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Behavioral Effects and Neurobiological Mechanisms of 3-Aminobenzimide in a Rodent Model of Chronic Psychological Stress

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Behavioral Effects and Neurobiological Mechanisms of 3-Aminobenzimide in a Rodent Model of Chronic Psychological Stress

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by

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ABSTRACT

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Major depressive disorder (MDD) is a leading cause of disability worldwide, with a lifetime prevalence rate of approximately 20%. Inadequate pharmacological treatment methods for MDD are a significant debilitating factor. Patient estimates suggest that the treatment resistance rate for pharmacological interventions is over 30%. Postmortem analyses of human tissue of individuals diagnosed with MDD have shown an increase in Poly (ADP-ribose) polymerase 1 (PARP-1) mRNA gene expression in prefrontal cortical white matter when compared to psychiatrically normal brain tissue. In order to further investigate this issue, the present study used the social defeat stress/chronic unpredictable stress (SDS + CUS) rodent model of depression to induce a state of chronic psychological distress. Rats were treated with either the PARP-inhibitor, 3-aminobenzamide (3-AB); a common selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX), or saline. During the stress manipulation we conducted the sucrose preference test, results revealed that saline-treated rats which had undergone SDS + CUS showed significant reductions in sucrose preference compared to all other groups. In addition, a social interaction test was conducted one day after the stress manipulation, and saline-treated stressed animals demonstrated less social interaction compared to all other groups, indicating the stress manipulation was effective. Neurobiological assays were conducted to examine PARP expression, microglial morphology, and proinflammatory cytokine expression. Though we

expected to find a decrease, results from immunofluorescence studies of tissue sections revealed an elevation of PARP-1 protein expression in prefrontal cortical gray matter in the FLX/Stress group compared with SAL/Stress group. Microglial morphological changes indicated that the SAL/Stress group had significantly more prolate microglia when compared to all other treatment groups, suggesting early activation of microglia, an indicator of neuroinflammation. Increases in IL-1β and TNF-α expression was observed in the hippocampus of the SAL/Stress group when compared to all other treatment groups. Interestingly, IL-6 expression was significantly elevated in the SAL/Stress group when compared to the FLX/Stress group and the CTRL/No stress group but did not significantly differ from the 3-AB/Stress group. This study revealed therapeutic potential of 3-AB for the treatment of stress-related disorders, as well as the neuroinflammatory mechanisms associated with chronic stress.

DEDICATION

This dissertation is dedicated to:

My husband, Gary Wills, who has always been my number one supporter throughout graduate school.

The best dogs in the world, Zoe & Buck, even on the worst of days I knew once I got home, they would cheer me right up.

My parents who have always pushed me to become the best version of myself and who have taught me the joys of learning.

My siblings, Nic, Elizabeth, & Jack, no matter how tough life gets whenever we get around each other we all start acting like kids again, cracking jokes and picking on each other.

Finally, the animals used in this project and all of scientific research without them scientific advancement would not be possible.

"The Harvard Law of Animal Behavior holds that under controlled experimental conditions of temperature, time, lighting, feeding, and training, the organism will behave as it damn well pleases."

- Joel Garreau

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Chapter 1. Introduction

Allostasis exemplifies the active physiologic process of maintaining homeostasis with the engagement of the nervous, endocrine, and immune systems while an organism interacts with the environment (McEwen, 2007; Smith & Vale, 2006; Sterling, 1988). When an organism encounters a perceived threat, a mild acute stress response is initiated causing behavioral and physiological changes (Chu et al., 2020). The stress response begins when activation of the sympathetic-adreno-medullary (SAM) axis notifies the adrenal medulla to release epinephrine and norepinephrine (NE), in order to initiate a state of temporary arousal. Subsequently, the hypothalamic-pituitary-adrenal (HPA) axis activates, leading the adrenal cortex to secrete glucocorticoids allowing the body to return to allostasis (Khan & Khan, 2017; Smith & Vale, 2006). A pivotal aspect of the normal stress response is that once the stressor is removed, the body will return to allostasis (Kalinichenko et al., 2019). However, exposure to chronic stress leads to deleterious physiological and psychological consequences, such as a compromised immune function (Dhabhar, 2009), diabetes (Chen et al., 2003), obesity (Razzoli & Bartolomucci, 2016), heart disease (Vitaliano et al., 2002), anxiety (Vyas et al., 2004), and depression (Brown et al., 2004; Drazen et al., 2001).

Stress and Immune Response

The immune and endocrine systems are very closely intertwined, so much so that immune cells contain "stress" hormone receptors. These hormones can modulate immune function through one of two ways: 1) directly, by binding to its respected receptor on the cell's surface or 2) indirectly, by initiating deregulatory cytokine production, such as interferon-γ (IFN- γ), interleukin-1 (IL-1), and tumor-necrosis factor (TNF; Glaser & Kiecolt-Glaser, 2005). Stressors can elicit several types of immune responses depending on certain factors, such as

duration of a stressor, the stressor's effect on leukocyte distribution in the body, and whether endogenous or synthetic glucocorticoids are involved (Dhabhar, 2014). An acute stressor is thought to activate an immunoprotective response to prepare the immune system for challenges (e.g., wound, surgery, infection; Dhabhar & McEwen, 1996). Studies have indicated that after a brief stressor, glucocorticoids can mediate the activity of leukocytes causing these cells to migrate from lymphoid organs to peripheral blood and skin (Dhabhar & McEwen, 1996; 1997). Contrarily, chronic stress has been shown to be immunosuppressive or immunopathological (Glaser & Kiecolt-Glaser, 2005). Epel and colleagues (2004) demonstrated that women who reported high levels of chronic stress had blood lymphocytes and monocytes with significantly shorter telomeres than women who reported low levels of stress. This suggests that chronic stress enhances the aging of immune cells and contributes to immune function dysregulation (Epel et al., 2004).

Chronic Stress and Oxidative Stress

Oxidative stress is indicative of cellular imbalance, in which reactive oxygen species (ROS) proliferation exceeds the neutralizing ability of antioxidants, leading to the damage of lipids, DNA, RNA, and other nearby molecules (Bettridge, 2000). Cortisol reactivity is thought to be a mediator of oxidative damage among people who are chronically stressed. Specifically, chronically stressed individuals show significantly elevated levels of cortisol and markers of oxidative damage when compared to low-stressed controls (Aschbacher et al., 2013). In a chronic unpredictable stress (CUS) rodent model of depression, it was demonstrated that chronically stressed rats had significantly increased levels of several markers of oxidative stress in similar brain regions as humans with major depressive disorder (MDD). In particular, the stressed rats showed a significant increase in lipid peroxidation in the prefrontal cortex and a

significant increase of mRNA levels of 8-oxoG glycosylase1 (*Ogg1*) and methionine sulfoxide reductase A (*MsrA*) in the hippocampus (Herbet et al., 2017). A collaborating laboratory reported similar results in human postmortem brain tissue, in that the brain tissue of humans with MDD had significantly shorter oligodendrocyte telomeres and increased *Ogg1* mRNA levels when compared to psychiatrically normal postmortem tissue (Szebeni et al., 2014).

Neuroinflammation and Depression

Inflammation is defined as the activation of the innate immune system. During an acute inflammatory response, the immune system must neutralize the threat and return the body to homeostasis as quick as possible (Chen et al., 2018). However, if the inflammatory response is activated for an extended period it begins to have a deleterious impact on the body. Neuroinflammation has been implicated in psychiatric illnesses, such as schizophrenia, bipolar disorder, and MDD (Swardfager et al., 2016). Over the past several decades there have been numerous studies that have indicated the critical role of inflammation in the development and severity of depression (Irwin & Miller, 2007; Schleifer et al., 1989; Zorrilla et al., 2001). Specifically, studies have shown that otherwise medically healthy individuals exhibited increased blood plasma levels of inflammatory markers, such as inflammatory cytokines, chemokines, and acute phase proteins (for review: Raison et al., 2006). Moreover, there is a high prevalence rate of MDD or depressive symptomology in autoimmune disorders, such as type-1 diabetes, HIV, and irritable bowel syndrome. This suggests that there may be a common pathological factor between these two types of seemingly different disorders (Arias et al., 2011; Sriram et al., 2015).

Initially, it was believed that the blood-brain-barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) prevented peripheral immune cells from entering the brain (Marques & Sousa,

2014). However, researchers have now demonstrated numerous ways in which the peripheral immune system interacts with the central nervous system (CNS). For example, afferent vagus neurons contain IL-1β and prostaglandin receptors, enabling inflammatory signals to transmit directly to the nucleus tractus solitarius of the brainstem (Olofsson et al., 2012). Peripheral cytokines may also enter the CNS by means of volume diffusion via the BCSFB of the choroid plexus or the ventricles (Dantzer, 2009). Additionally, the BBB can actively transport immune cells in and out of CNS via the inflammatory signaling molecule, nuclear factor- κB (NF-κB; Miller et al., 2009).

The release of proinflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) in the CNS impacts multiple molecular pathways. Namely, pro-inflammatory cytokines stimulate mitogen-activated protein kinase (MAPK), thus augmenting the quantity and activity of neuronal presynaptic reuptake pumps resulting in a reduction of monoamines in the synapse (Miller & Raison, 2006). Additionally, pro-inflammatory cytokines activate indoleamine 2,3 dioxygenase (IDO), an enzyme that metabolizes tryptophan into kynurenic acid, thereby compromising the availability of tryptophan to become serotonin (5-HT; Réus et al., 2017; Wichers & Maes, 2004).

Furthermore, psychological stressors can activate the innate immune system without a pathogenic challenge, a phenomenon known as "sterile inflammation" (Fleshner, 2013; Fleshner et al., 2017; Rock et al., 2010). When tissue damage is recognized, in the absence of pathogens, the innate immune system will initiate a sterile inflammatory response. This results in the release of damage-associated molecular patterns (DAMPs) such as S100 proteins, heat shock protein 72, and high-mobility group box-1(HMGB1). Once these endogenous molecules get released into the extracellular environment, they have the potential to activate local and systemic inflammatory responses (Campisi et al., 2003; Fleshner, 2013; Frank et al., 2019).

Glial Cells and Depression

Glial cells, derived from the Greek word for “glue”, were thought of as basic structural cells in the CNS, until recently. Now, it is known that these cells are profoundly complex and are key mediators in most, if not all, CNS activity (For review see Jäkel & Dimou, 2017). Studies are constantly unveiling glial cell dysfunctions at the core of many psychiatric disorders, including MDD (Rajkowska & Miguel-Hidalgo, 2007). There are three major types of glial cells that are thought to be dysregulated in depression: oligodendrocytes, astrocytes, and microglia.

Oligodendrocytes are specialized myelinating glial cells in which their membranes enwrap neuronal axons to enhance the saltatory conduction of action potentials (Domingues et al., 2016; Nave & Wener, 2014; Tasaki, 1939). Additionally, oligodendrocytes structurally control the diameter of axons and dictate the amount of spacing ion channels receive along the nodes of Ranvier, mediating the electrical properties of axons (Barres, 2008; Kaplan et al., 2001). Oligodendrocytes have the highest metabolic rate of any cell in the CNS, making them highly susceptible to oxidative stress (Connor & Menzies, 1996; McTigue & Tripathi, 2008). This is largely due to their prolific production of myelin membrane, possessing the ability to synthesize three-times the cell’s weight in membrane daily, eventually supporting a membrane that is 100-times the weight of the oligodendrocyte (McLaurin & Young,1995; McTigue & Tripathi, 2008). In order to perform this feat, oligodendrocytes need to consume copious amounts of ATP and oxygen to produce adequate amounts of energy. However, if the byproducts of ATP and oxygen are not metabolized properly this results in the creation of ROS, an increased risk of neuroinflammation, and the demyelination of neurons due to the death of oligodendrocytes (Bradl & Lassman,2010).

Astrocytes are the most abundant and multifaceted type of glial cells. They play a crucial

role in maintaining homeostasis in the brain, by preserving the integrity of the BBB, providing nutrients to neurons, and regulating oxidative stress (Rajkowska & Stockmeier, 2013; Sofroniew & Vinters, 2010). During optimal conditions, astrocytes provide an antioxidant response by breaking down and clearing out free radicals in the CNS. However, during pathological conditions, such as when the system is under stress, astrocytes begin producing ROS and other types of free radicals, thus increasing oxidative stress and DNA damage in the brain, leading to increased inflammation (Vainchtein & Molofsky, 2020).

Research has shown that astrocytes, along with microglia, are key mediators in CNS inflammation by releasing pro-inflammatory cytokines (e.g., IL-1, IL-6, TGF-β, and TNF-α). These cytokines, in turn, can recruit and activate peripheral cytokines to aid in the restoration of homeostasis. Unfortunately, excessive levels of these cytokines have been shown to cause neural injury, which has been correlated with MDD (Rajkowska & Stockmeier, 2013). Although it is known that all glial cells are critical in the maintenance of homeostasis, the current study will be focusing on microglial function and psychological stress.

Microglia

Microglia are the resident immune cells of the CNS and share a common origin with peripheral macrophages (For review see Kettenmann et al., 2011). Microglial cells are the primary means of protection in the brain. When in the sentinel state, microglia are actively surveying the microenvironment for threats by using their motile and ramified processes (Colton & Wilcock, 2010; Giulian et al., 1995 Jia et al., 2020). Other roles microglia aid in are associated with neurodevelopment, such as mediating neurogenesis (Al-Onaizi et al., 2020; McPherson et al., 2010), synaptic formation (Andoh & Koyama, 2021; Parkhurst et al., 2013), and synaptic pruning (Hong et al., 2016; Paolicelli et al., 2011).

When there is a disruption to cerebral homeostasis microglia transition into their active state changing their shape, gene expression, and functional behavior (Colton & Wilcock, 2010; Giulian et al., 1995; Kettenmann et al., 2011). Microglia have two known active states, classical activation (M1) and alternative activation (M2; Boche et al., 2012; Jia et al., 2020). During classical activation microglia mediate proinflammation by activating (NOD)-like receptor protein 3 (NLRP3) which in turn stimulates the secretion of cytokines (i.e., IL-1β, IL-6, TNF-α) and other mediators of inflammation (i.e., inducible nitric oxide synthase [iNOS], cyclooxygenase [COX], and matrix metalloproteinase [MMP]). In contrast, the alternative activation is responsible for resolution and repair by preventing neurotoxicity and promoting the release of anti-inflammatory properties (i.e., IL-4, IL-13, IL-10, and transforming growth factor [TGF-β]; Saijo & Glass, 2011; Tang & Le, 2016). A state of microglial decline or dystrophy has also been observed (Jia et al., 2020). In this state of decline, microglia are characterized as being swollen and de-ramified with decreased mitotic activity. Interestingly, this deactivation has been reported during depressive-like behavioral states and is thought to occur due to an over-activation of microglia during later stages of depression. The high metabolic load disturbs the balance between anabolism and catabolism in these cells, causing them to succumb to death (Jia et al., 2020; Streit et al. 2020; Streit et al., 2004).

Accumulating evidence suggests that chronic stress can prime microglia into a proinflammatory activation state that contributes to the onset of depression (For review see Jia et al., 2020; See also Dantzer, 2009; Frank et al., 2007; Frank et al., 2019; Wohleb et al., 2012). The exact mechanisms as to how psychological stress induces microglial activation is enigmatic however, several factors have been implicated. Stressors can initiate molecular patterns of threat such as pathogen-associated molecular patterns (PAMPS) and DAMPs leading to microglial

priming and sensitization towards a neuroinflammatory response (Fleshner, 2013; Fleshner et al., 2017; Jia et al., 2020). One study demonstrated that rats subjected to the inescapable tail shock (ITS) paradigm had significantly increased levels of extracellular hippocampal HMGB1, a DAMP that mediates proinflammatory microglial activation, compared to control rats. However, the administration of BoxA, an HMGB1 antagonist, prevented stress-induced sensitization of proinflammatory microglial activation (Weber et al., 2015). Thus, microglial activation may potentially be mediated by stress-induced molecular patterns (Frank et al., 2019).

Furthermore, stress-induced aberrant neuronal activity has been suggested to contribute to proinflammatory microglial activation. It is established that microglia have receptors for an agglomeration of neurotransmitters (e.g., GABA, glutamate; Liu et al., 2016; Mead et al., 2012; Noda et al., 2000). Rats that underwent acute stressors displayed transient microglial activation in the areas surrounding neurons expressing c-Fos, a protein marker for neuronal activity, in the periaqueductal gray, a region of the brain associated with the stress response. Thus, this suggests a correlation between neuronal activity and stress-induced microglial activation (Sugama et al., 2009).

Stressors have also been associated with reductions in fractalkine signaling, a crucial pathway in neuronal-microglial crosstalk (Cardona et al., 2006; Harrison et al., 1998; Winkler et al., 2017). Fractalkine (CX_3CL1) is a chemokine that is predominantly expressed in neurons and fractalkine receptors (CX_3CR1) are exclusively found on microglia. CX_3CL1plays an important role in attenuating microglial activation as a means to return the cells back to their sentinel state (Lyons et al., 2009). Socially defeated mice have displayed significant reductions in CX_3CL1 and CX_3CR1 expression, indicating that stress negatively impacts the production of this chemokine (Wohleb et al., 2013; Wohleb et al., 2014).

Major Depressive Disorder

Psychological stress transpires when the demands of environmental or internal stimuli exceeds one's perceived ability to cope (Cohen et al., 1991; Glaser & Kiecolt-Glaser, 2005). For decades, research has demonstrated that people diagnosed with MDD also exhibit dysregulation of the HPA axis, with increased levels of cortisol and corticotrophin-releasing hormone (Board et al., 1955; Nemeroff & Evans, 1984; Thai et al., 2021; Vreeburg et al., 2009). The dysfunction is associated with the glucocorticoid receptor signaling, contributing to the chronic hypersecretions of corticotrophin-releasing hormone (Nandam et al., 2019). Namely, neuropeptides, (e.g., corticotrophin-releasing hormone) fail to adapt to chronically stressful situations, as they would in a healthy individual (de Kloet et al., 2005). Preclinical researchers have observed when rodents are chronically administered corticosterone, the rodent homologue to cortisol, they manifest depressive-like phenotypes (Gourley et al., 2008; Sterner & Kalynchuck, 2010; Zhao et al., 2007), indicating that increases in corticosteroid levels may contribute to the development of major depressive disorder (MDD).

