NEURAL MECHANISMS UNDERLYING STRESS-INDUCED DEPRESSION AND ITS PREVENTION

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

by

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May, 2011
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ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Dr. John Johnson for the opportunity to work on this project and his guidance throughout its course. Without his patience, endless proofreading, corrections and encouragement, writing this thesis would have not been possible. I would like to thank Dr. Veronica Porterfield and the graduate students of the Johnson lab – Zachary Zimomra and Jennifer Remus, for being excellent resources. I would especially like to thank Rob Camp for teaching me all the techniques we used in the laboratory, answering my questions and helping me expand my knowledge in the area of study.

I would like to thank Dr. Doug Kline, Dr. Bansidhar Datta, Dr. Manfred Van Dulmen and Dr. John Johnson for spending their valuable time reading this thesis and serving as committee members.

Last but not the least; I would like to thank Simon Evangelista for all the support, encouragement and having faith in me when I had none.
CHAPTER I

INTRODUCTION

Project Overview

Psychological and physical stressors stimulate a neurotransmitter in the brain, called norepinephrine, which induces the release of certain chemicals, called cytokines, from the immune system. Cytokines are seen at the beginning of infection and are also implicated in the etiology of depression. Current antidepressants increase the levels of norepinephrine in the brain and oftentimes lead to worsening of depressive symptoms during early stages of treatment. This project focuses on preventing stress-induced depression by blocking the action of the norepinephrine that lead to immune system activation, by using a drug called propranolol.

Stress

Stress is an integral part of modern society. Exposure to mild stress leads to the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous systems, the two components of the body involved in facilitating the physiological responses necessary for fight or flight. The stress response is thought to have evolved to facilitate temporary fight-or-flight reactions (Sapolsky, 1992). The stress response is considered to promote adaptive coping by causing a shift in the body’s homeostasis. Acute stressors can be a source of motivation, but long-term exposure to stress can lead to impaired judgment, poor learning and memory and it can affect the quality of life. After prolonged exposure to chronic stress, animals experience reductions in food and water intake (Weiss & Simpson, 1968) as well as reduction in activities such as social interaction (Short & Maier, 1993) and exploration (van Dijken, Van der Heyden, Mos, & Tilders, 1992). Reductions in these behaviors are comparable to sickness behaviors exhibited at the time of infections and depression. Stress is thought to be a precursor for depression (Brown, 1993) and...
most episodes of depression are often preceded by an identifiable stressor (American Psychiatric Association, 1994; Kessler, 1997). Exposure to chronic stress and its association with depression can be seen in soldiers, of which 25% suffer from major depression and about 20% develop Post-Traumatic Stress Disorder (PTSD) (Tanielian & Jaycox, 2009).

**HPA Axis and Autonomic Nervous System**

Stress activates two primary physiological systems, the HPA Axis (Goshen et al., 2009) and the sympathetic nervous system. These two entities are involved in production of energy for defensive purposes necessary for survival or response to a stressor. The hypothalamus controls and regulates secretion of hormones from the pituitary gland. During times of stress, neurons in the hypothalamus release corticotrophin-releasing hormone (CRH) which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal glands to produce glucocorticoids that help release energy by the breakdown of stored energy via glucogenesis, gluconeogenesis and the breakdown of proteins, fats and lipids to react appropriately to the stressor [Figure 1] (Maier & Watkins, 1998). Energy liberation is not the only function of glucocorticoids. These hormones play a role in the regulation of several processes such as apoptosis, neurogenesis, and synaptic plasticity (Abraham, Harkany, Horvath, & Luiten, 2001). Reduction in neurogenesis and synaptic plasticity in the hippocampus due to the action of glucocorticoids is implicated in the etiology of depression in some studies (Goshen et al., 2008).

