone and ketamine administration as experimental factors, each with multiple levels, the current work sought a high-throughput behavioral assay, the forced swim test (FST) was chosen. The FST can be interpreted as a measurement of learned

helplessness where animals are placed, typically exactly 24 hours after drug administration, in an inescapable glass chamber filled with water. The degree to which the animal floats passively (i.e. is not trying to escape) is then deemed indicative of depressive-like behavior (Slattery & Cyan, 2012). Since its establishment by Porsolt and colleagues (1977; 1978), the FST has been used to assess antidepressant drug efficacy by thousands of researchers. However, as with many behavioral models, the perception of the test's validity is likely skewed to a more favorable one by publication bias (a.k.a. the file drawer problem). The FST has largely failed to produce novel antidepressants, has failed in some cases to produce statistically significant results using classical antidepressants(Jin et al., 2017; Porsolt et al., 1979; Suman et al., 2018), and has been criticized for having low construct validity for a plethora of reasons (Commons, Cholanians, Babb, & Ehlinger, 2017; Yankelevitch-Yahav, Franko, Huly, & Doron, 2015). Moreover, the FST is highly sensitive to species strain in both mice (Lucki, Dalvi, & Mayorga, 2001) and rats (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013; Tejani-Butt, Kluczynski, & Paré, 2003). Others have suggested that the FST is simply a model of adaptive behavior in the face of an inescapable acute stressor, where immobility is merely a switch from active to passive behavior (Molendijk & de Kloet, 2015). Indeed, Porsolt and colleages (2009) noted that the test has an inherent flaw: the dependent variable of interest, immobility, is a product of the test itself and does not exist outside the test's context. The FST should therefore be seen as a simple test for antidepressants, not a model of depression (Castagné, Moser, & Porsolt, 2009).

Originally, the FST was seen as a model of learned helplessness, such as the tail-suspension test, or foot-shock paradigms. It was initially hypothesized that learned helplessness is a state where an organism has learnt that its behavior (i.e. trying to escape an undesirable environment or sensation) and the consequences of its behavior (i.e. escaping) are independent

(Maier & Seligman, 1976). Today, it is understood that this is not the case. In fact, passive behavior in response to extended periods of aversive stimuli is the default response, and serotonergic signaling from the dorsal raphe inhibits escape. Whereas when animals learn that aversive stimuli can be controlled, medial PFC activity inhibits the dorsal raphe (Maier & Seligman, 2017). This represents a clear fundamental misunderstanding in interpreting the various behaviors exhibited during the FST.

Fortunately, a large body of evidence supports the idea that the FST is, at the very least, sensitive to both classical (Dulawa, Holick, Gundersen, & Hen, 2004; Jin et al., 2017; Lucki, Dalvi, & Mayorga, 2001; Mezadri et al., 2011; Tejani-Butt, Kluczynski, & Paré, 2003) and more novel antidepressant drugs, including ketamine (Burgdorf et al., 2013; Carrier & Kabbaj, 2013; Garcia et al., 2008; Gigliucci et al., 2013; Koike, Iijima, & Chaki, 2011; Koike, Iijima, & Chaki, 2013; Muller et al., 2013; Salat et al., 2015; Wang et al., 2011) . However, as noted by Willner's review paper (1984) many non-antidepressant compounds also cause immobility in the FST due to the suppression of overall locomotor activity. Therefore, for the sake of enhancing the FST's credibility, we also tested locomotor activity before and after the FST, as suggested by Yankelevitch-Yahav and colleagues (2015). Still, there are multiple conceptual issues with the test's clinical relevance.

One theory to consider when assessing work employing the FST as a model of depression is that it is perhaps a better model of anxiety. A paper recently published by Anyan & Amir (2018) illustrates why this might be the case. Animal models which purportedly measure depression-like and anxiety-like behaviors, such as the FST and the elevated plus maze, respectively, have found both no, or a negative, association between the two, whereas the two pathologies are highly comorbid in humans (Ho, Eichendorff, & Schwarting, 2002; Estanislau et

al., 2011; Lamers et al., 2011). What's more, antidepressants such as selective-serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants (TCAs) typically take several weeks of chronic administration to produce a therapeutic effect (Harmer, Goodwin, Cowen, 2009; Frazer, Benmansour, 2002). Individuals can however experience the onset, or worsening, of anxiety for a brief period soon after taking the antidepressant in question. This is especially true for SSRIs (Gorman et al., 1987; Westernberg & den Boer, 1989; Marcinkiewcz, et al., 2016). Both the classic and modified FST paradigms are designed in such a way that allows the researcher to observe behavioral effects 24 hours after administering the drug of interest. It is therefore possible that these findings are being misinterpreted as antidepressant-like where they should be seen as anxiogenic-like. This is supported by research showing that anxiogenic agents, such as β-carboline-3-carboxylic acid ethyl ester, an inverse agonist at the benzodiazepine binding site of GABA A receptors, have immobility-decreasing effects in the FST (Nishimura, Ida, Tsuda, & Tanaka, 1989). Furthermore, the α-adrenoreceptor antagonist yohimbine, which has been shown to have anxiogenic effects, also prolongs the effect of SSRIs on immobility in the FST (Dhir & Kulkarni, 2007). It is therefore important to look to other experimental paradigms modeling depression for future directions.