Depressive disorders are considered the most prevalent mental health condition, affecting approximately 300 million people globally (James et al., 2018; Liu et al. 2020). Currently, depressive disorders are a leading cause of health-related disability worldwide with a lifetime prevalence rate of approximately 20% (König et al., 2020; Roman & Irwin, 2020; Otte, 2016). The deplorable outcomes associated with depressive disorders are attributed to early age of onset, high prevalence rates, chronicity of the disease, and low remission rates (Van Dam et al., 2018). Specifically, over 50% of patients undergoing pharmacological treatment for MDD will never fully achieve remission even after being prescribed multiple first-line pharmacological treatment options (Akil et al., 2018; Rush, 2006; Williams et al., 2016). The lack of

pathophysiological understanding of MDD is a major cause for the deficit in pharmacotherapeutic agents (Roman & Irwin, 2020; Troubat et al., 2020).

Depressive disorders have a common feature surrounding the presence of despondence, worthlessness, or irritability, concatenated by physical and cognitive changes that significantly affect a person's ability to function (APA, 2013; Coryell, 2020). Of interest in the current study is the classical depressive disorder, MDD. The fundamental feature of MDD is the persistent state of low mood and/or anhedonia that has occurred for at least two weeks and is associated with a noticeable change in daily functioning. Additionally, at least three criterion symptoms must be present, such as changes in weight, sleep, or psychomotor activity as well as decreases in concentration and energy, feelings of worthlessness, and possible suicidal ideation (APA, 2013).

Etiology of MDD

Our current understanding of the manifestation of depression is somewhat limited. The estimated heritability of MDD is 30 - 40% (Geschwind & Flint, 2015) indicating that environmental factors play a significant role in the development of the disorder. There are several factors that have been associated with the increased risk of developing depressive symptoms, such as gender, adverse childhood experiences (ACEs), and chronic medical illnesses (CMIs).

Gender Differences

Beginning from late adolescence to approximately 55 years-of-age, women are twice as likely to report depressive symptoms and receive a diagnosis of depression than males (Girgus & Yang, 2015; Hyde et al., 2008; Nolen-Hoeksema & Hilt, 2009). However, after the age of 55 the gender differences become ambiguous; with some researchers suggesting that the gender

differences dissipate (Bebbington et al., 1998; Girgus et al., 2017; Pachana et al., 2012), while other researchers continue to report significant gender disparities among women (Anstey et al., 2007; Cheruvu & Chiyaka, 2019; Girgus & Yang, 2015). The inconsistencies found in these studies are most likely due to differences in the demographics of the sample population, instruments used to measure depressive symptomology, and/or sample size (Girgus & Yang, 2017).

Adverse Childhood Experiences

ACEs denote stressful or traumatic experiences occurring early in life, such as experiencing neglect, abuse, or living in a dysfunctional household (Boullier & Blair, 2018; Felitti et al., 1998; Tsehay et al., 2020). Studies have reported that ACEs are positively correlated with an increased risk and prevalence of negative health outcomes later in life. For instance, individuals exposed to ACEs have a two- to three-fold increase in developing MDD than the general population (Chapman et al., 2004; Li et al., 2020). Additionally, people with a history of ACEs are more likely to have increased symptom severity, risk of suicide, and treatment non-responsiveness than people with MDD without a history of ACEs (Otte et al., 2016).

Chronic Medical Illnesses

CMIs are disorders that last for more than a year or require continuous medical attention, such as diabetes mellitus, COPD, and arthritis (CDC, 2021). Research has demonstrated that chronic diseases are associated with a two- to four-fold increase in the manifestation of MDD than the general population (Katon, 2011; Khaledi et al., 2019, Moussavi et al., 2007). Although the specific mechanisms underlying the co-occurrence of depression and medical illnesses are unknown, there are several theoretical models that could explain the correlation between the two disorders. Specifically, medical treatment-pathways, behavioral connections, converging

biology, and psychosocial factors (For review see Gold et al., 2020). Risk factors that are correlated with CMI comorbid depression include lower treatment adherence (Grenard et al., 2011), worse prognosis (Simon, 2001), and increased mortality (Gold et al., 2020; Koyanagi et al., 2018). These vulnerabilities significantly reduce quality of life and is often associated with antidepressant treatment resistance (Gold et al. 2020).

Current Pharmacological Treatments

Monoamine Oxidase Inhibitors

Monoamine modulators have been the standard method of treatment since antidepressant side effects were observed in iproniazid, a drug developed to treat tuberculosis in the 1950s (Cole, 1959; Rosenblat & McIntyre, 2020). These antidepressant effects were due to the action iproniazid had on inhibiting monoamine oxidase, leading to the class of drug known as monoamine oxidase inhibitors (MAO-I). The discovery led to the formation of the monoamine hypothesis of depression, that suggests depression is a manifestation of neurobiological consequences rather than unresolved psychological tensions (Lopez-Munoz & Alamo, 2009; Rosenblat & McIntyre, 2020). MAOIs have potent antidepressant activity because they irreversibly inhibit the breakdown of monoamines, resulting in toxic levels of 5-HT and NE when the drug is combined with foods or medications that produce an increase in monoamines. Another detrimental side effect is that they can produce fatal hypertension (Julien, 2001).

Tricyclic Antidepressants

Coincidingly, tricyclic antidepressants (TCAs) were discovered in the 1950s along with MAOIs. Originally, TCAs were being investigated as a novel antipsychotic agent, but antipsychotic effects were not observed. Instead, researchers notice a marked reduction of negative symptoms (i.e., anhedonia, lethargy, avolition) in people with schizophrenia who were

administered TCAs (Millhouse & Porter, 2015). Upon further investigations in patients with MDD, TCAs showed robust antidepressant effects. The popularity of TCAs came from their similar effectiveness in the alleviation of depressive symptoms as MAOIs but without the life-threatening side effects. Although, it was later observed that TCAs could be lethal if overdosed, which is problematic when the medication is primarily used to treat patients with suicidal ideation as a cardinal feature of their disorder (Kerr et al., 2001).

Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) were discovered to be a safe and more tolerable alternative to MAOIs and TCAs. SSRIs work by selectively preventing serotonin transporters from removing 5-HT from the synaptic cleft (Millhouse & Porter, 2015). Since zimeldine was revealed to the European market in 1982 (Montgomery et al., 1989) and fluoxetine hydrochloride (Prozac; Eli Lilly) was approved by the USFDA in 1987 (Wong et al. 2005), SSRIs have been the golden standard for MDD treatment. SSRIs have exhibited mild anti-inflammatory properties in several studies which is suggested to aid in the antidepressant effects of these drugs (Gałecki et al., 2018; Sitges et al., 2014; Rafiee et al., 2016). However, a shortcoming of SSRIs is the slow onset of antidepressant action, taking 4-6 weeks to produce full antidepressant effects (Julien, 2001).

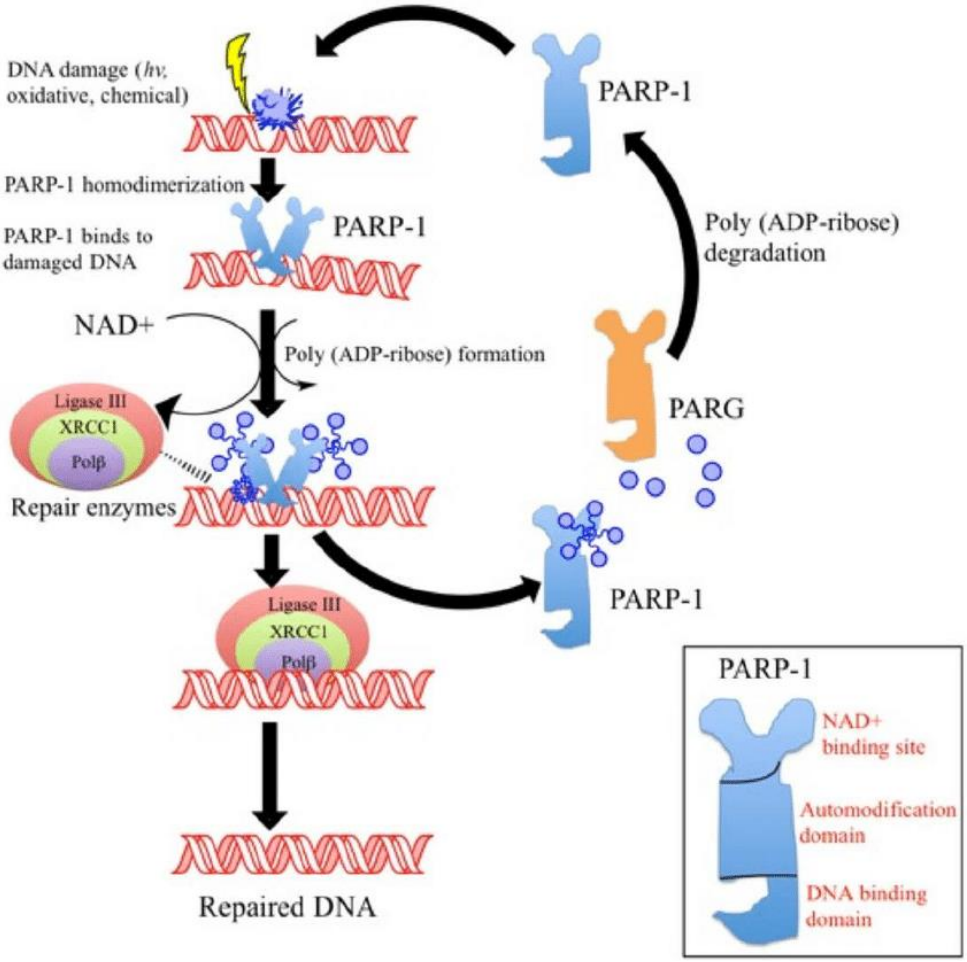
Poly (ADP-ribose) Polymerases

Poly (ADP-ribose) polymerases (PARPs) are nuclear enzymes that perform a variety of cellular processing tasks (e.g., DNA repair and transcriptional regulations; for review see Gibson & Kraus, 2012; Tang et al. 2010). Of the eighteen known PARP sequences, PARP-1 accounts for approximately 90% of total PARP activity and has been the most well documented (D'Amours et al., 1999; Kauppinen & Swanson, 2005). The functional properties of PARP-1 largely involve

post-transcriptional DNA modifications, such as DNA surveillance and repair by binding to DNA strand breaks during genotoxic events (D'Amours et al., 1999; Krishnakumar & Kraus, 2010). The activation of PARP-1 activity commences with the detection of DNA damage. PARP-1 binds to the DNA strand break and begins to synthesize poly (ADP-ribose; PAR) polymers by using nicotinamide adenine dinucleotide (NAD^+) as a substrate, a process known as PARylation. PARylation allows for the scaffolding of proteins such as DNA repair proteins, transcription factors, and chromatin modulators. (Bai & Virág, 2012; Javle & Curtin, 2011; Luo & Kraus, 2012). PAR is then rapidly degraded by PARG [poly (ADP-ribose) glycohydrase]. The generation and degradation of PAR need to occur quickly since defects in this process can result in increased DNA damage (Figure 1; Pazzaglia & Pioli, 2019).

Figure 1

Function of PARP-1 in DNA Damage



Note. This figure demonstrates the role of PARP-1 in DNA damage and repair. When DNA damage occurs PARP-1 will form a homodimerization and bind to the strand break. Once this occurs, PARylation will begin with PARP itself (known as automodification) or other DNA repair proteins. PARP will finally detach itself from the DNA and become degraded. PARP-1 = Poly (ADP-ribose) polymerase; XRCC1 = X-ray repair cross complementing 1; Polβ = DNA polymerase beta; PARG = Poly (ADP-ribose) glycohydrolase; NAD+ = Nicotinamide adenine dinucleotide. Adapted from "Clinical significance of PARP-1 inhibitors in cancer

chemotherapy," by S. Singh, K. Makwana, T. Buckley, A. Agrawal, S. V. Koduru, A. K. Tiwari, 2013, Translational Clinical Biology, 1(1), p.11. Reprinted with permission.

Studies have shown that PARP-1 plays a significant role in mediating stress and inflammation by regulating transcription factors, cytokines, chemokines, and associated gene transcriptions (Arruri et al., 2021; Bai & Virág, 2012; Hassa & Hottiger, 2002; Pazzaglia & Pioli, 2019; Phulwani & Kielian, 2009). PARP-1 can promote proinflammation by directly binding and activating the NF-kB transcription factors (Bai & Virág, 2012; Hassa & Hottiger, 1999; Hassa & Hottiger, 2002; Phulwani & Kielian, 2009). As shown in Figure 2, PARP-1 is a primary component in oxidative-stress induced inflammation, due to PARP-1 being activated by and further exacerbating the production of free radicals. This is exemplified in animal models of inflammation, in which the administration of a PARP-inhibitor has been shown to significantly reduce levels of inflammatory cytokines (Cuzzocrea, 2005; Giansanti et al., 2010; Koedel et al., 2002).

PARP-1 can activate microglial cells through several different mechanisms. If PARG fails to degrade PAR in a timely manner, extracellular PAR can initiate the activation of the innate immune response by becoming a DAMP or by activating the DNA damage response signal leading to the production of proinflammatory factors (Krukenberg et al., 2015). PARP-1 can also destabilize HMGB1 from the chromatin causing it to translocate to the cytosol. If the cell becomes necrotic, HMGB1 can leak into the extracellular space and serves as a DAMP, leading to the proinflammatory activation of microglial cells (Ditsworth et al., 2007; Luo & Kraus, 2012). Additionally, Martínez-Zaudio and Ha (2014) demonstrated *in vitro* microglial cell cultures exposed to PARP-1 led to significant increases in *Il-1β* and *Tnf* gene expression by

enhancing the recruitment of NF-κB to the promotor sites of the genes.

Figure 2 (Continued on next page)

Oxidative Stress and PARP Activation in Microglia

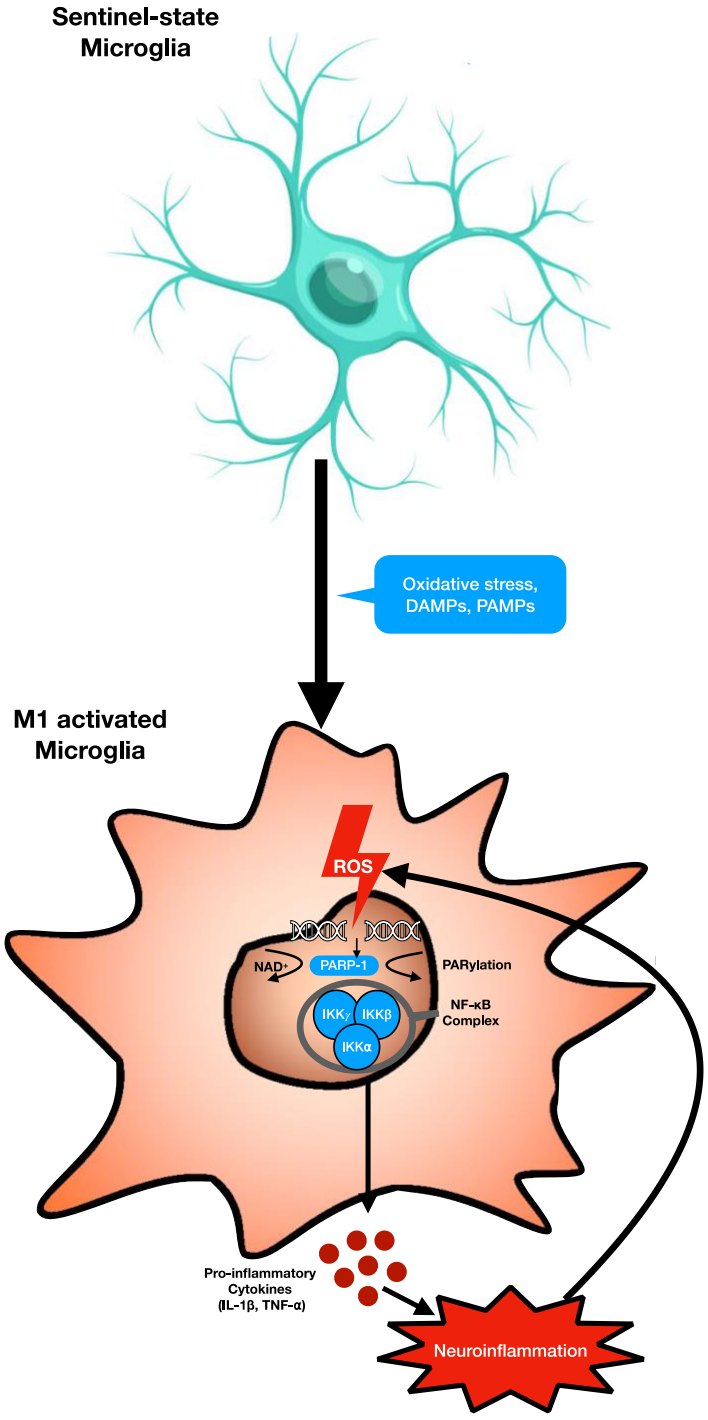


Figure 2 (continued)

Note. This figure denotes the hypothetical depiction of the interaction between oxidative-stress induced inflammation and PARP-1 activity in microglial cells. DAMP = Danger associated molecular pattern; PAMP = Pathogen associated molecular pattern; ROS = Reactive oxygen species; NAD+ = Nicotinamide adenine dinucleotide; NF-κB = Nuclear factor kappa B; IKK-α, β, γ = Inhibitor of NF- κB kinase; IL-1β = Interleukin-1β; TNF-α = Tumor necrosis factor-α. Microglia images were found on Google image search.

PARP Inhibitors as a Novel Antidepressant

The application of PARP-inhibitors is FDA approved as a form of chemotherapy in numerous types of cancers (Lin & Kraus, 2017; Papeo et al., 2013). However, studies have assessed the use of PARP-inhibitors in the prevention of inflammation in the CNS (Ha et al., 2002; Hamby et al., 2007; Hassa & Hottiger, 2002; Kauppinen & Swanson, 2005; Sriram et al., 2015). PARP-1 is a coactivating factor of NF-κB, which has been shown to regulate the microglial proinflammatory response by mediating microglial gene expression, changes in morphology, and migratory behavior (Hassa et al., 2003; Ullrich et al., 2001). The beneficial effect of PARP-inhibition has been demonstrated in a number of studies on neurological diseases, such as traumatic brain injury (d'Avila et al., 2012), Parkinson's disease (Kam et al., 2018), and stroke (Hamby et al., 2007). Additionally, in a lipopolysaccharide (LPS)-evoked depression model in mice the administration of 3-AB counteracted LPS-induced depressive-like behaviors (Sriram et al., 2015).