The sympathetic and parasympathetic nervous systems maintain a balance critical for homeostasis of physiological functions, and are controlled by neurons located in the hypothalamus. During a fight-or-flight response, the sympathetic nervous system (SNS) is activated which leads to increased heart rate and contractility, increased rate of breathing, vasodilation and increased blood flow to muscles, increased gluconeogenesis, lipolysis; all of which prepare the body for fight or flight. Norepinephrine is also released as a neurotransmitter in the central nervous system where its primary role involves regulation of arousal and
adaptation to internal and external stressors (Goddard et al., 2010). It acts on neurons with adrenergic receptors that are found in brain areas such as the locus coeruleus, hypothalamus, hippocampus and the amygdala (Fugge, Van, & Mijnster, 2004; Pacak & Palkovits, 2008). Stress responses, memory encoding, sleep/arousal, and fear-conditioning are mediated by these areas. Norepinephrine plays a critical role in stress responses by stimulating neurons in the hypothalamus. Neurons from the ventrolateral medulla and the nucleus of solitary tract directly innervate the paraventricular nucleus of the hypothalamus which is the seat of stress responses (Sawchenko & Swanson, 1982; Sawchenko, Li, & Ericsson, 2000). This stimulation activates the HPA axis to secrete glucocorticoids (cortisol in humans and corticosterone in laboratory rats) (Pacak et al., 2008). The effects of norepinephrine are mediated by several subtypes of adrenergic receptors. The α1, α2 and β receptors are most important in mediating modulatory activities of norepinephrine. α1 and β receptors mediate the post-synaptic effects of norepinephrine (Murugaiah & O’Donnell, 1995) whereas α2 receptors act as pre-synaptic autoreceptors which inhibit norepinephrine release by feedback inhibition.

Depression

Depression and stress show a strong correlation. Depressive disorders such as major depression are important health problems which not only affect those suffering from them, but also the families and well-wishers of the individuals (Sartorius, 2001). With current lifetime prevalence between 4.4% to 20% (Bakish, 2001), major depression is the leading cause of disability (WHO, 2008) in the United States affecting about 6.7% of the American population (Kessler et al., 2009). Depression is often associated with decreased quality of life, increased risk of suicide, loss of productivity, increased health care use, increased risk of drug abuse and dependence, and is often a doorway to other psychological disorders. The Diagnostic and Statistical Manual of Mental Disorders, 4th edition, (American Psychiatric Association, 2000) requires five out of nine symptoms of the following to be presented in the same two week period in order for an individual to
be clinically diagnosed with Major Depressive Disorder. The symptoms are as follows:

1. Depressed mood for prolonged periods in adults and irritable mood in children and adolescents.
2. Marked decrease in interest and/or pleasure in everyday activities.
5. Psychomotor agitation or retardation.
6. Loss of energy or fatigue.
7. Inappropriate guilt or feelings of worthlessness.
8. Diminished ability to think or concentrate.

A combination of the above symptoms can have a disabling effect on psychosocial functioning (Papakostas, Peterson, Mahal, Mischoulon, Nierenberg, & Fava, 2004). The most deleterious effect of depressive disorders is suicide, which accounted for 90% of the 33,000 deaths due to suicide in the United States in 2006 (Centers for Disease Control and Prevention).

There are several biochemical mechanisms which contribute to depression especially alterations in the levels of central neurotransmitters. Thus, most pharmaceutical treatments for depression target neurotransmitters and normalize behavior (Martindale, 2009).

**Antidepressants**

There are several classes of antidepressants which target different aspects of the nervous system. These are classified into first generation antidepressants, which include Tricyclic antidepressants (TCAs) and Monoamine Oxidase Inhibitors (MAOIs); and second generation antidepressants, which include Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin-Norepinephrine Reuptake Inhibitors (SNRIs). All of these antidepressants increase monoaminergic transmissions, primarily serotonin and norepinephrine (Meyer et al., 2006; Schildkraut & Kety, 1967). Extensive studies have shown that these antidepressants
alleviate depressive symptoms for only a third of the patients (Entsuah, Huang, & Thase, 2001; Kennedy, Andersen, & Thase, 2009; Cipriani et al., 2009; Hansen, Gartlehner, Lohr, Gaynes, & Carey, 2005; Machado, Iskedijian, Ruiz, & Einarson, 2006). There is a delay of about two weeks before any therapeutic benefits of these antidepressants are observed, and the maximum improvements of depressive symptoms are often seen only after an average of 6 weeks (Richelson, 1994). The mechanism behind their success is thought to lie in the downregulation of β-adrenergic receptors due to elevated norepinephrine levels (Hosoda et al., 1993; Seo et al., 1999). Glucocorticoid receptor antagonists such as Mifepristone have also been shown to function as effective antidepressants (Wulsin, Herman, & Solomon, 2010) again suggesting a potential role of glucocorticoids in mediating depression via increasing apoptosis and impairing neurogenesis in the hippocampus.

