THE MECHANISMS BY WHICH REPEATED STRESS EXPOSURE ALTERS BRAIN CYTOKINES: IMPLICATIONS FOR DEPRESSIVE-LIKE BEHAVIOR

A thesis submitted to the
Kent State University Honors College
in partial fulfillment of the requirements
for Departmental Honors

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May, 2013
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ACKNOWLEDGMENTS

I would like to acknowledge and thank my advisor, Dr. John Johnson, for everything he has done for me. The skills and knowledge he has graciously granted upon me are indispensable. I would also like to acknowledge and thank my thesis defense committee, Dr. Paul Sampson, Dr. Soumitra Basu, and Dr. Douglas Kline. Without these people and their help, this thesis would have not been possible.
CHAPTER I

SUMMARY

Major depression is a mental illness that can be debilitating to sufferers. Furthermore, it is relatively common and reoccurring. According to the Centers for Disease Control and Prevention, about one of ten American adults suffer from depression. Also, depression recurrence depends on the number of previous episodes you have had. The likelihood of an additional episode increases as the number of previous episodes increase. For people who have suffered from depression once, they have a 50% chance of experiencing a second episode. People, who have had two previous episodes of depression, have a 70% chance of a third episode. Regarding people who have had three or more episodes, all but about 10% will experience another episode. However, even with the prevalence of depression, antidepressants are found to be effective in only about 70% of cases (Leonard, 2010).

Antidepressants commonly target monoamine levels, such as serotonin. The decrease in serotonin levels is a common hypothesis of depression, but there are many other physiological explanations for depression, such as the link between the immune system and the brain. In the past, the brain was believed to be separate from the immune system and its effects due to the blood-brain-barrier, but that is not the case. Peripheral immune molecules have been shown to access the brain and therefore affect its activity, and ultimately cause mental illnesses, such as depression (Leonard, 2010).
The immune system’s goal is to protect and defend against foreign organisms, such as bacteria, viruses, and parasites. The immune system is comprised of many kinds of cells, which detect, neutralize, and ultimately kill disease-causing cells. The immune system consists of two responses, the innate immune response and the adaptive immune response. The adaptive immune system is the second-ordered response, which is slow and specific. The innate immune response is the first response, which is quick and used to protect the body against cuts and wounds, which allow pathogens to enter the body and infect. One common response by the innate system is to induce inflammation at the site of infection. The secretion of cytokines induces inflammation. Cytokines are signaling molecules released by cells that sense the damage caused by the wound and infection. After being released, cytokines signal to other immune cells, triggering a state of inflammation.

The family of cytokines is large, consisting of interleukins, lymphokines, and chemokines. Also, it was previously mentioned how cytokines induce inflammation, but it is important to note cytokines can have other roles, such as reducing inflammation. Cytokines can also affect the re-uptake of monoamine neurotransmitters, such as serotonin. In addition, cytokines are known to affect the release of certain hormones.

With the effects of cytokines being large, cytokines are also known to affect an animal’s behavior. Cytokines, such as the group interleukins, have been shown to induce sickness-like behavior, ultimately changing the behavior of an animal. Behavioral changes consist of decreases in food and water intake, decreases in social interaction and exploration, and cognitive impairment (Dantzer, 2001). With the understanding of
cytokines affecting the behavior of an animal, the macrophage hypothesis was introduced about thirty years ago to explain the onset of depression. The macrophage hypothesis is the link between the immune system and depression. Pathogens, or other molecules known to produce an immune response, induce brain cytokine levels leading to depressive-like behavior. Numerous studies have shown the association between depression and cytokines (Raison, 2006). Additionally, overall healthy individuals have shown an increase in cytokine levels and a single depression-related symptom, such as fatigue or insomnia (Raison, 2006).

With the link between the immune system and depression being examined, the question in how stress affects this link is raised. As an animal perceives a threat, the stress response promotes survival. The stress response involves the activation of two different systems, which will be discussed later in the thesis. For example, one of the systems causes the body to react by either fleeing the scene (or threat) or defending itself. This is the common phenomenon known as the flight-or-fight response.

Today, stress is all around us, but in different forms than what a wild animal may be exposed to. Today we have stressors such as financial or social. It is common for one to seek remedies to cope with such stressors, especially your everyday dosage. However, when the stress becomes chronic, or we are unable to cope, it may lead to depression.