Interestingly, in a finding from our lab, a case study on a 61-year-old female with ovarian

carcinoma and a history of TRD was prescribed a PARP-inhibitor, niraparib (300mg), and presented antidepressant and anxiolytic effects the following day (Jewett et al., 2020). Research in our lab has also demonstrated the antidepressant actions of the PARP-inhibitor, 3-aminobenzimide (3-AB), in a rat behavioral model of depression. In the study, we assessed the antidepressant activity of 3-AB in two rodent models of psychological stress. In the first model, rats were subjected to daily sessions of concurrent social defeat stress and chronic unpredictable stress for ten consecutive days. In the second model, rats underwent the forced swim test which is a measure of learned helplessness. Our results revealed that the administration of 3-AB was able to prevent the detrimental behavioral effects of psychological stress, as well as produce antidepressant-like activity (Ordway et al., 2017). These finding are paramount because we induced chronic stress without the administration of LPS, thus providing more translationally relevant evidence of the deleterious role that PARP plays in chronic psychological stress.

Modeling in Preclinical Research

Animal models are an invaluable method in allowing researchers the ability investigate possible physiological and molecular alterations that are associated with human disorders (Hollis & Kabbaj, 2014). However, modeling psychiatric disorders in animals can be challenging due to the heterogeneity and lack of biomarkers associated with them. Additionally, many of the symptoms attributed to these illnesses cannot be reasonably acquired from animals (e.g., delusions, suicidal ideation, hallucinations, feelings of inadequacy; Nestler & Hyman, 2010).

Chronic Social Defeat Stress

The social defeat stress (SDS; also known as the resident-intruder model) model mimics the pathogenesis of depression, since studies have indicated that repeated social stressors are a leading etiological factor of the disorder (Becker et al., 2021; Kessler, 1997; McGonagle &

Kessler, 1990). SDS is a paradigm that introduces a conspecific psychosocial stressor for several days in attempt to promote depressive-like phenotypes (Hollis & Kabbaj, 2014). Specifically in rats, the "intruder" is a singly housed male rat and the "resident" is a male rat that is cohabitating with a female rat. Prior to the commencement of the interaction between the two males the female is removed from the cage. Subsequently, the "intruder" rat is placed in the "resident" rat's home cage. The "resident" rat will investigate the "intruder" and begin initiating dominant behaviors (e.g., investigative sniffing, piloerection, tooth chattering) which often results in an attack (Blanchard et al., 1975; Blanchard & Blanchard, 1977). During this interaction the intruder displays classical submissive behaviors (e.g., flight, freezing, defensive upright posture, full submissive-supine posture; Blanchard & Blanchard, 1977; Koolhaas et al., 2013).

Socially defeated rats exhibit numerous behavioral and physiological impairments that are similar to humans with MDD (For review see Hollis & Kabbaj, 2014). Behaviorally, studies have demonstrated that SDS produces significant reductions on social interaction tasks (Berton et al., 2006; Hammels et al., 2015); increases in immobility time during the forced swim test, which is associated with behavioral despair and learned helplessness (Morais-Silva et al., 2019; Rygula et al., 2005); decreased preference towards a sucrose solution in the sucrose preference task, suggesting anhedonic behavior (Lui et al., 2017; Rygula et al. 2005); and decreased locomotor activity which demonstrates a reduction in incentive motivation (Iio et al., 2011; Rygula et al., 2005). Physiological impairments associated with SDS are decreased body weight (Iio et al., 2014; Pulliam et al., 2010), increased weight of adrenal gland (Becker et al., 2007), heightened levels of adrenocorticotropic hormone (Buwalda et al., 1999) and glucocorticoids (Lui et al., 2017; Tornatzky & Miczek, 1993), dysregulation of the PFC, increased amygdala activity (Planchez et al., 2019), pro-inflammatory cytokine release (Reader et al., 2015), and alterations

in neurotrophins (Berton, 2006). Significantly, these behavioral and physiological impairments can be reversed with the administration of antidepressant medication (Fuchs et al., 1996; Berton et al., 1999; Vialou et al., 2010).

There are some notable challenges with the social defeat paradigm. This paradigm has been proven difficult to implement in female rodents, however, recently, some researchers have reported the ability to socially defeat female mice (Harris et al., 2017; Newman et al., 2019). In humans, MDD has a greater prevalence rate among women (Girgus & Yang, 2015), thus by not including female rodents in this paradigm researchers are singling out the most vulnerable sex. Another disadvantage is that the activation of the stress response is largely reliant on how the "intruder" perceives the "resident". If the "resident" does not engage in dominant behavior, stress activation will not occur in the "intruder", thus it has been suggested to screen "residents" to ensure dominance (e.g., aggressive behavior; Golden et al., 2011; Hammels et al., 2015). Finally, animals have a differential response to the SDS paradigm, in that some are shown to be resilient to the socially defeating effects. The resilient animals do not show a deficit in sucrose preference compared to the susceptible animals and they also show increased K^+ channel expression in dopaminergic neurons residing in the VTA. However, it is unknown as to why there is a differential in tonic-phasic VTA neuronal firing rates among the resilient and susceptible animals (Krishnan et al., 2007).

Chronic Unpredictable Stress

The chronic unpredictable stress paradigm (CUS; also known as chronic mild stress) is a prototypical model of depression, in that a known risk factor is constant bombardment of minor stressors (For review see Willner, 2017b; see also Kessler, 1997; Willner et al., 1992). In this model, rodents are exposed to a series of socio-environmental stressors with the following

features: (1) they must be of mild intensity, meaning that none of the stressors alone could cause long lasting effects; (2) the paradigm is chronic in nature with stressors being repeated over weeks, acute application of the mild stressors would be ineffective; and (3) the administration of the stressors must occur unpredictably throughout the implementation of the study (Bondi et al., 2018; Gàll et al., 2020; Lu et al., 2006; Planchez et al., 2019).

Studies have demonstrated that rodents exposed to the CUS model have behavioral and physiological disparities that are also associated with MDD. Behaviorally, rodents have displayed a decrease in grooming behavior in the splash test, which is used as an index of self-care and motivational behavior (Hu et al., 2017; Kalueff & Tuohimaa, 2004; Machado et al., 2012); deficits in the sucrose preference test or cookie test, which are both used to measure hedonic behavior (Surget et al., 2011; Willner, 1987); increased immobility time in forced swim test (Hu et al., 2017; Molina et al., 1990); and decreases in locomotion and exploratory behavior in the open field test (Hu et al., 2017; Lin et al., 2005). Neurobiologically, rodents exposed to CUS display dysregulation of the HPA axis and an increase in corticosterone levels (Chen et al., 2016;). The elevated corticosterone levels contribute to synaptic disfunction in the hippocampus and decreases in hippocampal granule cells (Kvarta et al., 2015; Wu et al., 2007). Additionally, rodents exposed to CUS demonstrate enhanced microglial activation, reductions in 5-HT transmission, alterations in neurotrophins (e.g., BDNF), and dysregulations of the reward pathway (For review see Willner, 2017b).

Even though CUS is one of the most implemented rodent models, some researchers have struggled with the reliability of the model (For review see Sequeira-Cordero et al., 2019). Willner (2017a) discovered that approximately 25% of laboratories have experienced some reproducibility dilemmas with the CUS model. However, he attributed these issues to the

behavioral endpoint measure, the sucrose preference test, rather than the model itself. Labs were using variable sucrose solution concentrations, thus creating nonconformity in reproducibility (Willner, 2017a). An additional challenge with the CUS model is that the rodents must be exposed to the stressors for several weeks in order to exhibit depressive-like phenotypes (Bondi et al., 2018; Lu et al., 2006; Willner, 2017b).

Social Defeat & Chronic Unpredictable Stress

In the human condition, continuous social stressors and chronic mild stressors are leading contributors of MDD and are often cooccurring (Becker et al., 2021; Kessler, 1997; Willner et al., 1992). Thus, to form a preclinical model that exhibits both dimensions the "double-hit" stress paradigm was created (Szebeni et al., 2016). This model combines the SDS and CUS protocols by exposing rats to social defeat in the morning, followed by a mild stressor at random times in the afternoon and evening, for ten consecutive days. Previous research from our lab has demonstrated that rodents subjected to this paradigm had significantly decreased sucrose preference, significantly reduced social interaction times, and DNA oxidation in PFC white matter was significantly increased (Szebeni et al., 2016).

Purpose of Current Study

The lifetime prevalence rate of depressive disorders is 20% in the general population (König et al., 2020; Roman & Irwin, 2020; Otte, 2016) and these disorders commonly cooccur with other debilitating health issues, causing depression to be a leading contributor of disability worldwide (James et al., 2018). Additionally, remission rates for MDD are mediocre with approximately 50% of people seeking treatment unable to fully recover, even after trying several first-line pharmacological treatment options (Akil et al., 2018; Rush, 2006; Williams et al., 2016). Inadequate pharmacological treatment options are due to the limited understanding of the

underlying pathologies leading to MDD (Roman & Irwin, 2020; Troubat et al., 2020); thus, further research is necessary. This project seeks to further examine the antidepressant effects of the PARP-inhibitor, 3-AB, in the "double-hit" SDS+CUS paradigm in hopes to increase our understanding of the role PARP plays in MDD. The purpose of assessing the antidepressant effects of 3-AB in this model is to produce supplementary information on the aberrant pathways that intersect depression and neuroinflammation. Based on the literature review above the hypotheses of the current study are as follows:

Hypothesis 1: To analyze hedonic behavior in the rats we will implement the sucrose preference test during experimental days 8-10. The sucrose preference test is the hallmark test for measuring animal's hedonic behavior (For review see Liu et al., 2018). Diet-intake studies demonstrated that rodents have an innate proclivity to consume a sweet solution over water when given a two-bottle free-choice test (Grill & Norgren, 1978; Hasegawa & Tomita). Interestingly, this preference is diminished when rodents are exposed to stressed-based paradigms and the preference is restored with the administration of antidepressants (Katz, 1982; Lui et al., 2017; Rygula et al. 2005; Surget et al., 2011; Willner et al. 1987). Therefore, we hypothesize that the SDS+CUS rats that are administered either 3-AB or the SSRI, fluoxetine (FLX; tradename: Prozac) will show a significant preference to the sucrose solution when compared to the rats that were administered saline. The 3-AB and FLX administered rats will show a sucrose preference that is comparable to the no-stress group, and thereby these drugs will demonstrate significant protective effects against the anhedonic effects of stress exposure.

Hypothesis 2: Anxiety and depression have always been closely intertwined and the *DSM-5* recognized this by adding anxious distress (AD) as a specifier for MDD (APA, 2013). Since the publishing of the DSM-5, researchers suggest that the AD specifier has a high

prevalence rate, occurring in 50-75% of individuals diagnosed with MDD (Hasin et al., 2018; Rosellini et al., 2018; Zimmerman et al., 2014). To examine anxiolytic behavior in the rats, we will conduct the social interaction test during experimental day 11. The social interaction test is an ethological way to measure anxiety-related behaviors by viewing the amount of time a rat spends in a predefined interaction zone (For review see File & Smith, 2003; see also File & Hyde, 1978). Rats who have been exposed to chronic stressors have displayed significant reductions in time spent in the interaction zones (Avgustinovich & Kovalenko, 2005; Szebeni et al., 2016; Tsankova et al., 2006). Thus, we hypothesize that the SDS+CUS rats that are administered either 3-AB or FLX will spend significantly more time in the interaction zone when compared to the rats that were administered saline. The 3-AB and FLX administered rats will have similar times in the interaction zone as the non-stressed rats due to the protective mechanisms of these drugs against anxiogenesis.

Hypothesis 3: Potential mechanism for the antidepressant effects of 3-AB will also be investigated. The activation of PARP1 in prefrontal white and gray matter will be examined using immunohistochemistry (IHC) and Western Blots. PFC white matter will be assessed because we have previously demonstrated the increased activation of PARP-1 in postmortem white matter in the PFC of people with depression when compared to psychiatrically normal PFC white matter (Szebeni et al., 2016). Cortical white matter has an enhanced susceptibility to oxidative stress due to the high metabolic rate of myelinating oligodendrocytes and the reduced levels of antioxidant expression (Bradl & Lassman, 2010; Thorburne & Juurkink, 1996). Additionally, PFC gray matter abnormalities have been associated with chronic stress and MDD (Arnsten, 2015; Frodel et al., 2008). Oxidative stress forms a positive feedback loop with PARP1 (Bai & Virág,2012; Javle & Curtin, 2011; Tang et al., 2010). Thus, we hypothesize that rats

exposed to SDS+CUS will have an increased quantity of PARP-1 in the PFC.

Hypothesis 4: Cytokines play an important role in maintaining homeostasis in the CNS, although the chronic upregulation of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α has been shown to have a deleterious impact on the CNS (Irwin & Miller, 2007; Schleifer et al., 1989; Zorrilla et al., 2001). Neuroinflammatory markers, such as IL-1β, IL-6, and TNF-α protein expression will be examined as potential mediators of the manifestation or exacerbation of depressive-symptoms in the SDS + CUS rodent model. Previous researchers have demonstrated the anti-inflammatory properties of 3-AB in a mouse depressive model. However, the model required the administration of LPS, a known immunostimulant, to induce depressive-like symptoms (Sriram et al., 2015). Therefore, to demonstrate a more translational approach in assessing the anti-inflammatory properties of 3-AB, IL-1β, IL-6, and TNF-α protein analyses will be conducted on rat hippocampal tissue. We hypothesize that the SDS+CUS rats that are administered SAL will display a significant increase in IL-1β, IL-6, and TNF-α expression when compared to the rats in any other treatment group. The 3-AB administered rats will have similar IL-1β, IL-6, and TNF-α expression as the non-stressed rats.

Hypothesis 5: Microglial activation will be assessed by conducting IHC on PFC white and gray matter tissue for the microglial activation marker, ionized calcium-binding adaptor protein-1 (IBA-1). IBA-1 is ubiquitously distributed in microglial cells, thus making it the ideal IHC marker when observing both activated and ramified microglia (Ahmed et al., 2007). IBA-1 is a protein that regulates actin-cross-linking during the morphological transformation of ramified microglia to activated ones (Hovens et al., 2014; Sasaki et al., 2001). Therefore, microglial activation is associated with enhanced IBA-1 expression. Activated microglia secrete proinflammatory cytokines and contribute to enhanced oxidative stress, both of which occur in

MDD (Jia et al., 2020; Torres-Platas et al., 2014). Thus, we hypothesize that SDS+CUS rats that were administered saline will have significantly greater microglial activation thus exhibiting morphological changes that are associated with activation (e.g., increased soma size, increased soma elongation, retracted processes, & thicker processes) to the rats who were administered 3-AB or FLX.

Chapter 2. Methods

Animals & Housing Environment

All animals for this project were obtained from Envigo (Indianapolis, IN) and were approximately PND 60 upon arrival. Male Sprague-Dawley rats were randomly selected to serve as an experimental "intruder" and exposed to SDS+CUS or a control animal (no-stress group). Rats in the no-stressed group were group-housed while the rats in the SDS+CUS group were singly housed with environmental enrichment provided as specified in the NIH Guidelines of the Care and Use of Animals. The SDS+CUS rats were randomly assigned to three different treatment groups, in which they received daily administration of: 3-AB, FLX, or Saline. Additionally, male Long-Evans Hooded rats were used as the "aggressors/residents" in the social defeat paradigm. Each "resident" rat shared a cage with an ovariectomized (OVX) female Sprague-Dawley rat. Upon arrival, animals were given 1 week to habituate to their novel environment. Group assignments were based on stress exposure and treatment type. Animals were housed in a 12-h light/dark cycle with food and water provided *ad libitum*. All procedures obtained approval by East Tennessee State University Animal Care and Use Committee.

Drugs and Solutions

Fluoxetine HCl (FLX; 10mg/kg/day) and 3-Aminobenzamide (3-AB; 40mg/kg/day), were obtained from Sigma-Aldrich (St. Louis, MO) and diluted in a saline solution (0.9% sodium chloride) to create their respective concentrations.

Female Rat Ovariectomy

Female rats underwent surgery to prevent pregnancy while they mated with the aggressors. Prior to commencing surgery all surgical equipment and surgical field was sterilized and surgeries were documented. Female rats were anesthetized using isoflurane (USP; Trade

name: Isothesia; Henry Schein, Dublin, Ohio) and administered 1mg/kg meloxicam (S.C.; Covetrus, Portland, ME) as an analgesic. Once sedated, the lower back of the rat was shaved, and the surgical site was cleaned with betadine. A 2 cm midline dorsal incision was made in the lower back, directly below the last rib. Next, blunt nose hemostats were used to separate the fascia from the external oblique muscles. Then, a small (1cm) incision was made in the thin muscle layer of the lateral external oblique on each side, to enter the peritoneal cavity. Adison forceps were used to extract the ovarian fat pad to locate the ovaries. Once located, hemostats clamped onto the oviduct and 4-0 silk sutures were used to ligate the ovaries. Fat pad was reinserted into peritoneal cavity and 2-0 Maxon sutures were used to close muscle wall. Surgical staples were used to close skin together. Rats received a second dose of meloxicam 24h post-op and were provided with 7 days of recuperation prior to mating with the male aggressor.

Social Defeat Stress Paradigm (SDS)

Rats in the stress-exposed groups underwent 10 days of social defeat stress from 9am to 12pm, as described previously by (Ordway et al., 2017; Szebeni et al., 2016). In this paradigm, the OVX female is removed from the "resident" cage and the cage is brought to the testing room. The "intruder" is then placed in the "resident" cage for 5 minutes or until the "resident" performs a 2-second pin. Behaviors that we were observing from the intruder are boxing stance, freezing and crouching, vocal distress on-the-back, and standing on their hindlegs. Behaviors that we were observing from the "resident" rat are preattack behaviors such as approach and sniffing, piloerection, tooth chattering, and biting; attack behaviors such as chasing, lateral attack, and pinning.