**Depression and Cytokines**

Pro-inflammatory cytokines have been implicated in the etiology of depression. Pro-inflammatory cytokines, such as IL-1, IL-6, Tumor Necrosis Factors α and β, are low molecular weight proteins that are seen at the beginning of an infection and are necessary for recruitment of other immune cells. Most cytokines are named interleukins (ILs) indicating they are secreted by the leukocytes.

During infections, sickness behaviors are mediated by cytokines; these include increased sleep, anorexia, fatigue, decreased libido, decreased social interaction, and exploration (Kent, Bluthe, Kelley, & Dantzer, 1992). In order to mediate such behavioral changes, cytokines must communicate to the central nervous system. Direct communication to the brain can be ruled out as cytokines are lipophobic molecules that do not cross the blood brain barrier (BBB). Instead, it is thought that they influence the neuroendocrine regulations of the brain critical for changes in behavior by communicating with the brain in areas where the BBB is absent (Saper & Breder, 1994), such as the circumventricular organs, or via the induction of molecules that cross the BBB, such as prostaglandins (Van Dam, Brown, Man-
Cytokines can also affect afferent vagal fibers and transmit signals to the NTS which in turn can affect other brain areas such as the paraventricular nucleus (PVN) of the hypothalamus (Maier et al., 1998). Receptors specific to Interleukin-1 are densely distributed in the hippocampus (Cunningham & De Souza, 1993), as well as on glial cells (Ban, Sarlieve, & Haour, 1993) and neurons (Takao, Tracey, Mitchell, & DeSouza, 1990).

Interleukin 1 (IL-1) produced by the microglia in the brain plays an important role in neuroendocrine regulation. IL-1 has an extended family of proteins (Sims et al., 2002); IL-1α, IL-1β and IL-1 receptor antagonist (IL-1RA), IL-1rI, IL-1rII. IL-1RA binds to the receptors but does not trigger a signal, hence blocking the effects of IL-1 signaling (Eisenberg et al., 1990). The different receptors present (Loddick, Liu, Takao, Hashimoto, & DeSouza, 1998) are the IL-1r1 (transmembrane receptors that provides intracellular signaling) and IL-1r2 (decoy receptor that binds to IL-1 but does not produce a signal and hence antagonizes the effects of IL-1) (Colotta, 1993; Dinarello & Thompson, 1991).

The following studies associate pro-inflammatory cytokines with depression; (i) Medical conditions that are characterized by inflammation, especially that of the brain, report high incidences of depression (Yirimiya, 1997; Dantzer, Wollman, Vitkovic, & Yirmiya, 1999; Schiepers, Wichers, & Maes, 2005). (ii) Patients with Major or Minor depression show elevated levels of inflammatory markers which correlate with severity, duration, and age of disease onset (Hayley, Poulter, Merali, & Anisman, 2005; Levine, 1999; Anisman, Ravindran, Griffiths, & Merali 1999). (iii) Patients suffering from cancer or Hepatitis C also suffer from depression as a result of cytokine therapy, which is often reversed with antidepressants treatment (Musselman et al., 2001; Capuron & Miller, 2004). (iv) Genetic association between genes of inflammatory factors including polymorphisms of the IL-1 gene family, and the severity and responsiveness of the depression to antidepressants (Yu, Chen, Hong, Chen, & Tsai, 2003). (v) Onset of depressive mood in healthy subjects following experimental exposure to immune challenges (Reichenberg et al., 2001).
Cytokines have similar depressive effects in animals. Exposure to an immune challenge, or peripheral/central administration of IL-1 lead to depressive symptoms in rats (Yirimiya, 1997; Yirimiya, 1996), which can be attenuated by treatment with antidepressants (Yirimiya et al., 2001; Castanon, Bluthe, & Dantzer, 2001) or by pretreatment with IL-1RA (Pollak & Yirmiya, 2002).