Limitations

It is possible that the design of this research induced order-effects that were uncontrolled for. The initial locomotor chamber exposure and subsequent FST exposure 20 minutes later may have produced unknown effects on FST behavior during the test session. Controlling for this would have meant administering both drug and hormone at different time periods relative to all the behavior test sessions, essentially conducting a separate experiment. There are also several discrepancies between the human menstrual and rat estrus cycles that make the generalizability

of these results difficult. Other evidence suggesting that rodent models are problematic are the levels of differential sensitivity between the sexes. Female rodents have been reported as more sensitive to lower doses of ketamine (Carrier & Kabbaj, 2013; Franceschelli et al., 2015; Sarkar & Kabbaj 2016), whereas no such difference has been found among clinical trials, as previously discussed (Coyle & Laws, 2015; Romeo, Choucha, Fossati & Rotge, 2015).

Future directions & Conclusion

Future studies should conduct parallel *in vivo* and *in vitro* experiments, for the sake of establishing the relevance of the literature which much of the theoretical framework of ketamine is based on. One could easily determine whether the addition of P to a high E2-treatment yields greater microglial inflammatory activity. It may also be beneficial to attempt to replicate the findings of the current work in other species, or strains of rat. Lastly, given that with P replacement showed the greatest response to ketamine in the FST, it would be prudent to elaborate on this and administer a moderate P dose (i.e. 250ug) to observe any potential dose-dependent responses that might occur.

From a behavioral perspective, there are several models with more acceptable construct validity than the FST. Anhedonia, defined as a lack of ability to feel pleasure or lesser desire to engage in previously pleasurable activities, is a hallmark symptom of depression in the DSM-V (American Psychiatric Association, 2013). Of the minimum five symptoms an individual must have to be diagnosed with MDD, one must be either depressed mood or anhedonia (Tolentino & Schmidt 2018). Importantly, anhedonia is relatively easy to observe in animals. On the other hand, the FST been said to produce learned helplessness, as previously discussed, which does not accurately mimic how depression presents itself in clinical populations. Anhedonia in animals is much more translatable to depression in humans, although it of course only represents a single

symptom of a complex disorder. Models ranging from the more complex, such as intracranial self-stimulation, to those with higher throughput, such as the sucrose preference test (SPT), should be employed in the future to assess the interactive effects between ovarian hormones and ketamine.

Lastly, an important consideration for future experiments is to use tests like the FST or SPT as assays to assess the effectiveness of manipulations meant to elicit depressive-like behavior in the first place. Although it would have greatly increased the experimental timeline, it may have been prudent to expose the rats daily to some form of stressor to elicit depressive-like behavior, which could then be relieved by ketamine. Manipulations such as chronic unpredictable mild stress, social isolation, or social defeat stress are often used before the administration of an antidepressant manipulation (Donahue et al., 2014; Garcia et al., 2009; Sarkar & Kabbaj, 2016). This serves to produce neurobiological and behavioral changes which can be detected by behavioral assays like the FST and SPT, and then mitigated by ketamine.

In conclusion, there is a lack of research on the interaction between ketamine and ovarian hormones. The current study did not find any statistically significant effects in terms of this interaction. We showed that ketamine is most effective when P is on board, in agreement with previous literature, and that the FST was sensitive to ketamine among males, as others have shown in the past. Few pharmaceutical agents have been shown to have such robust effects in ameliorating suicidal ideation and depressive mood. With ketamine, this can occur sometimes within hours of administration (Berman et al., 2000; Zarate et al., 2006; Diazgranados et al., 2010; Murrough et al., 2013), representing one of the largest breakthroughs in treatment for depression in history. The consequences of developing a solid base of preclinical knowledge with regards to ketamine-hormone interactions is critical to our understanding of its mechanism

of action in females. It is critical because females will likely represent at least two thirds of individuals who wind up being prescribed ketamine in the future. Due to ketamine's lack of patentability it is promising that the pharmaceutical industry is likely racing to understand ketamine's mechanisms of action to produce even more effective antidepressants. But without understanding why these effects are taking place, our understanding regarding the nature of major depressive disorder will remain too superficial to make these leaps in treatment.

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Appendix A: Hormone vs. Ketamine 3x5 Two-way ANOVA & Effect sizes

Forced swim test total time spent immobile ANOVA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	η^2
Hormone	2435.55	2	1217.78	1.96	0.15	3.12	0.04
Ketamine	2360.51	4	590.13	0.95	0.44	2.49	0.04
Interaction	4904.42	8	613.05	0.99	0.45	2.06	0.09
Within	46586.15	75	621.15				
Total	56286.64	89					

Forced swim test latency to immobility ANOVA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	η^2
Sample	5753.37	2	2876.68	0.45	0.64	3.12	0.01
Columns	41965.48	4	10491.37	1.64	0.17	2.49	0.07
Interaction	32782.61	8	4097.83	0.64	0.74	2.06	0.06
Within	479448	75	6392.64				
Total	559949.5	89					

Appendix B: Hormone One-way ANOVA & Effect size

Forced swim test total time spent immobile ANOVA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	η^2
Between Groups	2626.64	2	1313.32	2.55	0.11	3.68	0.25
Within Groups	7711.69	15	514.11				
Total	10338.33	17					

Appendix C: Locomotor 3x5 Two-way ANOVA

Locomotor behavior post-ketamine ANOVA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	168241.57	2.00	84120.79	0.79	0.46	3.12
Columns	553625.30	4.00	138406.33	1.30	0.28	2.49
Interaction	514210.86	8.00	64276.36	0.61	0.77	2.06
Within	7957635.83	75.00	106101.81			
Total	9193713.57	89.00				

Appendix D: Male total immobility Welch's T-test

Welch's two-sample T-test

Total Immobility	*n*	*mean*	*SEM*	*T-stat*	*T-crit*	*p*	*d*
10mg/kg	10	10.47	2.50	-4.28	1.78	0.0005	1.09
Saline	10	30.61	6.58				