Chronic Unpredictable Stressors (CUS)

Rats in the stress-exposed groups underwent a series of unpredictable mild stressors for

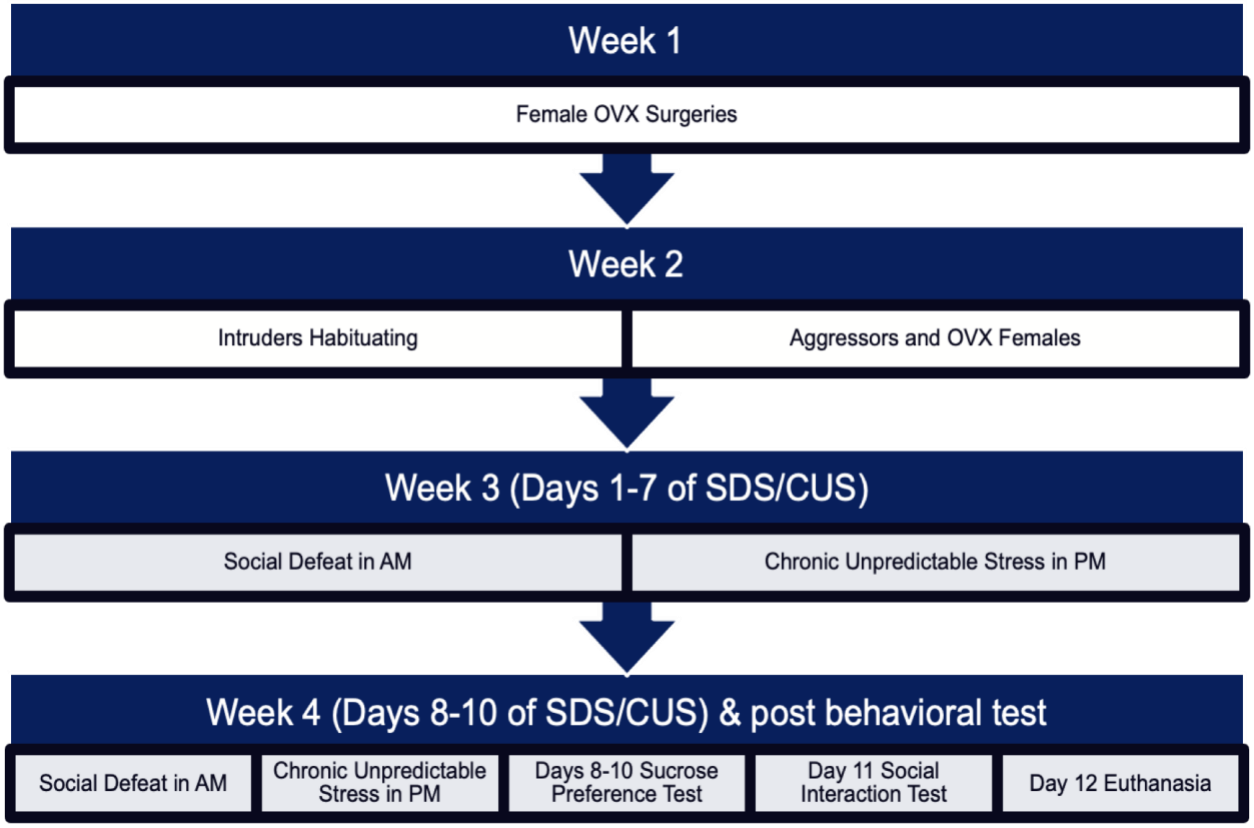
10 days, as described previously (Ordway, 2017; Szebeni et al., 2016). Unpredictable stressors include 30-minute restraint, 1h shaking and crowding, 10-minute cold water (18ºC) swim, 15-minute warm water (25 ºC) swim, and 24h tipped cage. Each CUS was performed twice over the 10-day period, during various hours of the day.

Length of Study

The study lasted 12 days. Days 1-10 the stress-exposed rats underwent SDS+CUS, on day 11 the social interaction test was conducted, and on day 12 the animals were euthanized (Figure 3).

Figure 3

Experimental Timeline



Note. The experiment was conducted over a 4-week period, as described above. OVX = Ovariectomy; SDS = Social defeat stress; CUS = Chronic unpredictable stress.

Sucrose Preference Test

The sucrose preference test was conducted on days 8-10 during the first 2 hours of the dark cycle (7pm-9pm). The reasoning for running this test during this time is because rats are nocturnal animals, and they complete most of their foraging during this time. The sucrose preference test is a measure of anhedonia since rodents have an innate proclivity for sucrose (Katz, 1982; Willner et al., 1987). By monitoring their responsiveness to a rewarding stimulus,

we can measure anhedonic behavior. Rats had two pre-weighed bottles placed on their cage, one containing water and the other containing 0.8% sucrose solution. The 0.8% sucrose concentration has been shown to provide optimal sucrose preference (Slattery et al., 2006). Sucrose preference will be calculated as a percentage by assessing sucrose consumption relative to total fluid consumption.

Social Interaction Test

The social interaction test was conducted on experimental day 11. The test was conducted in a locomotor arena (black plexiglass box with open top; $91cm^3$) divided in half by wire mesh (new measurements: 91cm tall x 91 cm wide x 45.5cm long) to allow sensorial contact but prevent any physical contact. The intruder and control rats were placed on one side of the locomotor arena, for a 10 min habituation trial to acclimate to the locomotor arena prior to the introduction of the aggressor. Immediately after the 10 mins, an aggressor was placed on the other side of the wire mesh to begin the interaction test. The interaction test lasted for 5min, in which the intruder rat's time spent in the interaction zone (area close to the wire mesh) was recorded using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL). Time spent in the interaction zone is a dependent measure for social avoidance.

Tissue Collection

Animals were euthanized on day 12 of the experiment via live decapitation. Brains were sectioned, in which ¼ of each brain was fixed and ¾ was frozen. The fixed portion of the brain was sectioned in which the sagittal cut was made along the longitudinal fissure, while the coronal cut was made at bregma. The ¼ of the brain was placed in a vial of 4% paraformaldehyde and stored in 4℃ for 24h, then the PFA was poured out and replaced with a 20% sucrose solution and stored in 4℃ until the tissue descended fully into the sucrose solution. Next, the 20%

sucrose solution was poured out and the brains were stored at -80℃. To freeze the ¾ portion of the brain, once the brain was harvested it was placed on a block of dry ice until completely frozen, at which time it was transferred into a vial and stored at -80℃ until they were analyzed.

Immunohistochemistry

PARP-1 Tissue Sectioning

The frozen portions of the brains were used for PARP-1 immunohistochemistry (IHC) and wester blots. Tissue was coronally sectioned using a Leica CM1950 (Buffalo Grove, IL). First, prefrontal cortical gray matter was sectioned at 50 µM to collect tissue punches using an Integra Miltex 2mm biopsy punch (York, PA). 10 mg of gray matter (~ 60 tissue punches) was collected in 1.5mL microcentrifuge tubes (Thermo Scientific, Waltham, MA) and stored in -80℃ until we conducted western blots.

Once the white matter of the anterior cingulate cortex began to appear we made 20µM sections. Every two sections were mounted on a Superfrost Plus Microscope Slide (Fisherbrand, Pittsburgh, PA). Eight sections of tissue were collected and mounted onto 4 slides. The slides were put into a slide box and the slide box was placed in a vacuum desiccator (Thermo Scientific, Waltham, MA) overnight at 4℃ to remove any moisture on the tissue. The following day the slides were transferred and stored in -80℃ until we began staining the tissue.

PARP-1 Fluorescent IHC Staining & Imaging

For each animal, two slides were picked at random to conduct PARP-1 IHC staining. Fixation of the tissue occurred by removing the slides from the -80℃ freezer and inserting them into a Coplin staining jar that was filled with cold 100% acetone and placed in the -20℃ freezer for 20min. Then, the slides were washed four times by filling the jar with 1X phosphate-buffered saline (PBS) and placing it on a rocker at room temperature (pH 7.4; 10 min each wash). Next,

the slides were block-permeabilized by adding 100µL of a buffer solution containing 1x PBS, 0.3% Triton x 100, and 1% bovine serum albumin (PTB) to each tissue section and the slides were left at room temperature for 1h. PTB was then dumped off and slides were quickly rinsed with PBS. The primary antibody, anti-PARP 1 polyclonal antibody (rabbit; Abcam, Cambridge, UK), was diluted to 1:500 in PTB and 100 µl of the primary antibody solution was added to 3 out of the 4 tissue samples, with the 4th sample only containing PTB to act as a negative control. Slides were then incubated overnight with primary antibody at 4℃.

The following day, slides were washed 3 times using the method described above (10 min each time). Then, the secondary antibody, donkey anti-rabbit alexa fluor-594 (Thermo Scientific, Waltham, MA), was prepared by diluting it to 1:1000 in a solution containing PBS and 0.3% Triton (PT). 100µl of diluted secondary was mounted onto each tissue sample and was incubated at room temperature for 1h. Slides were then washed in PBS, 3 times, using the washing techniques previously described above (10 min each wash). PBS was removed and a fluorescence mounting media containing DAPI was added followed by a coverslip. Finally, clear fingernail polish was applied on the sides of the coverslips to increase the preservation of the tissue. Tissue was stored at 4℃ until ready to conduct fluorescence imaging.

Cell visualization was conducted using an EVOS FL Auto Imaging System and software (ThermoFisher Scientific; Waltham, MA). Each tissue sample had approximately 20 images captured and stitched together to form one large mosaic of the white matter. Images were captured using the 20x objective lens and the red (TxRed) laser channel on to fluoresce the PARP-1 antibody at 594 nm. Once imaging was complete, cell quantification was performed using MCID Analysis Software (InterFocus Ltd; Linton, England).

PARP-1 Western Blot

Western Blotting technique was used to analyze PARP-1 protein expression in frontal cortical gray matter. Tissue was collected by using a 2mm biopsy punch and stored in microcentrifuge vial at -80℃ until ready to analyze, as previously mentioned above. Samples were then homogenized in lysis buffer containing protease and phosphatase inhibitor cocktail (PPC1010; Sigma-Aldrich). The samples were then loaded on a precast Tris-Glycine 4-20% gradient gel (NuSep; Germantown, MD) in an electrophoresis chamber and the electrical current was set at 150 V for 50 mins. After electrophoresis, gel was removed from cassette and proteins were transferred to methanol activated polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories; Hercules, CA) using Mini Blot Module (ThermoFisher Scientific; Waltham, MA) at 20V for 1h. The membranes were blocked using Tris-buffered saline (TBS) with 5% blocking buffer for 30 min at room temperature. Subsequently, the primary antibody, PARP antibody, (#9542, Cell Signaling Technology; Danvers, MA) was diluted 1:1000 in TBS. The PVDF membranes were submerged in the diluted antibody and incubated at 4℃ overnight.

The following day, PVDF membranes were blotted with secondary antibody, anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling Technology; Danvers, MA) diluted 1:5000 with TBS and incubated at room temperature for 1h. For loading controls β-actin Mouse monoclonal antibody (#3700, Cell Signaling Technology; Danvers, MA) was used as primary antibody and anti-mouse IgG, HRP-linked antibody (#7076, Cell Signaling Technology; Danvers, MA) was used as secondary. Western blots were visualized using G:Box Automated Imaging (Syngene, Frederick, MD). Band density was detected using semi-quantification analysis with GeneTools (Syngene, Frederick, MD).

IBA-1 Tissue Sectioning

The fixed portions of the brain were used for IBA-1 IHC immunofluorescent staining. Tissue was coronally sectioned at 50µm thickness using a Leica CM1950 (Buffalo Grove, IL). PFC sections were collected and stored as free-floating sections in 1x PBS (pH 7.4) until staining began.

IBA-1 Microglia IHC & Confocal Microscopy

The free-floating sections were washed in 1x PBS for 10 mins, repeated 4 times. Then, the sections were permeabilized in a solution containing 1x PBS with 0.4% Triton X-100 and 0.5% BSA at room temperature for 20 mins by adding 1.5mL of the solution per well. This solution was discarded and blocking solution containing 1x PBS with 0.4% Triton X-100 and 1.0% BSA was added to each well and incubate at room temperature for 2h. The primary antibody, IBA-1 polyclonal antibody (Rabbit; 019-19741; Fujifilm, Richmond, VA) was diluted 1:1000 in 1x PBS with 0.4% Triton X-100 and 1.0% BSA and 1mL was added to each well, except for the negative control well. Sections were left to incubate overnight at 4°C.

The following day, the primary antibody solution was removed from each well and sections were washed with PBS (4 times, 10 min each time). The secondary antibody, donkey anti-rabbit alexa fluor-488 (A-21206; ThermoFisher Scientific; Waltham, MA), was diluted 1:1000 in 1x PBS with 0.4% Triton X-100 and 1.0% BSA and 1mL was added to each well. Sections incubated for 2h at room temperature. The secondary was discarded, and sections were washed in PBS (3 times for 10 mins). Sections were mounted on charged slides in PBS. PBS was removed and 20µl of EverBrite™ Hardset Mounting Medium with DAPI (Biotium; Fremont, CA) was added followed by a coverslip. Finally, clear fingernail polish was applied on the sides of the coverslips to increase the preservation of the tissue. Tissue was stored at 4°C until ready to

conduct fluorescence imaging.

Cell visualization was conducted using an inverted confocal microscope (Leica TCS SP8; Wetzlar, Germany) with Leica LAS X software (version #: 3.5.5.19976). Each tissue sample had 6 images captured (3 PFC white matter & 3 PFC gray matter) with the imaging specifications (Scan speed – 8,000 Hz, Objective lens – HC PL APO CS2 40x/ 1.30 Oil, Zoom – 1.25x, Resolution – 1024 x 1024) remaining constant throughout the microscopy procedure. The HyD detector was selected to minimize the risk of photobleaching the tissue. Images were Z-stacked to better visualize the microglial processes. Sequential scanning was implemented to negate crosstalk between the two fluorochromes. The first sequence used the green (OP SL 488) laser channel to fluoresce IBA-1 antibody at 488nm with an intensity of 0.70%. The second sequence used the blue (Diode 405) laser was used to fluoresce DAPI at 405nm with an intensity of 5.05%. Once imaging was complete, cell quantification was performed using the microscopy image analysis software, Imaris (Oxford Instruments; Concord, MA). The Imaris software recreates a 3D rendering of the cells found in the image. Each image had 5 cells randomly selected to be analyzed.

Cytokine ELISA

To conduct the enzyme-linked immunosorbent assays (ELISA) tissue was collected from the frozen brain sections. Tissue was mounted and coronally sectioned on a cryostat (Leica CM1950). Once the ventral hippocampus was visible tissue was sectioned at 100 µM and hippocampal tissue was removed using an Integra Miltex 3mm biopsy punch. Approximately 30mg of hippocampal tissue (~ 90 punches) was collected from each animal and divided into three microcentrifuge tubes (1 tube per ELISA). Microcentrifuge tubes were weighed before and after tissue collection to ensure exact tissue weight was obtained. Microcentrifuge tubes were

then stored in -80℃ until we conducted the ELISAs.

TNF-α

To analyze TNF-α protein expression in the hippocampus we purchased a TNF-α ELISA kit (Cat # EKF57956) from Biomatik (Wellington, DE). To homogenate tissue, 500µl of RIPA cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1.0% NP-40, 0.5% sodium deoxycholate, & 0.1% sodium dodecyl sulfate) with protease and phosphatase inhibitors (P5726, P8340, P0044; Sigma-Aldrich, St. Louis, MO) was added in each microcentrifuge sample vial and a sonic dismembrator (Fisherbrand Model 505 Sonic Dismembrator; Waltham, MA) was used to mechanically disrupt cell structure. Homogenates were then centrifuged for 5 mins at 5,000g at 4℃. The 96-well plate was precoated with anti-TNF-α antibody in each well. The standard curve was made using the TNF-α standard solution (250 pg/mL) provided by Biomatik. The standard solutions were created via serial dilution and ranged from 3.906 pg/ml to 250 pg/ml. Tissue samples were also diluted (1:2) using the dilution buffer provided in the kit. All standards and tissue samples were pipetted into wells in duplicate. The filled plate was sealed and covered and incubated for 90 mins at 37℃. After the incubation, the plate was washed twice with the provided wash buffer. Then the biotin conjugated anti-TNF-α antibody was added to all the wells as detection antibodies. The plate was sealed and placed in incubator for 60mins at 37℃. Subsequently, the plate was removed from incubator and washed 3 times. HRP-streptavidin conjugate was then added to each well to amplify the signal of the detection antibody. The plate was sealed and incubated for 30 mins at 37℃. The cover was removed, and the plate was washed 5 times. Then, TMB substrate was added to the plate to generate a detectable signal from the HRP enzymatic reaction. The plate was covered and incubated at 37℃ in the dark for 10-20 mins. Then, the cover was removed and Stop solution was added to each well to stop the reaction

between HRP and TMB. The plate was immediately placed in the Bio-Tek ELx 800 microplate reader (Winooski, VT) to measure optical density at a 450-nm wavelength.

IL-1β

Analysis of IL-1β protein expression was conducted with an IL-1β ELISA kit (Cat # EKF57939) from Biomatik. To homogenate tissue, 500µl of RIPA cell lysis buffer with protease and phosphatase inhibitors was added in each microcentrifuge sample vial and a sonic dismembrator (Fisherbrand Model 505 Sonic Dismembrator; Waltham, MA) was used to mechanically disrupt cell structure. Homogenates were then centrifuged for 5 mins at 5,000g at 4℃. The 96-well plate was precoated with anti- IL-1β antibody in each well. The standard curve was made using the IL-1β standard solution (2,000 pg/mL) provided by Biomatik. The standard solutions were created via serial dilution and ranged from 31.25 pg/ml to 2,000 pg/ml. Tissue samples were also diluted (1:2) using the dilution buffer provided in the kit. All standards and tissue samples were pipetted into wells in duplicate. The filled plate was sealed and covered and incubated for 90 mins at 37℃. After the incubation, the plate was washed twice with the provided wash buffer. Then the biotin conjugated anti- IL-1β antibody was added to all the wells as detection antibodies. The plate was sealed and placed in incubator for 60mins at 37℃. Subsequently, the plate was removed from incubator and washed 3 times. HRP-streptavidin conjugate was then added to each well to amplify the signal of the detection antibody. The plate was sealed and incubated for 30 mins at 37℃. The cover was removed, and the plate was washed 5 times. Then, TMB substrate was added to the plate to generate a detectable signal from the HRP enzymatic reaction. The plate was covered and incubated at 37℃ in the dark for 10-20 mins. Then, the cover was removed and Stop solution was added to each well to stop the reaction between HRP and TMB. The plate was immediately placed in the Bio-Tek ELx 800 microplate

to measure optical density at a 450-nm wavelength.