**Stress, Cytokines and Depression**

Stressful situations have been shown to induce the expression and production of both central, as well as peripheral, IL-1 (Heinz et al., 2003). Stress-induced IL-1 has many behavioral and endocrinal effects. (i) Elevation of IL-1 in the hippocampus results in memory impairment (Goshen et al., 2007; Goshen et al., 2008). (ii) Brain IL-1 mediates cortisol secretion by hyperactivation of the HPA axis as well as suppression of hippocampal neurogenesis which are implicated in the etiology of depression (Goshen et al., 2008). Thus, researchers have proposed that exposure to stressful life events may lead to the onset of depression via induction of brain cytokines, particularly IL-1.

In animal models, stress can increase IL-1 production in brain areas such as the hypothalamus, hippocampus, brain stem, and the frontal cortex (Shintani et al., 1995; Nguyen et al., 1998; Deak, Bellamy, & D'Agostino, 2003; O'Connor et al., 2003). The increase in IL-1 in the hypothalamus activates the HPA axis through interactions with catecholaminergic projections from the nucleus of solitary tract and the locus coeruleus by the neurotransmitter norepinephrine (Ericsson, Kovacs, & Sawchenko, 1994; Buller, Xu, Dayas, & Day, 2001). Ablations of the noradrenergic projections from the locus coeruleus prevent stress-induced IL-1β productions (Johnson et al., 2005) suggesting a role of norepinephrine in regulating brain IL-1 production. Further studies have shown that administration of desipramine, a noradrenergic reuptake inhibitor, facilitates production of IL-1 during times of stress (Blandino, Barnum, & Deak, 2006). Administration of a β-agonist, such as isoproterenol, is sufficient in inducing secretion of
IL-1 in the absence of stress (Johnson, Cortez, Kennedy, Foley, Hanson III, & Fleshner, 2008). Administration of propranolol, a β-adrenergic receptor antagonist that prevents excessive binding of norepinephrine to its receptors [chemical structure in Figure 3], blocks stress-induced IL-1β production when administered prior to tailshock stress (Johnson et al., 2005). Therefore, norepinephrine secreted as a result of a stressor exposure is thought to stimulate IL-1 production via β-adrenergic receptors (Johnson et al., 2005).

Upon entering Johnson’s laboratory I participated in a study to examine the effects of chronic stress on brain cytokine production. Chronic mild stress was shown to produce a decrease in sucrose preference, exploratory behavior and social interaction [Figure 3] and an increase in IL-1β mRNA in the hippocampus. I proposed that propranolol, by blocking the action of norepinephrine on β-adrenergic receptors and thus preventing the induction of IL-1 in the hippocampus, could potentially be used in the blockade of the behavioral changes seen in stressed animals.
CHAPTER II

HYPOTHESIS

Administration of propranolol will prevent stress-induced depression by blocking central norepinephrine acting on β-adrenergic receptor, hence preventing the induction of IL-1β in the hypothalamus and hippocampus.

AIMS

**AIM 1:** To determine if propranolol prevents depressive symptoms in animals exposed to chronic mild stress.

**AIM 2:** To study the effects of propranolol on the HPA axis response to stress to determine the role of the glucocorticoids in depression.

**AIM 3:** To study the effects of propranolol on the induction of IL-1β, subunits of IL-1 (IL-1R1, IL-1R2) and other pro-inflammatory factors (IL-6, COX-2,) following exposure to chronic mild stress.

**AIM 4:** To determine physiological doses of the drug required for effectively preventing depressive symptoms.
CHAPTER III

METHODS

Subjects

Subjects were 40 adult male Fisher 344 rats (225-300g; Harlan, Inc., Indianapolis, IN, USA). Animal colonies were maintained in a facility with a 12 hour light/dark cycle with lights on at 7:00h. Each animal was housed individually in a Plexiglas cage with access to food and water ad libitum. The animals were allowed to habituate to the facility for one week and were handled briefly for 2-3 minutes for five days the following week in order to reduce stress to the animals. Baseline weights and baseline sucrose preference were tested prior to the surgeries. All experimental procedures, care, and use of the animals were in accordance with the Institutional Animal Care and Use Committee at Kent State University.

β-Adrenergic Receptor Antagonist Treatment

Osmotic mini-pumps (Alzet, Cupertino, CA, USA) were surgically implanted in anesthetized animals using inhalant isoflurane dosed to effect. The dorsal area was shaved and an incision (size of the mini-pump diameter) was made between the scapulae. The mini-pumps were implanted subcutaneously. These osmotic mini-pumps delivered saline or Propranolol (Sigma-Aldrich) in sterile, endotoxin free saline, heated to dissolve. Propranolol, a non-selective β-adrenergic receptor antagonist that readily crosses the blood-brain barrier, was delivered at the rates of 0, 0.5, 2, or 10 mg/day. Post-surgery, the subjects were housed singly and allowed to recover for four days before baseline behavior testing and sucrose preference.