Acute stressors, such as a one-time mild stressful event, have been shown to activate the immune system response, leading to a transient rise in brain cytokines. Therefore, it has been proposed that chronic stressor exposure may result in a more sustained elevation in brain cytokines, which ultimately leads to depression. While the
effect of acute stress is somewhat understood, chronic stress, or repeated stress, and its
effects on brain cytokines have barely been examined. This thesis will take a further look
at the complex relationship between chronic stress, the immune response, and depression.
CHAPTER II

INTRODUCTION

Stress

The stress response promotes survival as the body responds to a threat, either physiological or psychological. When an organism perceives stress, the body responds in such a manner to restore homeostasis and well-being. In response to the stress, the body reacts using two different response mechanisms, the central stress response and the peripheral stress response. The central stress response is mediated by the stimulation of neurons located in the locus coeruleus (LC), which in turn release norepinephrine (NE), a neurotransmitter that aids in the adaption of environmental stressors. This is known as the noradrenergic system, and is important for alertness and wakefulness (Itoi et al., 2011). The central stress response is also known to decrease pain sensitivity and increase aggression or defensive behaviors. The peripheral stress response consists of two systems, the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. As the SNS is stimulated, release of NE and epinephrine (adrenaline) is induced. The peripheral stress response causes an increase in glucose (for energy), circulation of oxygen and energy, increase in heart rate and blood pressure, and ultimately the activation of the fight or flight response. Stress can also activate the HPA axis, which causes the release of glucocorticoids by the adrenal glands (Figure 1). Glucocorticoids (cortisol in humans and corticosterone in rodents) are anti-inflammatory hormones, and are known to regulate metabolism, immunity, and are responsible for
feedback inhibition of stress hormones (Zunszain et al., 2011). Activation of the HPA axis and induction of glucocorticoids shuts down non-essential functions such as reproductive, inflammation, and growth in the favor of survival (flight-fight response). This is due to the acute stress response; however, chronic stress exposure resulting in repeated activation of these physiological stress systems has been shown to cause numerous health complications.

Chronic activation of the stress circuitry can lead to serious health complications and disease. Stimulation of the SNS and HPA axis can lead to hypertension, increase in cardiovascular disease, and long-term suppression of the immune system (Chrousos, 2000). As the stress response remains activated, the SNS is continuously stimulated, increasing anxiety. Exposure to chronic stress is associated with depression, due to the failure of reaching homeostasis and turning off the stress response. The pathway, which is stimulated by stress, leading to depression, is unknown and is the focus of the work in this thesis.

**Cytokines and Depression**

Major depression is one of the most common psychiatric disorders and only approximately a third of patients receive adequate treatment (Thase, 2006). Disturbances in the limbic system are associated with depression. The limbic system is composed of six brain regions: the amygdala, basal ganglia, cingulated gyrus, hippocampus, hypothalamus, and the thalamus. Of these six regions, three of them have important connections to an organism’s response to stress and depressive-like behavior. The amygdala has a role in emotional responses, such as fear. The hypothalamus plays a role
in emotional and behavioral responses, as well as part of the stress response (HPA axis). Last, the hippocampus is known to convert information from short-term memory to long-term memory. These three brain regions are of interest, in addition to the brain stem and prefrontal cortex. The brain stem controls basic functions, such as breathing. The brain stem is also the region in which the locus coeruleus is located. The locus coeruleus is a nucleus involved in the response of stress and a main site of NE production. Finally, the prefrontal cortex is the region associated with executive decisions, personality expression, and social behavior. While all five brain regions of interest modulate slightly different tasks, they collectively play important roles in stress and depression (Figure 2).

Studies have performed functional-MRIs on major depressed patients to analyze the condition of numerous brain regions. The prefrontal cortex is has been shown to decrease in functionality, while the amygdala and hypothalamus have been shown to increase in activity in depressed patients. Additionally, the hippocampus has been shown to decrease in size in depressed patients. (Anand et al., 2005; Wang et al., 2012) These brain regions, along with the brainstem, will be analyzed in this thesis when animals are exposed to chronic stress.

Depressive-like behavior, in both humans and animal models, has been shown to be induced by Interleukin-1 (IL-1), a proinflammatory cytokine. In both depressed humans and animal models of depression, higher levels of proinflammatory cytokines were found in the blood (Raison et al., 2006). The increase in proinflammatory cytokines has been shown to modify NE metabolism and play a major role in mood disorders, especially depression (Dantzer et al., 1999). In medically ill patients, proinflammatory
cytokines are elevated and correlate with symptoms of sickness behavior (Capuron et al., 2004). Therefore, it has been found that medically ill patients have depression 5 to 10 times more often than the general population (Evans et al., 1999). Patients diagnosed with major depression, which not medically ill, have been shown to activate inflammatory pathways with increased expression of cytokines (Maes, 1999). Stress has long been known to be associated with the onset of depression leading many researchers to propose that stressor exposure may induce depression by the stimulation of inflammatory responses (Raison et al., 2006).