IL-6

To analyze IL-6 protein expression in the hippocampus we purchased an IL-6 ELISA kit (Cat # EKE61991) from Biomatik. To homogenate tissue, 500µl of RIPA cell lysis with protease and phosphatase inhibitors was added in each microcentrifuge sample vial and a sonic dismembrator was used to mechanically disrupt cell structure. Homogenates were then centrifuged for 5 mins at 5,000g at 4℃. The 96-well plate was precoated with anti- IL-6 antibody in each well. The standard curve was made using the IL-6 standard solution (4,000 pg/mL) provided by Biomatik. The standard solutions were created via serial dilution and ranged from 62.50 pg/ml to 4,000 pg/ml. All standards and tissue samples were pipetted into wells in duplicate. The filled plate was sealed and covered and incubated for 90 mins at 37℃. After the incubation, the liquid was dumped from the plate and biotinylated detection antibody was added to each well. The plate was sealed and placed in incubator for 60 mins at 37℃. Subsequently, the plate was removed from incubator and washed 3 times. HRP-streptavidin conjugate was then added to each well and the plate was sealed and incubated for 30 mins at 37℃. The cover was removed, and the plate was washed 5 times. Then, TMB substrate was added to the plate then, the plate was covered and incubated at 37℃ in the dark for 10-20 mins. Then, the cover was removed and Stop solution was added to each well. The plate was immediately placed in the Bio-Tek ELx 800 microplate reader to measure optical density at a 450-nm wavelength.

Statistical Analyses

Throughout the study the alpha criterion was set at $p < 0.05$ to further investigate significant interactions. Post hoc tests were conducted using Dunnett's test (Dunnett, 1955) in

order to compare our treatment groups (3-AB, FLX, & SAL) with the control group while limiting the Type-I error rate. Additionally, the Dunnett's test results in narrower confidence intervals over tests of multiple comparisons (e.g., Bonferroni or Tukey; Dunnett, 1964). Outliers were determined using Grubbs' test (Grubbs, 1969). When outliers were identified they were eliminated from the dataset. Descriptive statistics tables and graphical representations of the data will use the mean and standard error of the mean (SEM) to summarize the data.

Sucrose Consumption Task

During the Sucrose Consumption Task, daily bottle weights were recorded before and after the task using Microsoft Excel (2021). To assess sucrose preference, the amount of sucrose water consumed was divided by the total amount of liquid consumed. Mean and standard errors of sucrose preference were calculated and used to create graphs in GraphPad Prism 6 (2017). Data was organized into R 4.0.4 (R Core Team, 2021) and a two-way ANOVA was fit to the data to investigate the effects of treatment type (3-AB, FLX, Saline, & Control) by Days (Day 1, 2, &3) of sucrose consumption.

Social Interaction Task

The Social Interaction test was conducted using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL). Once testing was complete data was exported and organized in Microsoft Excel. Social interaction was examined by calculating the amount of time the animal spent in the interaction zone compared to the total task time. Data was then imported into R and a one-way ANOVA was fit to the data to investigate the effects of drug treatment on time spent in the interaction zone.

PARP IHC

PARP-1 IHC imaging was conducted using an EVOS FL Auto Microscope (Zoom - 20x)

and image analysis was conducted on MCID. Three distinct regions were assessed in the PFC: layer 1 gray matter, layers 3-6 gray matter, and the forceps minor of the corpus callosum (FMI) white matter. Two gray matter areas were examined to investigate whether PARP-1 expression varied between cortical layers. The FMI region was examined because we previously demonstrated that rats had an increase in DNA oxidation after 10-days of SDS+CUS (Szebeni et al., 2016). Data was imported to GraphPad Prism to create graphs and run statistical analyses. A one-way ANOVA was fit to the data to examine the effects of drug treatment on PARP-1 expression.

PARP Western Blots

Western blot images were captured using G:Box Automated Imaging and semi-quantification analysis was performed with GeneTools (Syngene, Frederick, MD) to examine the optical protein density of PARP-1. PARP-1 protein amount was standardized by dividing the amount by the normalizing control β-actin. Once the data was gathered, it was imported into GraphPad Prism for data visualization and statistical analyses. An unpaired t-test was fit to the data to determine the effects of treatment on PARP-1 protein abundance based on relative densitometry.

IBA-1 IHC

Tissue stained for IBA-1 were imaged using the Leica SP8 confocal microscope, each animal had a total of 6 images collected (3 PFC white matter & 3 PFC gray matter). Images were then imported to the Imaris cell analysis software, in which the software creates a 3D rendition of the cell to allow cell quantification. Ten cell morphological parameters were examined: soma volume, soma area, soma sphericity, ellipticity (prolate), ellipticity (oblate), filament volume, filament area, filament length, number of dendrite branch points, and number of sholl

intersections. Following cell quantification, data was extracted and uploaded into Microsoft Excel and prepared for statistical analyses. One-way ANOVAs were conducted to investigate if treatment type had an effect on microglial morphology.

ELISAs

Once plates were read using Bio-Tek ELx 800 microplate reader, data was imported into Microsoft Excel. Wavelength was converted into pg/ml using the standard curve created by the standard solutions. Data was imported in GraphPad Prism for data visualization and statistical analysis. One-way ANOVAs were performed to view the effects of treatment type on cytokine levels (IL-1β, IL-6, & TNF-α).

Chapter 3. Results

Sucrose Preference Test

The Sucrose Preference Test was conducted on experimental days 8-10 from 7 PM – 9PM. Sucrose preference was calculated as a percentage of grams of sucrose water consumed over grams of total liquid consumed in the allotted time frame. The descriptive measurements for sucrose preference are presented in Table 1.

Table 1

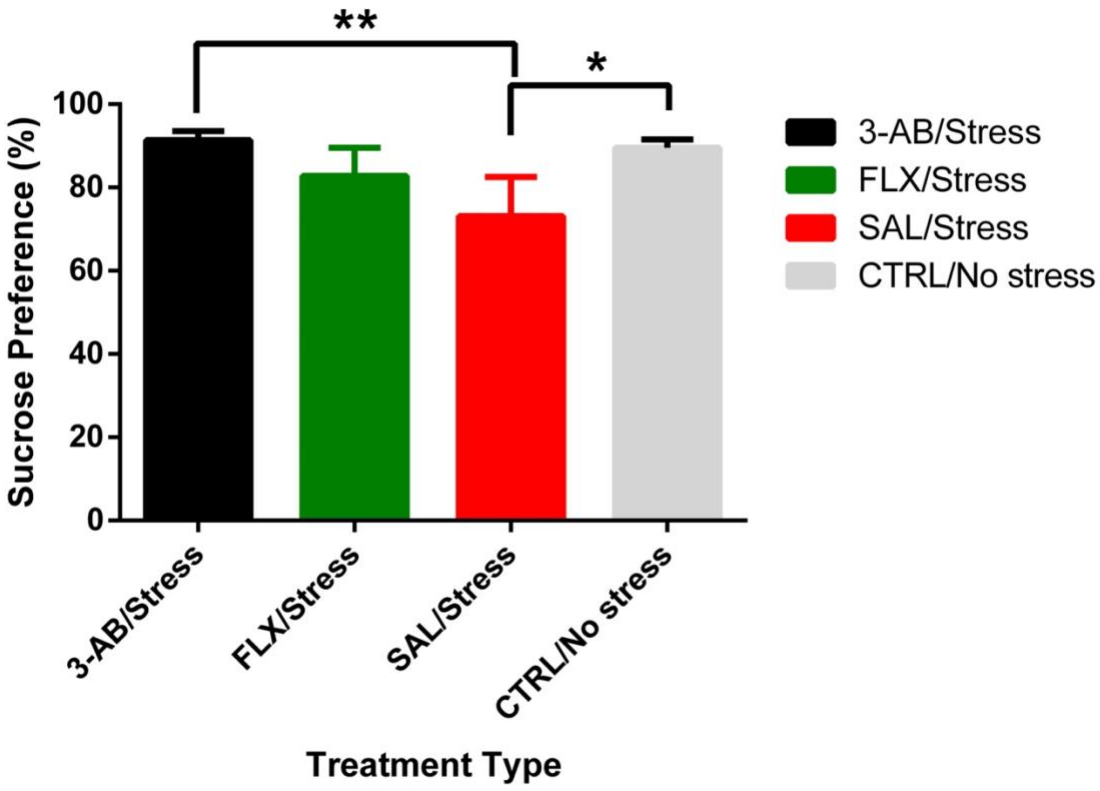
Descriptive Statistics for the Sucrose Preference Test

Treatment Type	n	Mean (%)	SEM (%)
Day 1			
3-AB	8	87.112	2.184
FLX	8	71.350	11.088
SAL	8	58.425	11.491
CTRL	8	85.646	3.779
Day 2			
3-AB	8	92.312	1.278
FLX	8	82.100	7.999
SAL	8	70.575	12.290
CTRL	8	91.625	1.580
Day 3			
3-AB	8	94.638	1.345
FLX	8	94.825	1.091
SAL	8	90.488	3.031
CTRL	8	91.439	1.797
Total			
3-AB	24	91.354	1.124
FLX	24	82.758	4.805
SAL	24	73.162	6.103
CTRL	24	89.570	1.538

A two-way ANOVA (Treatment Type by Days) with repeated measures was fit to the data. A change in sucrose preference was revealed with a significant between-subjects effect of Treatment Type ($F_{(3,84)} = 4.570$, $p = 0.005$, $\eta^2 = 0.096$) and within-subjects effect of Days ($F_{(2,84)} = 17.505$, $p < 0.01$, $\eta^2 = 0.245$); however, there was not an interaction effect of Treatment Type by Days. Pairwise comparisons using Dennett's test were conducted on Treatment Type to assess group differences (Figure 5). Post hoc comparisons indicated that the 3-AB group (M = 91.35, SD = 5.51) demonstrated a significantly higher ($p < 0.01$) sucrose preference as compared to the SAL group (M = 73.16, SD = 29.90) with a mean preference difference of 18.19%. The CTRL group (M = 89.57, SD = 7.53) showed a significantly higher ($p = 0.017$) sucrose preference than the SAL group with a mean difference of 16.41%. Pairwise comparisons using Dunnett's test were also conducted on Days indicating that the mean sucrose preference was significantly higher ($p < 0.001$) on Day 3 as compared to Day 1 with a mean difference of 17.21%.

Figure 4

Mean (± SEM) in Sucrose Preference (%)



Note. Percent sucrose preference is presented as a function of group. An asterisk (*) denotes that the CTRL/No stress group showed a significantly higher sucrose preference compared to the SAL/Stress group, $p < 0.05$. Double asterisks (**) denotes that the 3-AB/ Stress group showed significantly higher sucrose preference compared to the Saline/ Stress group, $p < 0.01$. Error bars show standard error of the mean.

Social Interaction Test

The social interaction test was conducted on experimental day 11. Social interaction was quantified as a percentage in the amount of time spent in the avoidance zone of the locomotor

arena. Prior to conducting data analysis assumptions were checked and met. Descriptive statistics for the social interaction test are provided in Table 2.

Table 2

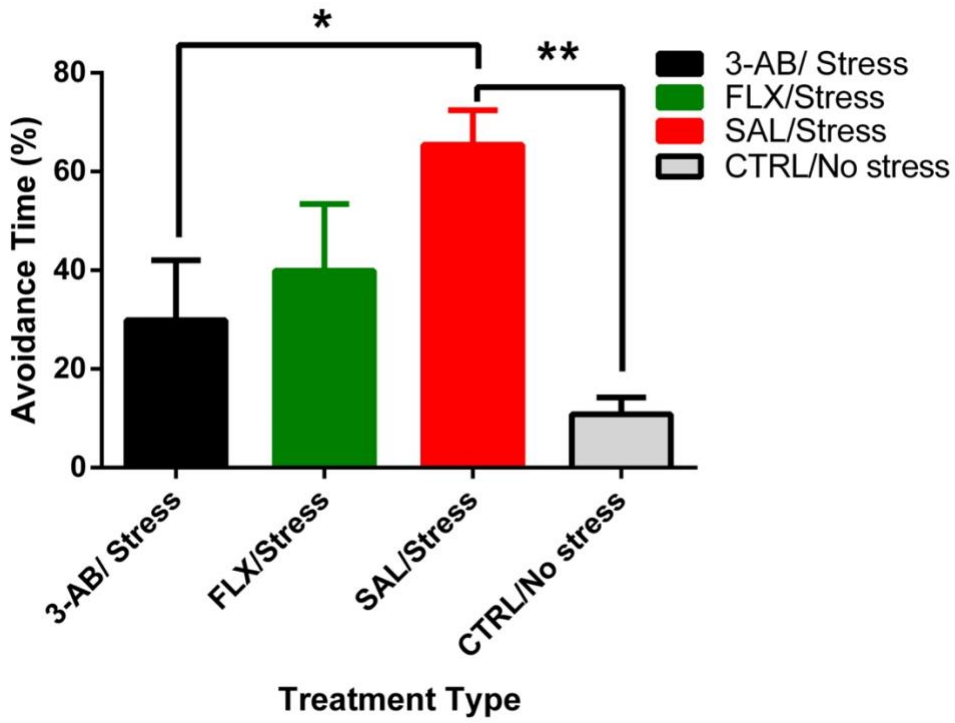
Descriptive Statistics for Time in the Avoidance Zone during the Social Interaction Test

Treatment Type	n	Mean (%)	SEM (%)
3-AB	8	29.877	12.163
FLX	8	39.901	13.561
SAL	8	65.412	7.041
CTRL	8	10.824	3.379

A one-way between subjects ANOVA was conducted to compare the effect of drug treatment (3-AB, FLX, SAL, & CTRL) on time spent in the interaction zone. There was a significant effect of drug treatment on the percentage of time spent in the avoidance zone, $F_{(3,28)} = 5.263$, $p = 0.005$, $\eta^2 = 0.36$. Pairwise comparisons using Dennett's test were conducted on Treatment Type to assess group differences (Figure 6). Post hoc comparisons indicated that the SAL group (M = 65.41, SD = 19.92) spent a significantly higher (p's < 0.045) percentage of their time in the avoidance zone as compared to the 3-AB group (M = 29.88, SD = 34.40) and the CTRL group (M = 10.82, SD = 9.56). The FLX (M = 39.90, SD = 19.91) group did not significantly differ from the SAL in time spent in the avoidance zone.

Figure 5

Mean (± SEM) amount of Time Spent in Avoidance Zone



Note. Percent of time spent in the avoidance zone is presented as a function of group. An asterisk (*) represents that the 3-AB/Stress group spent significantly less time in the avoidance zone as the SAL/Stress group, $p < 0.05$. Double asterisks (**) represents that the CTRL/No stress group spent significantly less time in the avoidance zone as the SAL/Stress group, $p < 0.01$. Error bars show standard error of the mean.

PARP-1 IHC

IHC was performed to view whether treatment during chronic stress affected the expression of PARP-1. We examined three distinct regions of the PFC: layer 1 (L1), layer 3-6

(L3-6), and the FMI. Table 3 presents the descriptive statistics for PARP-1 area fraction in each brain region. PARP-1 area fraction is determined by dividing the stained tissue area by the total tissue.

Table 3

Descriptive Statistics of PARP-1 Area Fraction

Treatment Type	n	Mean	SEM
PFC L1 Region			
3-AB	5	0.061	0.030
FLX	8	0.266	0.086
SAL	8	0.076	0.035
CTRL	8	0.097	0.027
PFC L3-6 Region			
3-AB	6	0.133	0.037
FLX	8	0.196	0.050
SAL	8	0.057	0.023
CTRL	8	0.053	0.011
PFC FMI			
3-AB	6	0.229	0.092
FLX	8	0.251	0.068
SAL	8	0.088	0.029
CTRL	7	0.048	0.016

Note. PARP-1 immunoactivity was utilized to assess area fraction in each brain region.

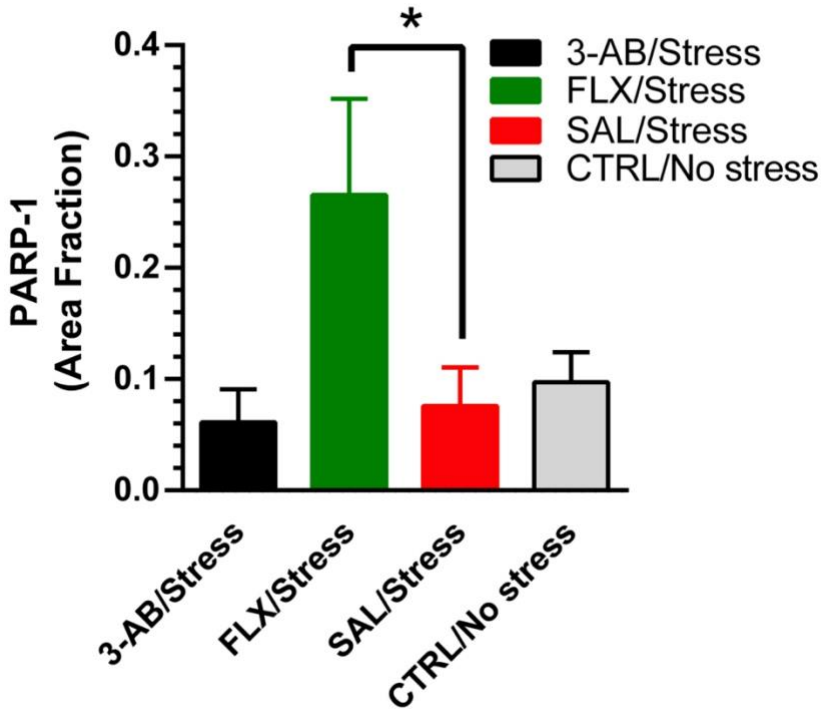
PFC L1

PARP-1 IHC was conducted in the PFC gray matter layer 1 to determine amount of

protein present in the region. Grubb's test revealed 3 outliers in the 3-AB/ Stress group, so they were eliminated from the dataset. A one-way ANOVA was fit to the data to assess the effects of Treatment (3-AB, FLX, SAL, CTRL) on PARP1 levels. The ANOVA revealed a significant difference in PARP-1 levels ($F_{(3,25)} = 3.11$, $p = 0.045$, $\eta^2 = 0.272$). Post hoc comparisons using Dunnett's test demonstrated a significant increase ($p = 0.043$) in PARP-1 expression in the FLX/Stress group ($M = 0.266$, $SD = 0.244$) when compared to the SAL/Stress group ($M = 0.076$, $SD = 0.099$) with a mean PARP-1 expression difference of 0.190 (Fig. 7).

Figure 6

Group Means (± SEM) of PARP-1 in L1 Region



Note. PARP-1 area fraction in layer 1 of the PFC is presented as a function of group. Area fraction represents the ratio of PARP-1 immunoreactivity to the total area of the tissue section.