Stress Procedures

The animals were divided into five groups. Two groups received 0mg of Propranolol – one serving as the non-stressed controls and the other serving as the stressed controls. The three other groups received 0.5mg, 2mg and 10mg of
Propranolol. These three groups, as well as the 0mg stressed group, were exposed to chronic stressors paradigm for 4 days. The other 0mg group served as the undisturbed, non-stressed control and these animals were left undisturbed in their home cages. The chronic stress paradigm (Willner et al., 1997, Willner et al., 2005) consists of exposing the animals to a variety of different stressors each day. In this study the following stressors were used to produce depressive symptoms: Day 1: Restraint in DecapiCones (Bioseb) for an hour prior to which tail vein blood was collected for baseline corticosterone readings. Blood samples were collected 30 minutes and 60 minutes into the restraint stress to assess the amount of glucocorticoids present following acute restraint stress. Blood samples were also collected from the non-stressed controls at 30 minutes and 60 minutes. Food was deprived that night. Day 2: The animals were exposed to two footshocks that were two minutes apart and left in the operant box for 30 minutes for fear conditioning. They were left in constant light the following night. Day 3: The cages were tilted at 45 degrees for a 3 hour period following which 1 liter of water was added to the cages. The animals were exposed to the wet bedding for a period of 20 hours. Day 4: The animals were re-exposed to operant box for 30 minutes with 2 footshocks. These animals as well as the controls were given a sucrose preference test that night [Figure 4].

Behavioral Testing

Behavior testing consisted of sucrose preference test, open field exploration for ten minutes, and social interaction with juvenile for five minutes. For the sucrose preference test, animals were given a choice between water and 1% sucrose solution overnight. Baseline readings were taken four days after surgical implantation of osmotic mini-pumps and post-stress readings were taken 24 hours after the last stressor. A reduction in sucrose preference indicated anhedonia (Willner, 1997). Sucrose preference was calculated as a percent of sucrose consumption out of total drinking volume. For the open field exploration and social interaction, animals were placed in new Plexiglas cages and allowed to move
freely for 10 minutes. Followed immediately was the social interaction test in which a juvenile male rat was placed in each cage for five minutes. Each session was videotaped and scored at a later time. The time spent in exploration, was calculated by the animal moving, standing up on its hind limbs or sniffing its surrounding. Time spent in social investigation marked by anogenital sniffing (contact between the nose of the mouse and the pup). All animals were sacrificed 24 hours after the post-stress behavioral assessment.

**Brain Tissue, Peripheral Organs and Blood Collection**

The animals were decapitated and trunk blood and brain areas were collected. The trunk blood from each animal was collected in two 10ml EDTA-vacutainer tubes (BD PharMingen, Franklin Lakes, NJ, USA) – one for measurement of cytokines and the other containing glutathione (Sigma G-4251) for measurement of catecholamines (glutathione reduced oxidation of catecholamines). Each sample was kept on ice until it was ready to be centrifuged. After centrifugation at 10000 rpm for 10 minutes, 1ml of plasma was collected from the vacutainers and stored in microfuge tubes at -20ºC until time for the assay. The hypothalami and hippocampi were isolated by free hand dissections on a frosted plate kept on crushed ice. The samples were stored separately in microfuge tubes and frozen immediately in liquid nitrogen and stored in -80ºC until they were ready to be processed. The left and right adrenal glands as well as the spleen were harvested, weighed and discarded.