**Stress and Cytokines**

During times of stress IL-1 induction is due to the activation of the SNS and subsequent activation of beta-adrenergic receptors (β-ADR), specifically β2-ADRs. This activation causes the intracellular stimulation of cyclic adenosine monophosphate (cAMP), which acts in a Protein Kinase A-independent pathway. This pathway involves extracellular signal regulated kinase (ERK) and p38 signaling, inducing IL-1 production (Tan et al., 2007). Studies have demonstrated that NE binds to β-ADRs, inducing IL-1 production (Johnson et al., 2005; Johnson et al., 2008).

Alternatively, stimulation of the HPA axis during times of stress is known to suppress IL-1 induction. The HPA axis leads to the release of glucocorticoids, which bind to glucocorticoid receptors. The activation of glucocorticoid receptors leads to the suppression of IL-1 production (Amano et al., 1993; Goujon et al., 1995; Knudsen et al., 1987) (Figure 3).
The link between stress and cytokines is quite complex because in addition to the acute, transient effects of stress on cytokine production, stress exposure can also prime the immune system to future stimulation. Priming is the sensitization of a cell, which are microglia cells in this case. Microglia cells are known to produce and release cytokines in the brain (de Pablo et al., 2006). Acute stress enhances some features of the immune system when pathogens are administered after the stressor exposure, such as increasing levels of nitric oxide, a bactericidal mediator (Campisi et al., 2002), that facilitates recovery from bacteria-induced inflammation (Campisi et al., 2012). Additional studies administered lipopolysaccharide, a component in the cell wall of bacteria that binds to toll-like receptor 4 (TLR4) on microglia cells and causes an immune response, 24 hours to 4 days after tailshock stressor exposure and observed enhanced proinflammatory cytokines and sickness responses (Johnson et al., 2002; Wohleb et al., 2011; Frank et al., 2007). Studies have also shown this priming effect can be blocked by administrating a drug to block glucocorticoid receptors (Frank et al, 2010; Munhoz et al., 2006). This is due to high levels of glucocorticoids upregulating TLR4 expression. By blocking glucocorticoid receptors, TLR4 expression is no longer increased, and therefore, LPS cannot readily bind to TLR4.

The regulation of brain cytokines by NE and corticosterone may change following repeated stressor exposure. To date, the opposing regulation of brain cytokines by NE and corticosterone has only been investigated following acute stressor exposure, yet chronic stress is more highly associated with depression. Recent data from the laboratory of Dr. Johnson has shown that exposing animals to repeated stressors gradually increases
basal circulating corticosterone (Camp et al., 2012) and increases NE turnover at some
limbic areas (e.g. prefrontal cortex, hypothalamus, and amygdala) (Porterfield et al.,
2012) (Figure 4). How the changes in stress hormones alter brain cytokine levels is
unclear and will be investigated in the studies in this thesis.

**Hypothesis**

We hypothesize that chronic stressor exposure results in changes in the regulation
of brain cytokines that leads to greater cytokine production in the brain. To test this
hypothesis, we constructed three studies that will be thoroughly explained in later
sections.

*Study 1 Hypothesis:* We have hypothesized that chronic stress results in
sensitization of β-ADRs causing induction of IL-1 mRNA.

*Prediction:* To test this hypothesis we administered saline (as control),
propranolol (a β-ADR antagonist), or corticosterone block (metyrapone and
aminoglutethimide) and propranolol to control and chronically stressed animals, and
measured IL-1 mRNA. We predicted chronically stressed animals which received
propranolol would have significant decreases in IL-1 mRNA compared to non-stressed
control animals. Also, chronically stressed animals that received propranolol and
corticosterone block were predicted to have no changes in IL-1 mRNA levels compared
to non-stressed control animals because of the “ying-yang” relationship between the SNS
and HPA axis. With both the β-ADRs and corticosterone being blocked, IL-1 mRNA
levels would neither increase nor decrease.
**Study 2 Hypothesis:** We have hypothesized that chronic stress results in sensitization of glucocorticoid receptors causing suppression of IL-1 mRNA.

**Prediction:** To test this hypothesis we administered either saline (as control) or corticosterone block (metyrapone and aminogluthethimide) to control and chronically stressed animals, and measured brain IL-1 mRNA. We predicted chronically stressed animals would have significant increases in IL-1 mRNA compared to non-stressed control animals.

**Study 3 Hypothesis:** We have hypothesized that chronic stress primes inductions of IL-1 protein via the activation of β-ADRs.