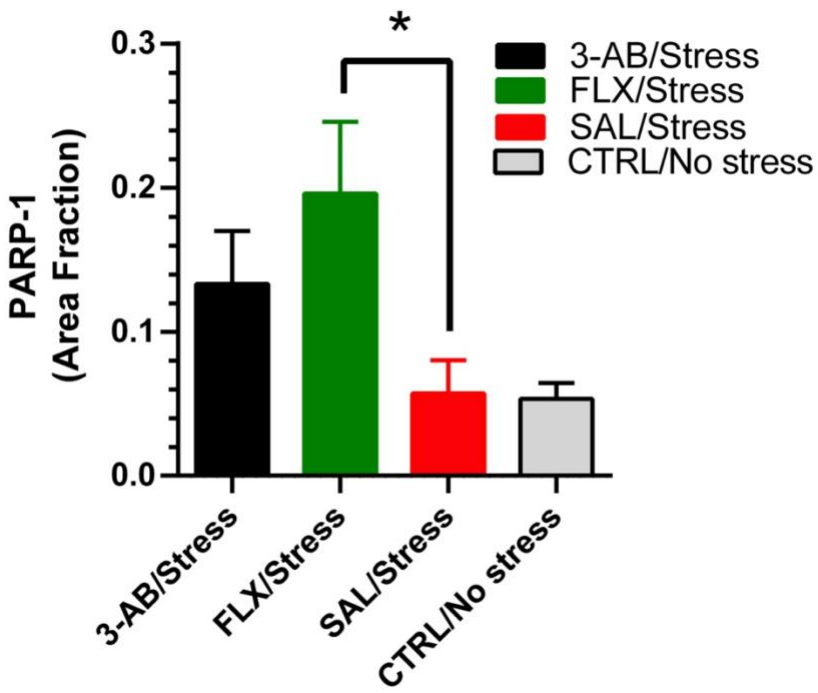
An asterisk (*) represents a significant increase in PARP-1 area fraction in the FLX/Stress group compared to the SAL/Stress group, $p < 0.05$. The error bars represent standard error of the mean.

PFC L3-6

PARP-1 IHC was conducted in the PFC gray matter layers 3-6 to determine amount of protein present in the region. Grubb's test revealed 2 outliers in the 3-AB/ Stress group, so they were eliminated from the dataset. A one-way ANOVA was fit to the data to assess the effects of Treatment (3-AB, FLX, SAL, CTRL) on PARP-1 levels. The ANOVA revealed a significant difference in PARP-1 levels ($F_{(3,26)} = 4.446$, $p = 0.012$, $\eta^2 = 0.339$). Post hoc comparisons using Dunnett's test demonstrated the FLX/Stress group ($M = 0.1960$, $SD = 0.140$) showed a significantly higher ($p = 0.012$) in PARP-1 levels than the SAL/Stress ($M = 0.057$, $SD = 0.065$) group with a mean difference of 0.139 (Fig. 8).

Figure 7

Group Means (± SEM) of PARP-1 Area Fraction in L3-6 Region



Note. PARP-1 area fraction in layers 3 - 6 of the PFC is presented as a function of group. Area fraction represents the ratio of PARP-1 immunoreactivity to the total area of the tissue section. An asterisk (*) represents a significant increase in PARP-1 area fraction in the FLX/Stress group compared to the SAL/Stress group, $p < 0.05$. Error bars show standard error of the mean.

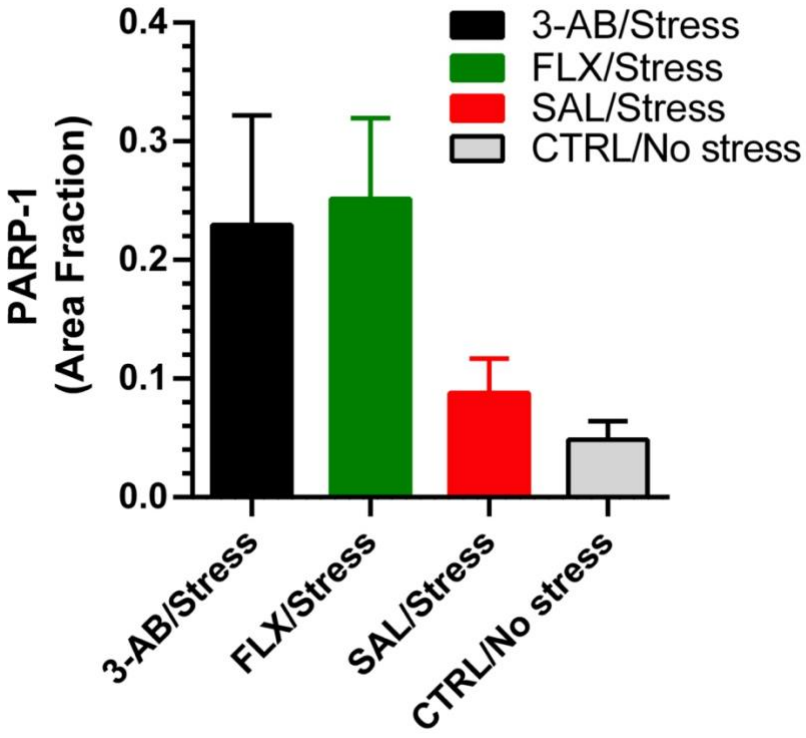
Forceps Minor of the Corpus Callosum

PARP-1 IHC was conducted in the PFC white matter to determine amount of PARP-1 protein present in the region. A sample was eliminated from the CTRL/ No stress group and 2 samples were removed from the 3-AB/ Stress group because they were determined to be outliers using Grubb's test. A one-way ANOVA was fit to the data to assess the effects of Treatment (3-

AB, FLX, SAL, CTRL) on PARP-1 levels. A change in PARP-1 levels was revealed ($F_{(3,25)}$ = 3.24, p = 0.039, η^2 = 0.280). Post hoc comparisons using Dunnett's test did not show any significant differences when comparing the treatment groups against the SAL/Stress group (Fig. 8).

Figure 8

Group means (± SEM) of PARP-1 Area Fraction in the FMI Region



Note. PARP-1 area fraction in the forceps minor of the corpus callosum of the PFC is presented as a function of group. Area fraction represents the ratio of amount of PARP-1 immunoreactivity to the total area of the tissue section. There were no significant differences found when using Dunnett's test to compare treatment groups against SAL/Stress. Error bars show standard error of

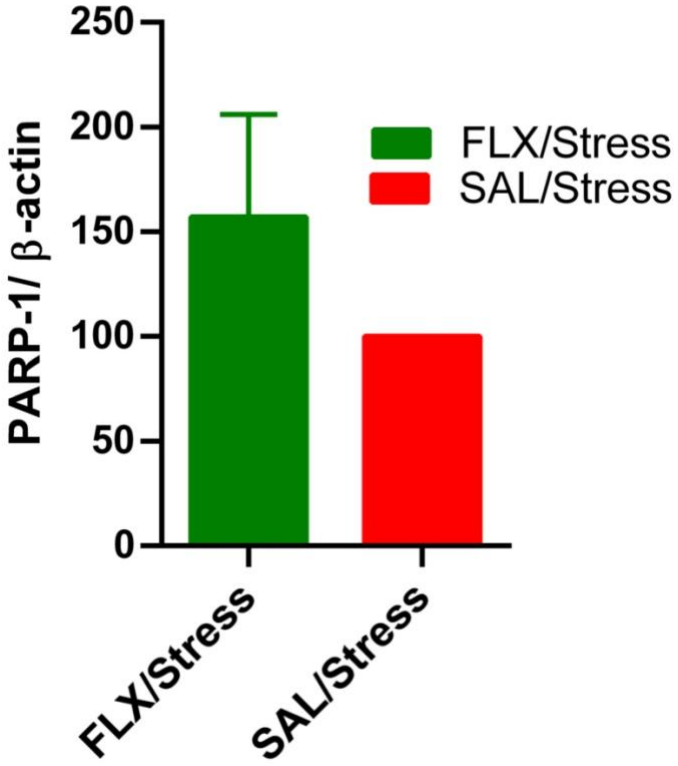
the mean.

PARP-1 Western Blots

PARP-1 Western Blots were conducted on PFC gray matter of 4 animals in the FLX/Stress group and 4 animals in the SAL/Stress group as a confirmatory measure for the PARP-1 IHC. Each sample was performed in quadruplicate and normalized with β-actin. An unpaired t-test was fit to the data to assess the effects of Treatment (FLX & SAL) on PARP-1 protein levels based on relative protein abundance. PARP-1 protein expression was not significant between the treatment groups ($t_{(6)} = 0.1219$, $p = 0.91$, $\eta^2 = 0.0025$; Fig. 9).

Figure 9

Western Blot Group Means (± SEM) of PARP-1



Note. Percentage of normalized PARP-1 protein abundance as a function of group. PARP-1 was normalized with the house-keeper protein, β-actin. Each sample was performed in quadruplicate. There was no significant difference in percent protein abundance between the FLX/Stress group and the Sal/Stress group. Error bars represent standard error of the mean.

IBA-1 IHC

To examine the effects that treatment type had on microglia morphology, microglia in white and gray matter were investigated. Ten parameters were analyzed to assess microglial soma and filament morphology. Soma area (μm^2) was determined by measuring the surface area of the object. Ellipticity refers to if the object has an ellipsoid shape, this was determined by calculating the lengths of the three radii of the soma. A prolate ellipsoid is "cigar-shaped" while an oblate ellipsoid is "disk-shaped". Soma sphericity is determined by calculating the ratio of a sphere, with a comparable surface area to the object, to the surface area of the object. Soma volume (μm^3) was determined by measuring the amount of space the object occupies. Filament area (μm^2) was defined as the sum of the surface area of all the dendritic segments of a given cell. Filament length (μm) is the sum of all the dendritic segments of a cell. Filament volume (μm^3) was determined by the sum of the space that all the dendritic segments occupy in a given cell. Number of dendritic branch points was calculated by counting the branching points of the dendrites. The number of sholl intersections was determined by placing concentric spheres around the cell body and counting the number of dendrite intersections that intersect these spheres. Descriptive Statistics for each variable and condition are presented in Tables 4 – 7.

Table 4

Descriptive Statistics of Gray Matter Microglia Soma Morphology Using IBA-1 IHC

Treatment Type	n	Mean	SEM
Soma Volume (um^3)			
3-AB	8	255.126	13.111
FLX	8	284.571	14.887
SAL	8	265.213	18.742
CTRL	8	270.650	20.505
Sphericity			
3-AB	8	0.876	0.008
FLX	8	0.876	0.011
SAL	8	0.877	0.007
CTRL	8	0.873	0.012
Ellipticity (Prolate)			
3-AB	8	0.410	0.006
FLX	8	0.412	0.018
SAL	8	0.453	0.011
CTRL	8	0.400	0.014
Ellipticity (Oblate)			
3-AB	8	0.412	0.010
FLX	8	0.396	0.009
SAL	8	0.367	0.009
CTRL	8	0.395	0.007
Soma Area (um^2)			
3-AB	8	225.609	6.811
FLX	8	237.416	7.958
SAL	8	226.892	9.255
CTRL	8	229.566	9.402

Note. IBA-1 immunoreactivity was used to quantify microglial morphology.

Table 5

Descriptive Statistics of Gray Matter IBA-1 Filament Morphology Using IBA-1 IHC

Treatment Type	n	Mean	SEM
Filament Area (um^2)			
3-AB	8	1906.958	126.723
FLX	8	2077.308	170.445
SAL	8	1958.809	134.372
CTRL	8	2132.016	172.806
Filament Length (um)			
3-AB	8	418.646	37.634
FLX	8	458.938	52.768
SAL	8	436.148	32.436
CTRL	8	446.076	44.130
Filament Volume (um^3)			
3-AB	8	860.739	54.508
FLX	8	911.627	60.068
SAL	8	862.856	69.142
CTRL	8	946.633	69.901
No. of Branch Points (count)			
3-AB	8	29.108	2.366
FLX	8	34.625	5.256
SAL	8	31.867	2.531
CTRL	8	33.667	4.020
No. of Sholl Intersections (count)			
3-AB	8	274.033	17.525
FLX	8	312.408	34.260
SAL	8	292.192	20.515
CTRL	8	301.592	28.332

Note. IBA-1 immunoreactivity was used to quantify microglial morphology.

Table 6

Descriptive Statistics of White Matter IBA-1 Soma Morphology Using IBA-1 IHC

Treatment Type	n	Mean	SEM
Soma Volume (um^3)			
3-AB	8	233.125	11.301
FLX	8	244.961	11.088
SAL	8	232.590	9.403
CTRL	8	256.489	17.509
Sphericity			
3-AB	8	0.810	0.065
FLX	8	0.869	0.011
SAL	8	0.862	0.009
CTRL	8	0.872	0.012
Ellipticity (Prolate)			
3-AB	8	0.405	0.014
FLX	8	0.434	0.013
SAL	8	0.434	0.014
CTRL	8	0.417	0.014
Ellipticity (Oblate)			
3-AB	8	0.397	0.016
FLX	8	0.371	0.013
SAL	8	0.389	0.011
CTRL	8	0.370	0.007
Soma Area (um^2)			
3-AB	8	213.837	7.170
FLX	8	216.486	7.156
SAL	8	210.404	5.181
CTRL	8	226.015	8.344

Note. IBA-1 immunoreactivity was used to quantify microglial morphology.

Table 7

Descriptive Statistics of White Matter IBA-1 Filament Morphology Using IBA-1 IHC

Treatment Type	n	Mean	SEM
Filament Area (um^2)			
3-AB	8	2049.226	191.655
FLX	8	2001.328	68.145
SAL	8	2060.371	69.363
CTRL	8	2201.194	137.225
Filament Length (um)			
3-AB	8	439.380	54.206
FLX	8	428.308	22.467
SAL	8	436.315	22.355
CTRL	8	472.644	32.775
Filament Volume (um^3)			
3-AB	8	882.949	65.863
FLX	8	893.158	35.874
SAL	8	872.697	29.484
CTRL	8	960.753	61.620
No. of Branch Points (count)			
3-AB	8	32.483	4.306
FLX	8	33.650	2.847
SAL	8	32.183	1.621
CTRL	8	35.667	2.941
No. of Sholl Intersections (count)			
3-AB	8	294.292	34.036
FLX	8	297.542	19.931
SAL	8	291.258	14.503
CTRL	8	316.842	20.751

Note. IBA-1 immunoreactivity was used to quantify microglial morphology.

Gray Matter

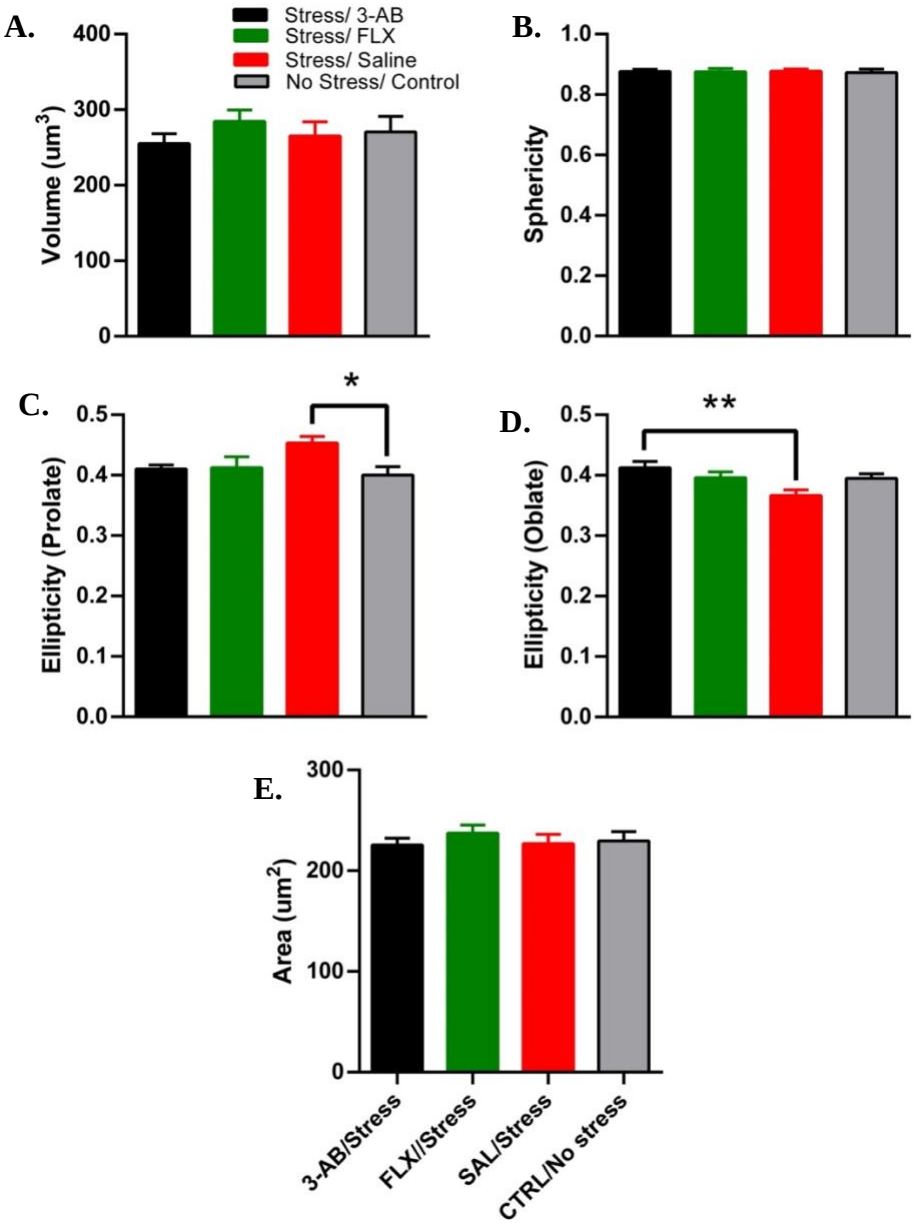
One-way ANOVAs were fit to each of the morphological measurements to investigate if Treatment type affected microglia cells. There were no significant differences found in microglia soma area, sphericity, soma volume, or filament morphology (p's > 0.05). However, significant main effects were found in ellipticity (oblate; $F_{(3,28)} = 4.507$, $p = 0.011$, $\eta^2 = 0.326$) and ellipticity (prolate; $F_{(3,28)} = 3.227$, $p = 0.038$, $\eta^2 = 0.257$). Post hoc comparisons using Dunnett's test demonstrated that the 3-AB/ Stress group (M = 0.413, SD = 0.099) had microglia that were significantly more oblate (p = 0.003) than the SAL/ Stress group (M = 0.367, SD = 0.093) with a mean difference of 0.046. Post hoc comparisons using Dunnett's test demonstrated that the CTRL/ No Stress group (M = 0.400, SD = 0.122) had microglia that were significantly less prolate (p = 0.020) than the SAL/ Stress group (M = 0.453, SD = 0.139) with a mean difference of 0.053 (Figure 12).