**Brain Tissue Processing and mRNA Extraction**

The hypothalami and hippocampi samples were thawed briefly and mRNA was extracted using the PrepEase™ RNA Spin Kit (Affymetrix / USB). 350ul of RA1 buffer and 3.5 μl b-Mercaptoethanol was added to the thawed sample and the tissue was dissociated using a sonic dismembrator (Fisher Scientific). The dissociated sample was transferred onto the purple PrepEase filter unit and centrifuged at 11000xg for one minute. The filter unit was discarded and the sample was prepared for RNA binding by adding 350ul of 70% Ethanol to the flow through and mixed by vortexing. The sample was transferred to the light blue
PrepEase RNA Spin Column to bind the RNA and centrifuged at 8000xg for 30 seconds. In order to allow for greater efficiency of DNA digestion the membrane was desalted by adding 350ul of Membrane Desalting Buffer and was centrifuged at 11000xg for one minute. To digest the DNA, 95ul of rDNase reaction mixture was added directly over the column and allowed to incubate for 15 minutes at room temperature (the DNase Reaction Mixture was prepared by mixing 10ul of rDNase stock solution and 90ul of DNase Reaction Buffer). The column was then washed and dried as follows: the column was first washed with 200 μl of RA2 Buffer and dried by centrifugation at 8000xg for 30 seconds. After placing the column in a new collecting tube it was washed with 600ul of RA3 Buffer and dried at 8000xg for 30 seconds. The column was placed in a new collecting tube and the last wash was with 250ul of RA3 Buffer and the column was dried by centrifugation at 11,000xg for 3 minutes. The samples were eluted with RNase-free water - 30ul was added directly over the columns containing the hypothalami samples and 60ul was added to the hippocampi containing columns. The samples were incubated at room temperature for one minute and were then centrifuged at 11000xg for one minute. RNA concentration was measured with a spectrometer using the relationship 1 O.D. = 40ug/ml. The samples were stored at -80ºC until ready to be used.

Measurement of Cytokines

The mRNA samples from the brain tissue were converted to cDNA by using the High Capacity RNA-to-cDNA Master Mix (Applied BioSystems). 2ul of 100ng/ul mRNA template (diluted with Nuclease-free water from initial concentration) was added to 18ul of the Master Mix and the mixture was put into a thermal cycler set for 20ul according to cycles specified by the manufacturer. qPCR was performed using the plate reader, using a universal master mix and gene expression probes (IL-1β, IL-6, COX-2, IL-1R1, IL-1R2 and GAPDH). The samples were assayed in triplicate and Livak and Schmittgen’s 2-ΔΔCT (Livak & Schmittgen, 2001) was used for analysis.
Measurement of circulating Epinephrine

Circulating epinephrine was analyzed by ELISA (Rocky Mountain Diagnostics, BA 10-1500) for which the samples were diluted at 1:10. The assay was run according to the manufacturer’s recommendations.

Measurement of Plasma Corticosterone

The amounts of circulating glucocorticoids were assessed by Enzyme Immunoassay (Enzo). Plasma samples from the trunk blood were allowed to thaw and were diluted at the ratio 1:50 with the dilutant and the steroid binding proteins displaced by heat treatment in a water bath instead of using the Steroid Displacement Reagent included in the kit (interferes with the assay which contains IgG). The assay was run according to the manufacturer’s recommendations. The standards were run in duplicates and the samples were run in individual wells. The plates were read at optical density of 405nm with correction between 570 and 590nm.

Statistics

A one way analysis of variance (ANOVA) was used to assess changes in each group’s weights, sucrose preference, open field exploration, social interaction, and brain cytokines with Least Significant Differences to determine where differences lie between groups. Repeated measures ANOVA was performed to analyze corticosterone levels from trunk blood samples collected by repeated tail-bleeds. DeltaGraph was used to plot the results.
CHAPTER IV

RESULTS

Effects of Propranolol and Chronic Mild Stress on Behavior

To determine the potential effects of propranolol on behavior, baseline exploration, social interaction, and sucrose preference were assessed four days after osmotic minipump implantation. All animals showed high levels of sucrose preference, exploratory behavior and social interaction regardless of propranolol treatment. A one-way ANOVA between treatment groups revealed no significant differences in baseline behavior between groups in exploration[F(4,39)=0.805, p=0.530], social interaction[F(4,39)=0.209, p=0.932], and sucrose preference [F(4,39)=0.137, p=0.967] (Data not shown).