**Prediction:** To test this hypothesis we administered either saline (as control) or isoproterenol, a beta-adrenergic receptor agonist, to non-stressed control and chronically stressed animals and measured brain IL-1 protein. We predicted chronically stressed animals would have primed cytokine production, causing significant increases in IL-1 compared to control animals.
Figure 1. Due to stress exposure, corticotrophin-releasing hormone (CRH) is secreted within the brain and onto the pituitary gland. In return, this stimulates the release of adrenocorticotropic hormone (ACTH). ACTH acts on the adrenal glands, releasing glucocorticoids (cortisol in humans and corticosterone in rats). Glucocorticoids inhibit further release of CRH and ACTH.
Norepinephrine (NE) is released by neurons of the locus coeruleus (LC), found in the brainstem. NE is released to brain regions of the limbic circuit, such as the prefrontal cortex, hypothalamus, hippocampus, and amygdala. Each of these brain regions are responsible for different responses and behaviors.

**Figure 2.** Norepinephrine (NE) is released by neurons of the locus coeruleus (LC), found in the brainstem. NE is released to brain regions of the limbic circuit, such as the prefrontal cortex, hypothalamus, hippocampus, and amygdala. Each of these brain regions are responsible for different responses and behaviors.
Acute Stressor Exposure

Figure 3. Acute stressor exposure stimulates both the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. The SNS results in the release of the norepinephrine (NE), which binds to beta-adrenergic receptors, and ultimately induces the release of IL-1. The HPA axis involves the release of glucocorticoids (cortisol or corticosterone), which binds to glucocorticoid receptors. This leads to the suppression of IL-1.
Chronic Stressor Exposure

Figure 4. Chronic stressor exposure also causes both the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis to be stimulated. However, unlike acute stressor exposure, there is a greater effect witnessed. Norepinephrine (NE) is released in great amounts, binding to beta-adrenergic receptors and causing a large induction of IL-1. At the same time, the HPA axis is causing glucocorticoids to be released in large amounts, binding to glucocorticoid receptors and suppressing IL-1.
CHAPTER III

MATERIALS AND METHOD

Animals

Adult male Fischer 344 rats (Harlan) were used for all studies in this investigation. Animals (200-275 g) were single housed in Plexiglas cages with food and water available ad libitum. All animals were housed in a pathogen-free area and with a 12 h light-dark cycle beginning at 07:00h. Rats were given 7 days to habituate to colony facility before experiments began. After habituation, animals were handled daily for 5 days. Protocols approved by Kent State University Institutional Animal Care and Use Committee were met for the care and use of the animals.

Chronic Stress Paradigm

Rats were exposed to stressors for four days, with two stressors daily. One stressor was held in the morning and one in the evening, except for day four which allowed for behavioral testing in the evening. On day one of the stress paradigm, rats were exposed to restraint stress for 1 h in a decapi-cone bag, followed by food deprivation for 15 h overnight. On day 2, rats were exposed to two (2s, 1.5mA) foot shocks in an operant box. Rats remained in the operant box for thirty minutes, followed by constant light conditions overnight. Day 3 consisted of exposing the rats to cage tilts (at 45°) for 3 h and wet bedding for 15h overnight. On day 4 rats were again exposed to
one (2s, 1.5mA) foot shock and remained in the operant box for 30min. Control, non-stressed, animals resided in home cages throughout the chronic stress paradigm.

**Tissue Collection**

All studies consisted of euthanizing animals on day five, 24 h after the last stressor. All brains were placed in a chilled matrix, which an plastic instrument that has slits throughout, 1mm apart. The matrix allows for dissections of consistent thickness of the brain regions. The prefrontal cortex, hypothalamus, amygdala, hippocampus, and brain stem were microdissected on a chilled glass plate. Olfactory bulbs were eliminated preceding the dissection of the medial prefrontal cortex from a 2 mm thick slice ranging from approximately 5 mm to 3 mm anterior of Bregma, where approximately 1 mm of tissue was dissected on each side of midline. Hypothalamus was isolated from a 2 mm think slice ranging from approximately -1.2 mm to -3.2 mm posterior of Bregma where the slice was further dissected in half at the top of each side of midline. The amygdala was collected out of the same anterior-posterior slice by removing the cortex from the tissue section remaining just lateral to the optic tracks. Hippocampus was isolated from a 4 mm thick section ranging from approximately -3.2 mm to -7.2 mm posterior of Bregma where the hippocampus was separated from cortex and underlying brain structures. Finally, the brainstem was isolated from the remaining tissue by removing the cerebellum and 5 mm section of brainstem was collected -9 mm to -14 mm from Bregma and sectioned in half to collect the dorsal side of brainstem that contains locus coeruleus and nucleus of the solitary tract neurons. Each brain section was placed in snap-cap
Eppendorf tubes and rapidly frozen in liquid nitrogen. Tubes were stored at -80°C until samples were processed.