White Matter

One-way ANOVAs were fit to each of the morphological measurements to investigate if Treatment type affected microglia cells. There were no significant differences found in microglia soma or filament morphology (p's > 0.05; Figures 14 & 15).

Figure 10

IBA-1 IHC Microglia Soma Morphology in PFC Gray Matter

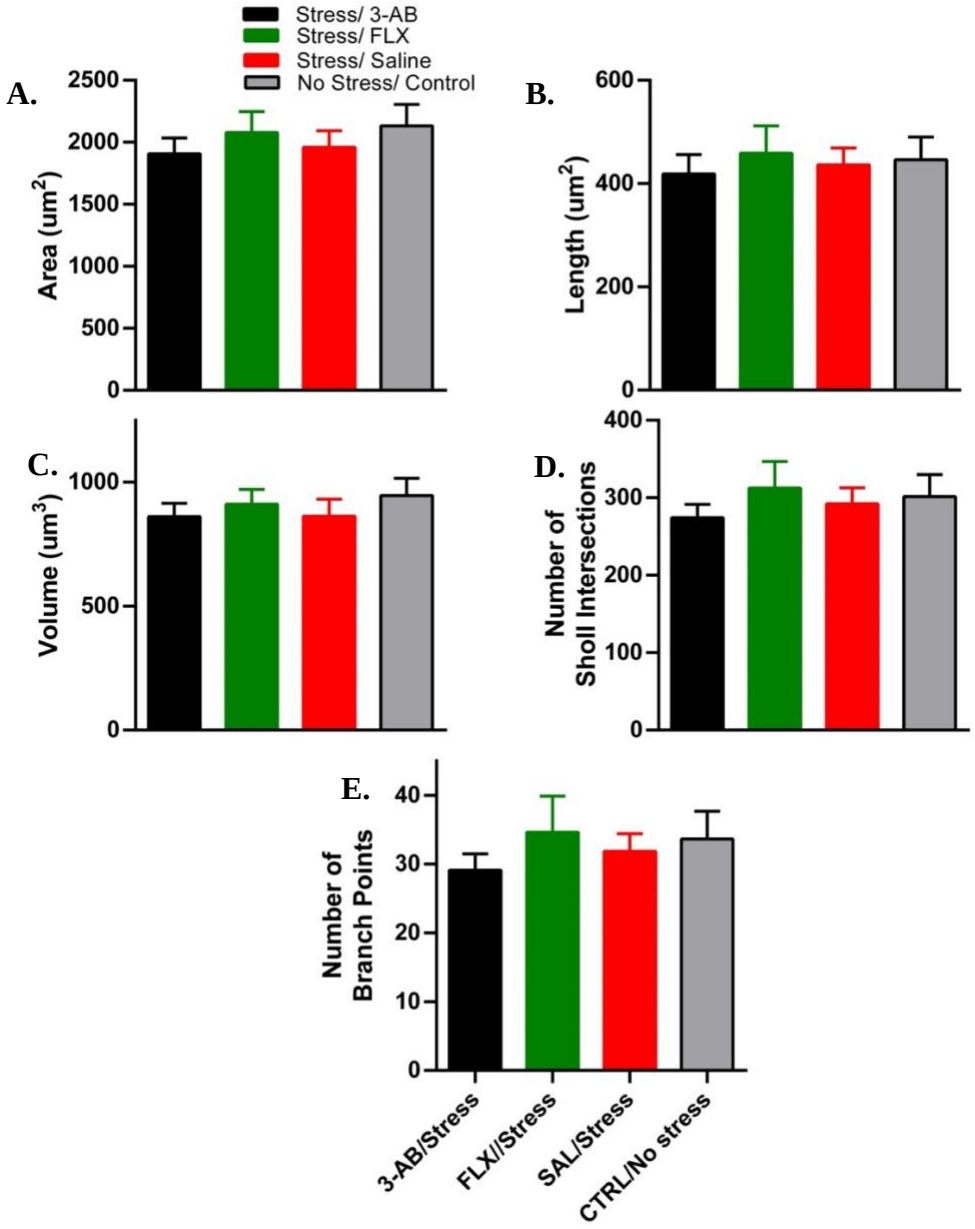


Note. Gray matter prefrontal cortical microglia soma morphological parameters were determined using Imaris. IBA-1 IHC was performed to stain PFC tissue. Microglia soma volume (μm^3) is presented as a function of group. Soma volume was calculated by determining the amount of

space the soma occupied B) Microglial soma sphericity is presented by treatment group. Sphericity was determined as the ratio of a sphere to the volume of the soma. C) Microglia soma prolate ellipticity is presented as a function of group. An asterisk (*) denotes that the SAL/Stress group had microglia soma with significantly more prolate ellipticity than the CTRL/ No stress group, $p < 0.05$. D) Microglia soma oblate ellipticity is represented by treatment group. Double asterisks (**) refer to the 3-AB group having significantly more oblate somas than the SAL/ Stress group, $p < 0.001$. E) Microglia soma area (μm^2) is presented as a function of group. Soma area was determined by calculating the surface area. The error bars represent standard error of the mean.

Figure 11

IBA-1 IHC Microglia Filament Morphology in PFC Gray Matter

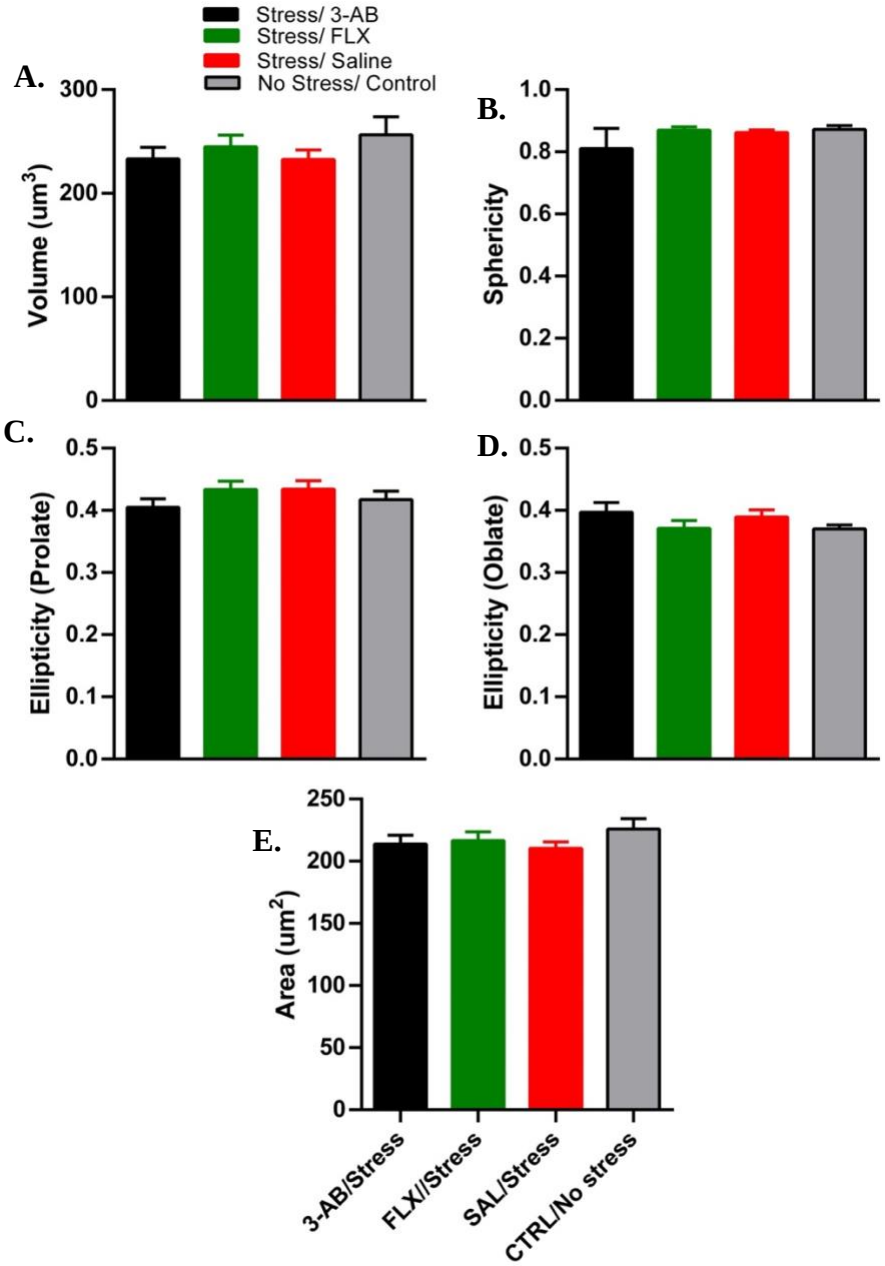


Note. A) Gray matter prefrontal cortical microglia filament morphological parameters were determined using Imaris. IBA-1 IHC was performed to stain PFC tissue. A) Microglial filament area (μm^2) is presented as a function of group. The sum of the surface area of all

dendritic segments was calculated to determine filament area. B) Microglia filament length (µm) is displayed by treatment group. The length of all dendritic segments was calculated to determine filament length. C) Microglia filament volume (μm^3) is presented as a function of treatment group. Filament volume was calculated by the sum of the volume of all dendritic segments. D) Microglia number of sholl intersections is displayed by group. E) Microglia number of branch points is presented as a function of group. Number of branching was calculated by the sum of branching points. The error bars represent standard error of the mean.

Figure 12

IBA-1 IHC Microglia Soma Morphology in PFC White Matter

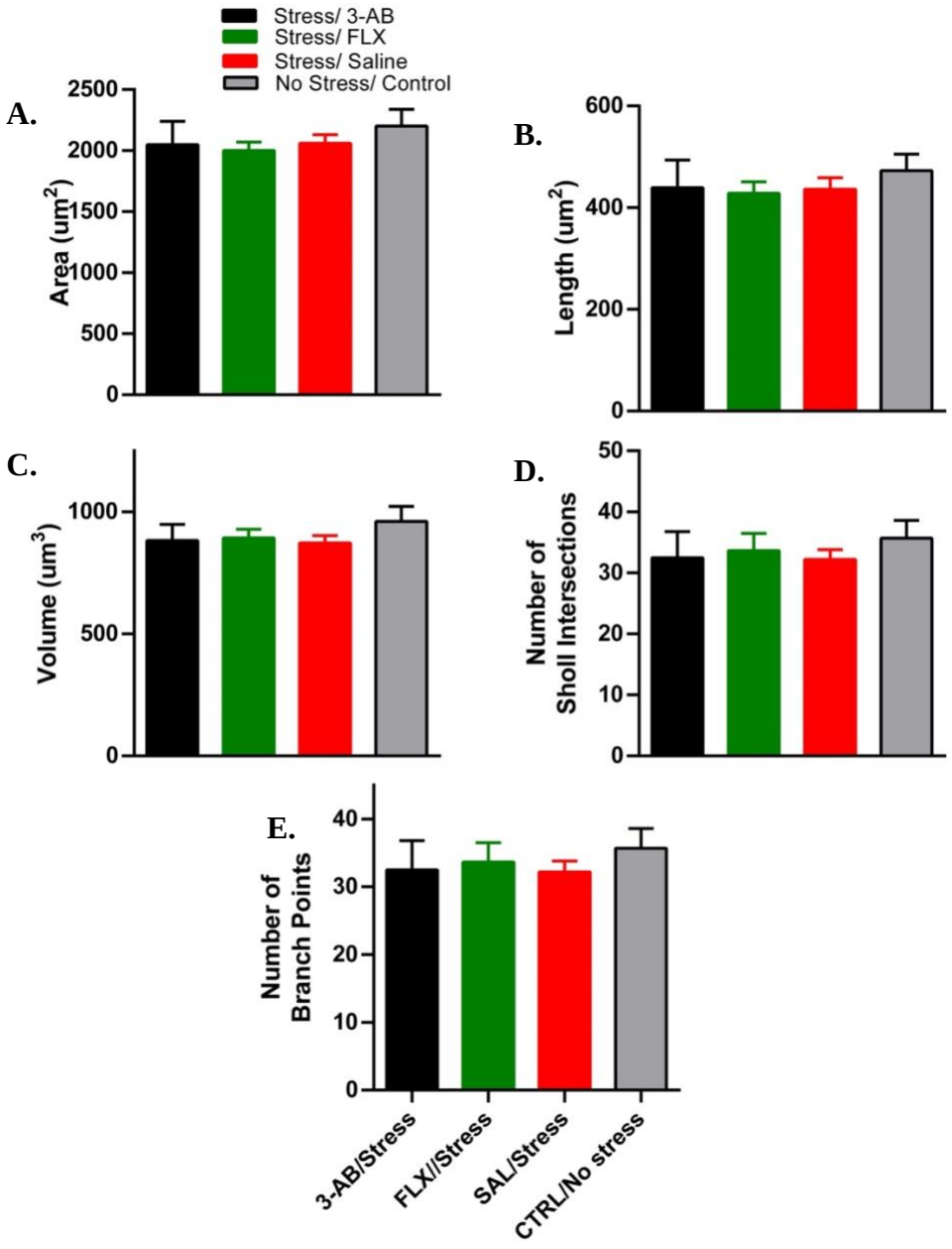


Note. White matter prefrontal cortical microglia soma morphological parameters were determined using Imaris. IBA-1 IHC was performed to stain PFC tissue. A) Microglia soma

volume (μm^3) is presented as a function of group. Soma volume was calculated by determining the amount of space the soma occupied B) Microglial soma sphericity is presented by treatment group. Sphericity was determined as the ratio of a sphere to the volume of the soma. C) Microglia soma prolate ellipticity is presented as a function of group. D) Microglia soma oblate ellipticity is represented by treatment group. E) Microglia soma area (μm^2) is presented as a function of group. Soma area was determined by calculating the surface area. The error bars represent standard error of the mean.

Figure 13

IBA-1 IHC Microglia Filament Morphology in PFC White Matter



Note. A) Gray matter prefrontal cortical microglia filament morphological parameters were determined using Imaris. IBA-1 IHC was performed to stain PFC tissue. A) Microglial

filament area (μm^2) is presented as a function of group. The sum of the surface area of all dendritic segments was calculated to determine filament area. B) Microglia filament length (μm) is displayed by treatment group. The length of all dendritic segments was calculated to determine filament length. C) Microglia filament volume (μm^3) is presented as a function of treatment group. Filament volume was calculated by the sum of the volume of all dendritic segments. D) Microglia number of sholl intersections is displayed by group. E) Microglia number of branch points is presented as a function of group. Number of branching was calculated by the sum of branching points. The error bars represent standard error of the mean.

ELISAs

Table 8

Descriptive Statistics of Cytokine Protein Expression (pg/mL)

Treatment Type	n	Mean	SEM
TNF-A (pg/mL)			
3-AB	8	187.788	13.266
FLX	8	167.678	11.588
SAL	8	274.512	38.745
CTRL	12	179.116	9.271
IL-1B (pg/mL)			
3-AB	8	35.389	1.104
FLX	8	34.255	2.990
SAL	8	48.127	4.162
CTRL	12	32.935	0.783
IL-6 (pg/mL)			
3-AB	8	7.914	1.995
FLX	8	5.540	0.771
SAL	7	11.505	2.062
CTRL	11	3.810	1.071

Note. ELISAs were utilized to measure cytokine protein expression and protein concentrations were compared against a standard curve.

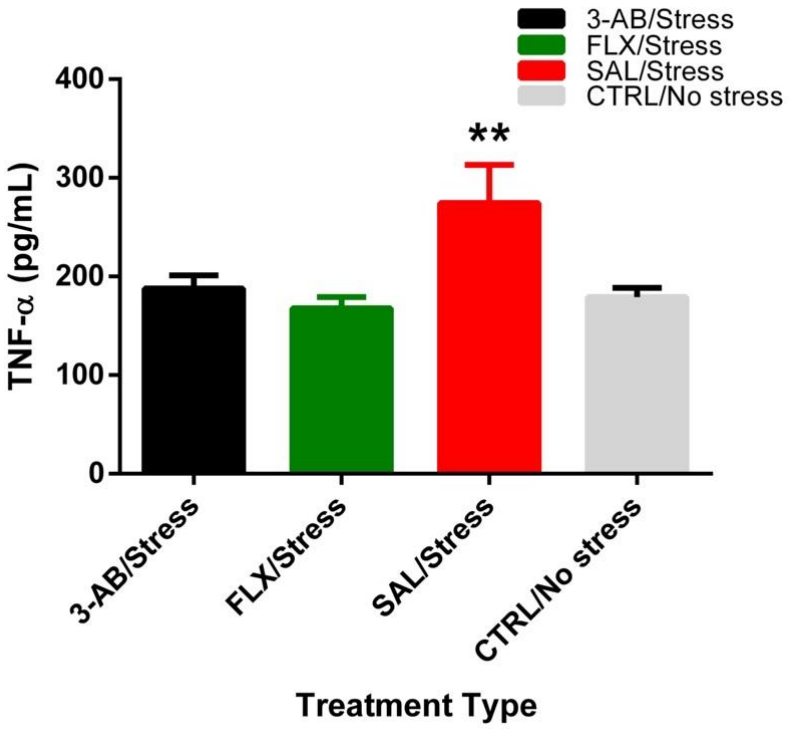
TNF-α

A one-way ANOVA was fit to the data to assess the effects of Treatment (3-AB, FLX, SAL, CTRL) on TNF-α levels in the hippocampus. A significant change in TNF-α levels was observed ($F_{(3,32)} = 5.60$, $p = 0.003$, $\eta^2 = 0.334$). Post hoc comparisons using Dunnett's test demonstrated the SAL/Stress group (M = 274.51, SD = 109.59) had significantly higher (p's < 0.008) TNF-α protein levels than the 3-AB/Stress group (M = 187.79, SD = 37.52), FLX/Stress

group (M = 167.68, SD = 32.78), & CTRL/No stress group (M = 179.12, SD = 32.12; Fig. 14).