Four days of chronic mild stress, resulted significant differences between treatment groups in exploration [F (4,39)=5.716, p=0.001] and social interaction[F(4,39)= 3.554, p= 0.016]. Post-hoc analyses revealed that there was a significant decrease in exploration in the 0mg-stress (p=0.001), 0.5mg-stress (p=0.012), and 10mg-stress (p<=0.001) groups compared to the non-stressed control animals, but there were no differences between the non-stressed controls and 2mg-stress group (p=0.216) [Figure 5A]. Post-hoc analyses also revealed that there was a significant decrease in interaction with a juvenile in the 0mg-stress group (p=0.001) compared to the non-stressed controls. There were no differences in social interaction between the non-stressed controls and 0.5mg-stress (p= 0.227), 2mg-stress (p= 0.616) and 10mg-stress (p=0.148) groups [Figure 5B].

Although there were no significant differences between groups for sucrose preference [F(4,39) = 2.238, p=0.085], the apriori hypothesis that chronic stress would decrease sucrose preference and propranolol would block this decrease was tested by running post-hoc analyses. These analyses revealed a significant difference between the non-stressed
controls and 0mg-stress animals (p=0.013) and 0.5mg-stress animals (p=0.021), but no
difference between the 2mg-stress (p=0.135) and 10mg-stress (p=0.269) groups [Figure 5C].

**Effects of Propranolol on Corticosterone Levels on Exposure to Acute Stress**

Animals were restrained for one hour and tail vein blood samples were collected
at 0, 30 and 60 minutes to tests the effects of stress and propranolol on acute stress-
induced glucocorticoids production. Repeated measure analysis of variance revealed
a significant effect of corticosterone across time [F(1,38)= 6.019, p=0.019]. Individual
one-way analysis of variances and post-hoc tests were run at each time-point to
examine the differences between the groups. At zero minutes, there were no significant
differences between the non-stressed control and the stressed groups [F(5,39) = 1.290,
p= 0.288]. At 30 minutes, there was a significant increase in the amount of corticosterone
[F(5,36)=8.790, p<= 0.001] between the non-stressed controls and all the stressed groups
(p<=0.001 each). Similarly, at 60 minutes, there was a significant increase in the amount
of corticosterone [F(5,32)=9.761, p<=0.001] in the stressed animals as compared to the
non-stressed controls (p<= 0.001 each). Propranolol had no effect on corticosterone
levels at either 30 or 60 minutes as compared to the 0mg stress animals [Figure 6A].

**Effects of Propranolol and Chronic Stress on Stress Hormones**

To determine the effects of propranolol on changes in HPA axis regulation
following chronic stress, trunk blood was collected 48 hours after the last stessor
for the measurement of basal corticosterone levels. A one-way ANOVA between
treatment groups revealed no significant differences between the different groups
in basal corticosterone levels [F(4,38) = 2.019, p=0.114] [Figure 6B]. A one-way
analysis of variance between the control group and all the stressed groups, revealed
a significant effect of stress on the levels of corticosterone [F(1,38)=6.019,p=0.019].

Trunk blood was collected 48 hours after the last stessor and the levels of
catecholamines present were assessed to determine the role of propranolol on circulating
epinephrine. Again, a one-way ANOVA between treatment groups revealed no significant differences between groups in the levels of epinephrine \[F(4,35)=2.366, p=0.074\] [Figure 8]. A one-way analysis of variance between the control group and all the stressed groups revealed a significant increase in the levels of epinephrine as a result of stress \[F(1,35)=4.525, p=0.041\].

**Effects of Propranolol and Chronic Stress on Organ and Body Weight**

Exposure to chronic stress often leads to loss in body weight, hypertrophy of the adrenal glands, and spleen atrophy. To test the effects of propranolol on these three indicators of stress, body weights of the animals were measured 24 hours after the last stressor and the spleens and the adrenal glands were harvested and weighed 48 hours after the last stressor. A one-way ANOVA between treatment groups revealed significant differences between the pre-treatment and post-stress body weight \[F(4,35) = 5.457, p=0.002\], adrenal gland weight \[F(4,39)=3.512, p=0.016\], and spleen weight \[F(4,39)= 7.338, p<=0.001\]. An increase in the combined adrenal glands weight was observed between the non-stressed control group and the 0mg stressed group \(p=0.013\), 0.5mg stressed group \(p=0.024\), 2mg stressed group \(p=0.004\), and the 10mg stressed group \(p=0.005\) [Figure 7A]. Post-hoc analyses revealed a significant decrease in body weight between the non-stressed control group and the 0mg stressed group \(p=0.005\), 0.5mg stressed group \(p=0.001\), 2mg stressed group \(p<=0.001\), and the 10mg stressed group \(p=0.004\) [Figure 7B]. There was a decrease in spleen weight between the non-stressed controls and the 0mg-stress \(p=0.014\) and 0.5mg-stress \(p=0.015\) groups, no difference in spleen weight between the non-stressed controls and the 2mg stressed group \(p=0.907\), and an increase in spleen weight between the non-stressed controls and the 10mg stressed group \(p=0.024\) [Figure 7C].