**Drugs**

All pharmacological agents were purchased from Sigma-Aldrich and dissolved in sterile saline. Propranolol, a beta-adrenergic receptor antagonist, was dissolved at a concentration of 1 mg/ml and 10 mg/kg was administered per rat. Isoproterenol, a beta-adrenergic receptor agonist (induces IL-1 levels), was dissolved at a concentration of 1 mg/ml and 1 mg administered per rat. Isoproterenol, unlike the other drugs, is not administered per kg (weight) because it is administered directly into the cerebrospinal fluid, rather than peripherally. Metyrapone and aminoglutethimide, a corticosterone blocker, was dissolved at a concentration of 10 mg/ml and 100 mg/kg administered per rat. Metyrapone inhibits hydroxylation, which impairs the synthesis of cortisol/corticosterone. Aminoglutethimide inhibits two enzymes necessary for cortisol/corticosterone synthesis, the side chain cleavage enzyme and the enzyme aromatase. All doses of each drug were chosen based on prior studies in our laboratory (Johnson et al., 2005).

**Measurement of Cytokines**

Samples needed to measure protein cytokine levels were homogenized with Iscove’s solution containing 2% aprotinin (Sigma-Aldrich) was added in volume of 250 or 500 µl. Aprotinin inhibits proteases, preventing the break down of proteins and preserving the tissues. Tissues were homogenized using a sonic dismembrator (Fisher
Scientific). Samples were centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was extracted and used to measure cytokine levels. Protein cytokine levels were measured from tissue samples using commercially available Enzyme-linked immunosorbent assay (ELISA) (R&D systems). ELISAs use the binding of antibodies and color change to detect prevalence of a substrate. The ELISA was performed according to the manufacturer’s instructions, except tissues were incubated for 45 minutes in the substrate solution instead of the recommended 30 minute period to ensure complete incubation. Messenger RNA cytokine levels were measured by isolating RNA, creating copy DNA, and performing quantitative polymerase chain reaction (qPCR). RNA was isolated using USB PrepEase RNA Spin Kit, which involved homogenizing the tissues according to the manufacturer’s instructions. Instructions were followed accordingly, except columns were centrifuged an extra time to ensure samples were fully dry before analyzing the RNA for quality and quantity readings. In order to obtain readings of RNA quality, RNA samples were analyzed using a Nanodrop, at 260/280 and 260/230 ratios. All RNA samples were diluted to the same concentration to create cDNA. Diluted RNA samples were mixed with cDNA Master Mix (ABI High Capacity Kit) in separate RNA-free snap-cap Eppendorf tubes. CDNA Master Mix is comprised of reverse transcriptase and necessary proteins and primers to convert RNA to cDNA. 20µl of total solution (18µl of Master Mix and 2µl of RNA sample) was mixed by the use of a vortex and placed in a Thermo Cycler with the settings of a volume at 20µl cDNA. To perform quantitative PCR (qPCR), Master Mix solution was created by adding Master Mix, RNase free water, and the corresponding probe. Master mix solution (Agilent Technologies) was added at
an amount of 30µl per each sample into a snap-cap Eppendorf tube along with RNase free water (25µl per each sample) and probe (3µl per each sample). The solution was made in separate tubes for each of the necessary probes. GAPDH and IL-1 probes were used and 2µl of cDNA was added, creating 20µl triplets of master mix solution and cDNA. Samples were pipetted into a 96-well reaction plate, with 20µl per well, and plate was capped. The plate was tapped to remove bubbles and centrifuged at 4000rpm for one minute until all bubbles were gone. The plate was ran in qPCR machine (Agilent Technologies), using MxPro program. A qPCR machine amplifies and detects DNA by cycling through extremely hot and extremely cold temperatures. Furthermore, it is also capable of detecting the DNA by measuring fluorescence.

Procedure

Study 1: A study was performed which consisted of a control group and a chronically stressed group, which within both of these groups, rats (n=13-14 /group) received injections of either saline, propranolol, or propranolol, metyrapone and aminoglutethimide. Control animals remained in home cages during the course of the experiment until the fifth day. Chronically stressed animals were stressed using the 4-day stress paradigm described previously. On the fifth day, rats were injected with an intraperitoneal (IP) injection of saline, an IP injection 10 mg/kg propranolol or an IP injection of 10 mg/kg propranolol and a subcutaneous (SC) injection of 100 mg/kg metyrapone and aminoglutethimide. Two hours later, rats were decapitated and brain regions collected for measurement of cytokine mRNA. The time frame of 2h was used due to past studies, which showed this to be the best time period to measure cytokine
levels (Johnson et al., 2005). Brain regions collected were the amygdala, hypothalamus, and hippocampus. RNA was isolated, cDNA was made, and quantitative polymerase chain reaction (qPCR) was performed to measure IL-1 mRNA.