Figure 14

TNF-α Protein Expression in the Ventral Hippocampus



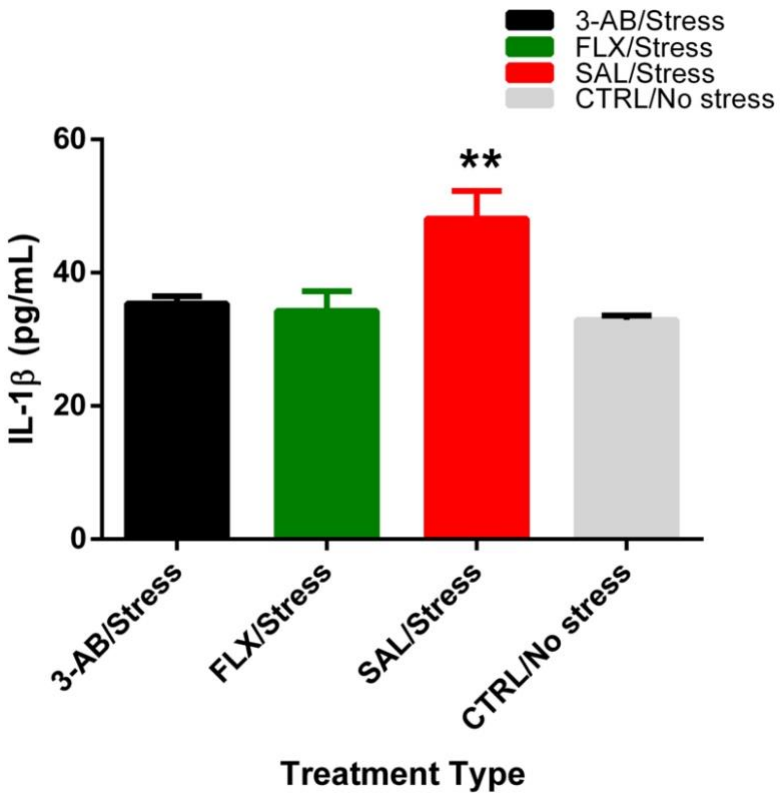
Note. TNF-α protein expression (pg/mL) in the ventral hippocampus is displayed as a function of treatment group. Double asterisks (**) represents that the SAL/Stress group demonstrated aa significant increase in TNF-α expression than all other treatment groups, $p < 0.01$. Error bars represent standard error of the mean.

IL-1β

A one-way ANOVA was fit to the data to assess the effects of Treatment (3-AB, FLX, SAL, CTRL) on IL-1β levels in the hippocampus. A significant change in IL-1β levels was observed ($F_{(3,32)} = 8.356$, $p < 0.001$, $\eta^2 = 0.439$). Post hoc comparisons using Dunnett's test demonstrated the SAL/Stress group (M = 48.13, SD = 11.77) had significantly higher (p's < 0.002) IL-1β protein levels than the 3-AB/Stress group (M = 35.39, SD = 3.122), FLX/Stress group (M = 34.25, SD = 8.46), & CTRL/No stress group (M = 32.94, SD = 2.71; Fig. 15).

Figure 15

IL-1β Protein Expression in the Ventral Hippocampus



Note. IL-1β protein expression (pg/mL) in the ventral hippocampus is displayed as a

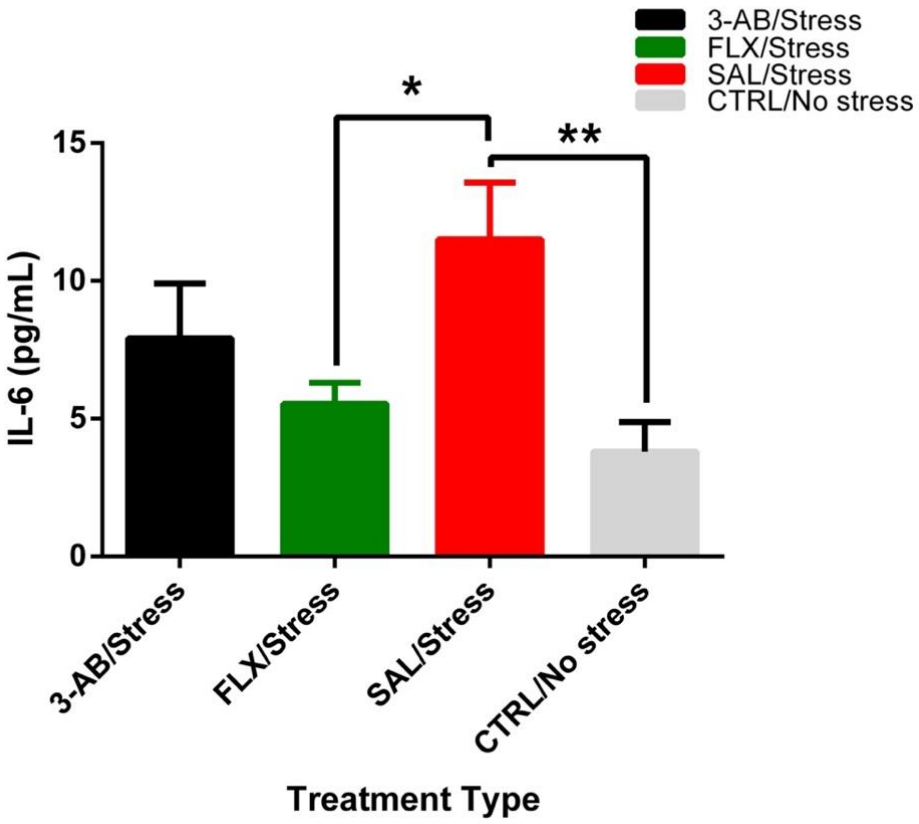
function of treatment group. Double asterisks (**) represents that the SAL/Stress group demonstrated a significant increase in IL-1β expression than all other treatment groups, $p < 0.01$. Error bars represent standard error of the mean.

IL-6

A one-way ANOVA was fit to the data to assess the effects of Treatment (3-AB, FLX, SAL, CTRL) on IL-6 levels in the hippocampus. A significant change in IL-6 levels was observed ($F_{(3,30)} = 4.92$, $p = 0.007$, $\eta^2 = 0.330$). Post hoc comparisons using Dunnett's test demonstrated the SAL/Stress group ($M = 11.50$, $SD = 5.45$) had significantly higher (p's < 0.02) IL-6 than the FLX/Stress group ($M = 5.54$, $SD = 2.18$) and the CTRL/No stress group ($M = 3.81$, $SD = 3.55$; Fig. 16).

Figure 16

IL-6 Protein Expression in the Ventral Hippocampus



Note. IL-6 protein expression (pg/mL) in the ventral hippocampus is displayed as a function of treatment group. An asterisk (*) denotes that the SAL/ Stress group had significantly higher IL-6 expression than the FLX/Stress group, $p = 0.016$. Double asterisks (**) represents that the SAL/Stress group demonstrated a significant increase in IL-6 expression than the CTRL/No stress treatment group, $p = 0.001$. Error bars represent standard error of the mean.

Chapter 4. Discussion

The aim of this study was to analyze the interaction of neuroinflammation and chronic stress through the inhibition of PARP activity. PARP is a DNA repair enzyme and is often activated in cases of increased ROS, which is typically increased in response to stress (Bai & Virág, 2012; Pazzaglia & Pioli, 2020). Our lab has previously reported that PARP-1 gene expression was elevated in postmortem brain tissue from individuals diagnosed with MDD (Szebeni et al., 2016). Additionally, in rodent models of chronic stress, the administration of a PARP inhibitor has shown similar antidepressant (AD)-like effects that are similar to FLX (Ordway et al., 2017; Sriram et al., 2015). PARP-inhibitors have been shown to suppress proinflammatory microglial activation by blocking NF-κB mediated responses (Chiarugi & Moskowitz, 2003; d' Avila et al., 2012; Kauppinen et al., 2009) and aberrant microglial functioning is associated with chronic stress and depression (Dantzer, 2009; Frank et al., 2019; Sugama et al., 2009); thus, we hypothesized that deleterious PARP activation is a contributing factor to microglial dysfunction. The current study substantiates previous findings in that 3-AB produces AD-like effects and protects against neuroinflammation.

Sucrose Preference Test

The sucrose preference test is an established method to measure anhedonic behavior in rodents (Liu et al., 2018; Willner et at., 1987). Animal studies have demonstrated chronic stress exposure induces a decreased preference to a 0.8% sucrose solution, a typically rewarding stimulus. The 0.8% concentration was discovered to be the threshold of when non-stressed rats can detect sucrose, a natural reward (Berridge, 1996). This decreased preference is reversed by the administration of antidepressants (Katz, 1982; Rygula et al., 2005). Results from the sucrose preference test, in the current study, revealed that the PARP-inhibitor, 3-AB, exhibits

antidepressant-like effects (See Fig. 5). The 3-AB/Stress rats had sucrose preference scores equivalent to the CTRL/No Stress rats and both of these groups had scores significantly greater than the SAL/ Stress rats. These findings are consistent with previous findings that 3-AB has the capability to prevent the development of anhedonia in chronically stressed rats (Ordway et al., 2017).

Contrary to our hypothesis, the FLX/Stress rats were not statistically significant from the SAL/Stress group. This finding did not replicate our previous work (Ordway et al., 2017). The FLX/ Stress rats, in the current study, exhibited a wide variation in drinking behavior suggesting that perhaps there was treatment resistance in regard to the sucrose preference test. Prior studies have revealed significant changes in gene regulation of rats that were treatment resistant to SSRIs (Bergström et al., 2007; Christensen et al., 2011). It is possible that the variance in the FLX group is due to similar gene regulatory changes.

The sucrose preference test measures a behavioral response to a stimulus (e.g., consuming sucrose water) and from that response it is inferred that the animals are interested in the rewarding stimulus and that consummatory pleasure is homogeneous. However, animals are heterogeneous and there will always be some natural variability when conducting animal research (Planchez et al., 2019). Finally, in studies in which stress is the manipulation, there tends to be increased resilience to stress (Nestler & Waxman, 2020), which may have occurred in the present study. In future work, we will increase the number of subjects per group to try to account for this increased variability on different behavioral endpoints.

Social Interaction Test

The social interaction test is a widely used paradigm to measure anxiolytic behavior in rodents (File & Smith, 2003). Results from the current study partially supported the hypothesis,

in that the SAL/Stress group displayed enhanced social anxiety, as they spent significantly more time in the avoidance zone when compared to the 3-AB/ Stress and the CTRL/No stress groups. However, the FLX/Stress rats did not significantly differ from the SAL/Stress group in their social interaction times. Prior studies conducted in our lab have shared similar results, insofar as rats exposed to the SDS+CUS paradigm spent significantly less time in the interaction zone than non-stressed controls or treated-stressed rats (Ordway et al., 2017; Szebeni et al., 2016). Interestingly, these findings suggest that 3-AB has anxiolytic properties considering that the 3-AB/ Stress and CTRL/No stress groups did not statistically differ in interaction time.

PARP-1 IHC & PARP-1 Western Blots

The IHC and western blot results showed that the FLX/Stress group had a significant increase of PARP-1 expression in both gray matter brain regions (L1 & L3-6; Figures 6 & 7) when compared to the SAL/Stress group (Figure 9). Regardless, both assays did not support the hypothesis, since we hypothesized that the SAL/stress group would demonstrate an increase in PARP-1 expression in both gray and white matter relative to all other treatment groups.

Most notably, recently findings have suggested that SSRIs, namely FLX, have cytotoxic effects on astrocytes (He et al., 2021). Astrocytes are the most abundant cell type in the CNS, and they aid microglia in the neuroinflammatory response. The study demonstrated that FLX, among other SSRIs, had a paradoxical effect on astrocytic immune response, in that SSRIs promote the production of iNOS and nitric oxide (NO) but inhibited the production of IL-6 and IL-1β. Currently, the astrocytic cytotoxicity associated with the administration of SSRIs does not seem to interfere with the efficaciousness of treatment (He et al., 2021). The mechanisms as to how SSRIs cause cytotoxicity is unknown but research on the use of FLX as a treatment for glioblastomas, a type of malignant astrocyte, could provide insight. The study alluded the ability

of FLX to directly bind to the GluR1 binding subunit of AMPA receptors and evokes transmembrane calcium (Ca^{2+}) influx, thereby causing increased cytotoxic activity in astrocytes. The increase in intracellular Ca^{2+} concentrations can trigger mitochondrial overload and the activation of caspase-9, caspase-3, and PARP (Liu et al., 2015). Perhaps, in the current study FLX is binding to astrocytic AMPA receptors to create an increase in free radical production, in turn producing an increase in PARP-1 protein expression. Future studies should be conducted to further investigate FLX interaction with astrocytes.

IBA-1 IHC

IBA-1 IHC results revealed morphological differences in microglial cells among treatment type. The data indicated that morphological changes occurred in the ellipticity of microglia residing in the gray matter. Specifically, the soma of the microglia in the SAL/Stress group were significantly more prolate than the soma shape in the CTRL/ No stress group. The elongation of the somas in the SAL/Stress group is a notable phenotype associated with early microglial activation (Kettenmann et al., 2011). Additionally, the microglial soma of the 3-AB/Stress group were significantly more oblate than the soma in the SAL/Stress group, suggesting that 3-AB may be blocking this activation. A previous study (Zhou et al., 2019) showed similar findings, in that inactivated microglia were significantly more oblate than activated microglia. Taken together, this would suggest that the microglia in the SAL/Stress group appeared on the activation spectrum, since activation is not a binary process (Frank et al., 2019).

Contrary to what we expected, we did not see a change in white matter microglia. Our lab has previously showed that stressed rats have significantly elevated levels of 8-OHdg, a marker for DNA oxidation, compared to non-stressed rats (Szebeni et al., 2016). Perhaps the lack of

difference in white matter microglial morphology between groups can be attributed to the latency period amid the last stressor and euthanasia, giving the microglia enough time to transition back to their sentinel state. Even though the microglia did not differ morphologically, there is evidence that exposure to stress will prime microglia leading to exaggerated proinflammatory responses to future stressors (Calcia et al., 2016; Frank et al., 2019; Franklin et al., 2018). Future work could examine microglia at a more distant time point since the current data suggests that microglia may not activate rapidly after chronic stress.

Analyses of Cytokines

Increases in proinflammatory cytokine expression has been demonstrated in humans with depressive disorders as well as chronically stressed rodents (Irwin & Miller, 2017; Reader et al., 2015; Swardfager et al., 2016). In order to view whether our model stimulated proinflammatory cytokine release we examined ELISAs of hippocampal tissue on IL-1β, TNF-α, and IL-6 using hippocampal tissue. Our results demonstrated differential proinflammatory cytokine expression in the hippocampi of the treatment groups. The TNF-α and IL-1β data were consistent with our hypothesis in that the SAL/Stress group showed significantly elevated levels compared to all other treatment groups (Fig. 14 & 15).

Contrary to our hypothesis, the IL-6 data indicated that the SAL/Stress group did not significantly differ from the 3-AB/Stress group. The 3-AB/Stress group did exhibit modest reductions in IL-6 although these reductions were not statistically significant (Fig. 16). The lack of a significant difference between the 3-AB/Stress group and the SAL/Stress group could have been due to the small sample size.

Limitations

Attempting to model a psychiatric disorder will always prove to be challenging. A major limitation in the current study is the inability to assess subjective attributes that are associated with chronic stress (e.g., feeling of worthlessness, suicidal ideation, uncontrollable worrying, etc.; Nestler & Hyman, 2010). We attempted to mediate this challenge by using established behavioral paradigms that are used to objectify these internal symptoms, such as the sucrose preference task in measuring anhedonia and the social interaction task in measuring anxiogenesis.

The current study sought to assess the mechanisms involved in the AD-like effects of 3-AB. However, the idea of a PARP inhibitor being regarded as an antidepressant is a relatively novel concept and PARP is found ubiquitously in every cell in the body. Therefore, it is difficult to hypothesize where the protein dysregulation may reside. The PFC is a prime brain region of interest because previous studies in our lab demonstrated an elevation of oxidative stress in both human postmortem PFC tissue with MDD and SDS+CUS rats (Ordway, 2017; Szebeni, 2016).

The current study demonstrated minimal changes of microglia morphology in gray matter PFC and no changes in white matter PFC. Perhaps this was due to the limited duration of our paradigm restricting the chronic activation of microglia. Alternatively, the microglial cells may have already transitioned back to their quiescent state because of the 48h lapse between the final stressor and euthanasia. Also, due to time constraints and tissue availability we only examined the PFC for changes in microglia morphology. Microglial populations show physiological differences depending on their location in the CNS (For review see Tan et al., 2020). Therefore, I anticipate that microglial activation is more severe in more subcortical areas, such as the amygdala, hypothalamus, or the hippocampus. Therefore, future studies should assess more

subcortical brain areas and increase the latency period between the final stressor and euthanasia in order to give the microglia enough time to chronically activate. In addition, the number of animals should be increased in order to account for heterogeneity within and across treatment groups.

Conclusion

Persistent exposure to stress is associated with the increase in ROS, DAMPs, and proinflammatory cytokines which can lead to DNA strand breaks and PARP-1 activation (Bettridge, 2000; Herbet et al., 2017; Sriram et al., 2015). Furthermore, PARP-1 is also a coactivator of NF-κB and allows NF-κB transcription factors to bind to DNA strand breaks that lead to activation of a cascade of inflammatory processes (Hassa et al., 2003; Sriram et al., 2014). Thus, the administration of a PARP-inhibitor, such as 3-AB, can provide neuroprotection by disrupting the activation of PARP-1 and thereby reducing deleterious proinflammatory activity. The current study provides additional evidence towards the therapeutic potential of 3-AB and suggests that these effects could be due to its ability to reduce neuroinflammation in the PFC and hippocampus, two regions that are thought to be dysregulated due to chronic stress. We were able to demonstrate that the administration of 3-AB prevented disrupted behaviors produced by chronic stress and simultaneously produced reductions in neuroinflammation. Specifically, treatment with 3-AB prevented stress-related increases in PARP-1 abundance, microglia activation, and proinflammatory cytokine expression.

A finding that we did not expect was that the FLX/Stress group displayed an increase PARP-1 expression compared to the SAL/Stress group. However, a novel study demonstrated the ability for FLX to cause cytotoxicity in astrocytes (He et al., 2021), which could explain the increased levels of PARP-1 in our FLX/Stress animals. This intriguing finding will need to be

further investigated in future studies.

Depressive disorders are a leading cause of health-related disability worldwide (James et al., 2018), primarily due to inadequate understanding of the dysregulated pathophysiology associated with these disorders (Roman & Irwin, 2020; Troubat et al., 2020). These disorders frequently coexist with chronic medical illnesses leading to the presumption that inflammation is associated with the manifestation of depression (Gold et al., 2020). The present study provides insight on the neuronal mechanisms affected by chronic psychological stress. Furthermore, this is the first study to assess the effects of 3-AB on neuroinflammation and microglia activation following a chronic stress paradigm. Future studies will be needed to increase our knowledge on the therapeutic mechanisms of 3-AB in chronic stress.

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