Study 2: A study was performed which consisted of a control group and a chronically stressed group, which within both of these groups, rats (n=19-20/group) received injections of either saline or metyrapone and aminoglutethimide. Control animals remained in home cages during the course of the experiment until the fifth day. Chronically stressed animals were stressed using the 4-day stress paradigm described previously. On the fifth day, rats were injected with either a SC injection of saline or a SC injection of 100 mg/kg metyrapone and aminoglutethimide. Two hours later, rats were decapitated and brain regions collected for measurement of cytokine mRNA. Brain regions collected were the amygdala, hypothalamus, and hippocampus. RNA was isolated, cDNA was made, and qPCR was performed to measure IL-1 mRNA.

Study 3: A study was performed which consisted of a control group and a chronically stressed group, which within both of these groups, rats (n=15-17/group) received injections of either saline or isoproterenol. Control animals remained in home cages during the course of the experiment until the fifth day. Chronically stressed animals were stressed using the 4-day stress paradigm described previously. On the fifth day, rats were injected with either an intra-cistera magna (ICM) injection of saline or an ICM injection of 1 mg isoproterenol. Two hours later, rats were decapitated and brain regions collected for measurement of cytokine proteins. Brain regions collected were the
amygdala, hypothalamus, hippocampus, prefrontal cortex, and brainstem. ELISA kit assay (R&D systems) was performed to measure IL-1 protein.

**Statistics**

Data from each experiment was statistically analyzed. Data from the experiment examining the effects of propranolol, or propranolol, metyrapone and aminoglutethimide on cytokine mRNA levels was analyzed using a 2-way ANOVA between stress treatment (Control vs. Chronically Stressed) and drug treatment (Vehicle vs. Propranolol vs. Propranolol, metyrapone and aminoglutethimide). Post hoc analysis was performed by using a one-way ANOVA. Data from the experiment examining the effects of metyrapone and aminoglutethimide on cytokine mRNA levels was analyzed using a 2-way ANOVA between stress treatment (Control vs. Chronically Stressed) and drug treatment (Saline vs. Metyrapone and aminoglutethimide). Post hoc analysis was performed by using a Tukey test. Data from the experiment examining the effects of isoproterenol on cytokine protein levels was analyzed using a 2-way ANOVA between stress treatment (Control vs. Chronically Stressed) and drug treatment (Saline vs. Isoproterenol). In all experiments p<0.05 was used for the level of confidence for acceptance of significance.
CHAPTER IV

RESULTS

Role of β-adrenergic Receptors with the Induction of IL-1 mRNA

Study 1 aimed to determine the β-adrenergic receptors’ role in the induction of IL-1 mRNA levels when animals are exposed to chronic stress. Animals were exposed to a 4-day chronic stress paradigm. On the fifth day, animals were administered saline, propranolol, or propranolol + corticosterone block (Prop + CB). Propranolol is a β-adrenergic receptor antagonist and was administered via intraperitoneal (IP) injections. Blockade of corticosterone production was achieved via the subcutaneous injections of metyrapone and aminogluthethimide. To ensure the corticosterone was blocked, a corticosterone assay (Enzo Life Sciences) was performed on plasma samples of all rats. Rats were decapitated two hours after injections and brain regions were collected for brain cytokine mRNA measurements. Figures 5A, 5B, and 5C present fold change of IL-1 mRNA compared to controls in the amygdala, hippocampus, and hypothalamus, respectively. As previously reported, chronic stress over a 4-day period did not significantly alter IL-1 mRNA expression in any brain area compared to controls as demonstrated in saline injected animals. Administration of propranolol had no effect in control animals; however, it significantly decreased IL-1 mRNA expression in chronically stressed animals in the amygdala and hippocampus. A significant interaction between stress and drug treatment was observed in both the amygdala \[F (2, 26) = 7.384; p = 0.003\] (Fig. 5A) and hippocampus \[F (2, 33) = 5.599; p = 0.008\] (Fig. 5B) with
additional analyses revealing a significant decrease in IL-1 mRNA expression following propranolol treatment in animals exposed to stress but not in control animals. The decrease in IL-1 mRNA in the amygdala and hippocampus following propranolol administration in stressed animals was blocked by administration of metyrapone and aminoglutethimide as IL-1 mRNA values in Prop + CB injected animals was similar to saline injected controls.

**Role of Glucocorticoid Receptors with the Effect on IL-1 mRNA**

Study 2 aimed to determine the role of glucocorticoid receptors in the suppression of IL-1 mRNA levels after chronic stress exposure. Animals were exposed to a 4-day chronic stress paradigm. On the fifth day, animals were administered metyrapone and aminoglutethimide, a corticosterone blocker (CB). Animals were administered saline or CB via subcutaneous injections two hours prior to decapitation. Brain regions were collected for brain cytokine mRNA measurement. Figures 6A, 6B, and 6C present fold change of IL-1 mRNA compared to controls in the amygdala, hippocampus, and hypothalamus, respectively. Again, chronic stress over a 4-day period did not significantly alter IL-1 mRNA expression in any brain area compared to controls as demonstrated in saline injected animals. No significant interaction between CB and stress found; however, a significant main effect of the drug was observed with further analysis. Further analysis was performed by conducting a 2 x 2 ANOVA, and revealed a significant main effect of metyrapone and aminoglutethimide, in all brain regions, amygdala (p = 0.001) (Fig. 6A), hypothalamus (p = 0.002) (Fig. 6C), and the hippocampus (p = 0.007) (Fig. 6B).
Effect of β-adrenergic Receptor Stimulation on IL-1

Lastly, study 3 aimed to determine the effect of stimulating β-adrenergic receptors and analyze possible priming of IL-1 production. Animals were exposed to a 4-day chronic stress paradigm. On the fifth day, animals were administered saline or isoproterenol, a β-adrenergic receptor agonist, into the cisterna magna (ICM). Two hours after injections, animals were decapitated and brain regions were collected for analysis of brain cytokine protein levels. Figures 7A, 7B, 7C, 7D, and 7E present IL-1 protein measurements in the amygdala, hippocampus, hypothalamus, prefrontal cortex and brain stem respectively. Overall, no changes in basal IL-1 production followed chronic stress exposure while isoproterenol administration increased IL-1 mRNA in all animals. A 2 x 2 ANOVA revealed a significant main effect of the drug, isoproterenol, in four brain regions; amygdala [F (2, 44) = 13.352; p = 0.000], hypothalamus [F (2, 43) = 40.071; p = 0.000], prefrontal cortex [F (2, 45) = 37.752; p = 0.000], and brain stem [F (2, 42) = 15.036; p = 0.000]. More importantly, with the administration of isoproterenol, a significant increase in IL-1 production in stressed animals compared to controls was observed in both the amygdala [F (2, 44) = 4.456; p = 0.017] (Fig. 7A) and hypothalamus [F (2, 43) = 4.551; p = 0.016] (Fig. 7C). However, IL-1 levels of chronically stressed animals were equal to IL-1 levels in control animals in the other brain regions (Figures 7B, 7D, and 7E).
Figure 5. Role of beta-adrenergic receptors with the induction of mRNA IL-1 after chronic stressor exposure. After 4 days of stress, on day 5, rats were injected with saline, 10mg/kg propranolol (Prop), or 100mg/kg corticosterone block/propranolol (CB + Prop). Figures represent IL-1 mRNA fold change from control +/- SEM in the (A) Amygdala, (B) Hippocampus, and (C) Hypothalamus. * Represents significant difference (p < 0.05) between propranolol-injected stress animals and saline-injected stress animals. ** Represents significant difference (p < 0.05) between propranolol-injected stress animals and propranolol-injected control animals. # Represents significant difference (p < 0.05) between propranolol-injected stress animals and corticosterone block/propranolol-injected stress animals.
Figure 6. Role of glucocorticoid receptors with the effects on IL-1 mRNA after chronic stressor exposure. On day 5, rats were injected with either saline, or 100mg/kg corticosterone block (CB). Figures represent IL-1 mRNA fold change from control +/-SEM in the (A) Amygdala, (B) Hippocampus, and (C) Hypothalamus. * Represents significant main effect of drug (p < 0.05).
Figure 7. Effect of beta-adrenergic receptor stimulation on IL-1 after chronic stressor exposure. On day 5, rats were injected with either saline or 1mg isoproterenol. Figures represent mean IL-1 values +/- SEM in the (A) Amygdala, (B) Hippocampus, (C) Hypothalamus, (D) Prefrontal Cortex, and (E) Brain stem. * Represents significant main effect of drug (p < 0.05). ** Represents significant difference (p < 0.05) between isoproterenol-injected stress animals and saline-injected stress animals.
CHAPTER V

DISCUSSION

The studies presented examined the hypothesis that chronic stressor exposure results in changes in the regulation of brain cytokines that leads to greater cytokine production in the brain. In order to test this hypothesis, we constructed three studies. Specifically, each study concentrated on a different aspect of brain cytokine regulation.

Study 1 analyzed the role of β-adrenergic receptors with the induction of IL-1 mRNA after chronic stress exposure. We administered propranolol to chronically stressed and control animals and found that administration of propranolol, a β-adrenergic receptor antagonist, resulted in significant decreases in chronically stressed animals in the amygdala and hippocampus, compared to non-stressed controls. This decrease in IL-1 mRNA was not found in control animals, compared to chronically stressed animals, due to control animals having less corticosterone because they were not stressed. Furthermore, non-stressed animals did not exhibit significant decreases in IL-1 mRNA levels due to However, administration of drugs that prevented corticosterone synthesis (i.e. metyrapone and aminoglutethimide) completely prevented the decrease in IL-1 mRNA levels in stressed animals. Taken together, these data suggest stimulation of β-ADR is necessary for the stimulation of IL-1 mRNA. Also, these data suggest that propranolol may be blocking the induction of IL-1 mRNA by corticosterone block, attenuating IL-1 mRNA levels. Findings by other laboratories have found similar data, showing stimulation of β-ADR alone stimulates cytokine production (Tan et al., 2007). Similarly,
our laboratory, in the past, has shown propranolol to block cytokine production with an immune stimulus (Johnson et al., 2008).

Study 2 analyzed the role of glucocorticoid receptors with the effect on IL-1 mRNA after chronic stress exposure. We administered corticosterone block (i.e. metyrapone and aminogluthimide) to chronically stressed and control animals and found that administration of corticosterone block resulted in increased IL-1 mRNA levels found in both control and chronically stressed animals. Further analysis also showed a main effect of the drug. While we predicted to find the stress animals to solely have an increase in IL-1 mRNA, previous studies can explain this finding. Previously, our laboratory showed animals that are chronically stressed have a decrease in β-ADR expression (Porterfield et al., 2012). This decrease in β-ADR expression may explain why no difference in IL-1 mRNA levels is shown between stressed and non-stressed animals. With β-ADR expression decreasing, a large induction in IL-1 mRNA levels, compared to controls, is not expected in stressed animals.

Study 3 analyzed the effect of β-ADR stimulation on IL-1 after chronic stress. To test this relationship we administered isoproterenol to chronically stressed and control animals and found control animals had slight increases in IL-1 levels; however, stressed animals showed a significant increase in IL-1 levels in the amygdala and hypothalamus. Further analysis also showed a main effect of the drug. Importantly, until now previous data has yet to show regional changes in β-ADR signaling that favors greater brain cytokine production.
In the past, only widespread priming has been noted due to an immune stimulus. Numerous studies have found microglia cells, cells which produce IL-1 (de Pablo et al., 2006), to be primed by immunogens, such as lipopolysaccharide (LPS). LPS is a component in the cell wall of gram negative bacteria which binds to toll-like receptor 4 on microglia cells. This induces an immune response, causing the microglia cells to produce and release IL-1. One study involved acutely stressing animals, by a session of inescapable tailshock, and administrating LPS 24 hours later (Johnson et al., 2002), followed by measuring cytokines. This study showed a higher increase in cytokine levels of animals that received LPS and were stressed, compared to animals that were solely stressed. Another study acutely stressed animals and isolated microglia cells. 24 hours after stress exposure, microglia cells were administered LPS ex vivo and cytokine levels were measured (Frank et al., 2007). This study ensured the microglia cells themselves were being primed when exposed to LPS, an immune stimulus.

Other studies have also shown similar data, where cytokine levels are primed by an immune stimulus after stress exposure. My study, with the administration of isoproterenol, differed by priming the microglia via a β-ADR agonist, which stimulates the effect stress has on β-ADRs, rather than administrating an immune stimulus, such as LPS. This data also suggests this priming effect takes place due to stressor exposure and not solely exposure to immunogens. Also, previous studies have only looked at widespread cytokine levels, but this study looked at levels in five brain regions.

Brain cytokines, particularly IL-1, are believed to cause depression. While the mechanism is unclear, the macrophage hypothesis persists and is readily supported.
Although the mechanism, whereby stress stimulates β-ADR-mediated IL-1 induction is unclear, the data presented here supports this theory and provides further information. Our data indicates stimulating and blocking β-ADRs results in changes of IL-1 levels in the brain, such as decreases or increases in levels of mRNA or protein. More importantly, there are changes in the levels of IL-1 mRNA indicating chronic stress may alter different aspects of transcription, the process that converts DNA into RNA. Our data also shows chronic stress primes IL-1 in certain brain regions indicating depression may be a result of priming. Furthermore, it is important to note that the reasons for the regional differences are not yet known. These regional differences may be due to anatomy or other factors that have yet to be examined. While acute stress is relatively understood, chronic stress and its effects on β-ADRs and IL-1 levels have barely been studied.

To better understand this relationship, future studies may consist of examining the intracellular mechanisms of IL-1 production, specifically looking at cyclic adenosine monophosphate (cAMP) levels. CAMP is a key component in the intracellular mechanism of IL-1 production within a microglia cell. We expect to see cAMP levels to increase while IL-1 levels increase as well. To carry out this future study, we plan to isolate and examine microglia cells, which are known to produce IL-1 in the brain.
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