**Effects of Propranolol and Chronic Stress on Cytokines in the Hippocampus and Hypothalamus**

To test the hypothesis that propranolol will block the induction of cytokines, brain regions were harvested 48 hours after the last stressor and the amount of cytokine
mRNA present in the samples was determined. A one-way ANOVA between treatment groups revealed no differences between groups in IL-1 [F(4,28) = 1.165, p=0.351], IL-1R1 [F(4,28) = 0.484, p=0.748], IL-1R2 [F(4,28) = 0.245, p=0.910], IL-6 [F(4,28) = 0.135, p=0.968], and COX-2 [F(4,28)=0.877, p=0.492] mRNA levels in the hypothalamus [Figure 10 (A-E)]. Neither were differences were observed between treatment groups in IL-1R1 [F(4,28) = 1.876, p=0.147], IL-1R2 [F(4,28) = 2.475, p=0.071], IL-6 [F(4,28) =0.192, p=0.940], or COX-2 [F(4,28)=0.839, p=0.514] mRNA levels in the hippocampus except in the levels of IL-1 [F(4,28)=5.706, p=0.002] [Figure 9 (B-E)]. Post-hoc analyses revealed no differences between the non-stressed controls and the 0mg-stress (0.37), 0.5mg-stress (0.286), and the 2mg-stress (0.152) groups, but there was a significant increase between the non-stressed control group and the 10mg-stressed group (0.001) [Figure 9A].
CHAPTER V
DISCUSSION

Summary of Results

The aim of this study was to block the β-ADR during exposure to stress, assess its effects on depressive symptoms induced by stress, and its effect on the physiological response of the body to the stressors. Propranolol blocked depressive behavior in animals exposed to chronic stress without affecting the HPA responses generated by stress.

Primary Findings

Propranolol, β-ADR antagonist, blocked the onset of depressive behavior in animals exposed to chronic mild stress. Blocking the β-ADR during stressor exposure has been shown to block the induction of IL-1 in the brain (Johnson et al., 2005). Hence, these results are consistent with our hypothesis that propranolol may prevent stress induced depression by blockade of cytokine induction by norepinephrine. The effectiveness of the drug depends on its dosage. 2mg propranolol appears to be the most effective dose to block the decrease in exploration, decrease in interaction with juvenile was blocked by 0.5mg, 2mg and 10mg doses of propranolol, and decreased sucrose preference is blocked by 2mg and 10mg doses of propranolol. These dose dependent variations could be due to the varying quantities of β-ADR present in different brain regions or ability of propranolol to diffuse to different brain regions, which could help in explaining the variability of the effectiveness of the drug on different behavioral measures. It is known that noradrenergic neurons are found in small clusters in the brainstem, groups A1 to A2. These groups project their axons on different areas of the brain such as the hypothalamus, hippocampus, prefrontal cortex, amgydala, thalamus and the spinal cord (Flugge, Van, & Mijnster, 2004; Pacak et al., 2008). These areas specialize in the type of functions they perform and depending on
the amount of noradrenergic innervations, depressive behavior associated with functions of these brain areas could be affected differently. The highest dose of 10mg failed to block the effects of stress on exploratory behavior. This could be attributed to the fact that basal levels of norepinephrine are crucial for proper signaling and the high dose might be most effective in blocking more of the receptors impairing basal signaling. Administration of the 10mg dose may also result in upregulation of β-ADR increasing the sensitivity of the receptor, thereby more easily stimulating IL-1. This upregulation often leads to increased sensitivity of the receptor to the neurotransmitter and could produce an exaggerated IL-1 induction in selective brain areas resulting in depressive behaviors that appear more severe.

Results presented here defy current antidepressant treatment strategies which increase the amount of norepinephrine between synapses. For example, MAOIs increase the amount of norepinephrine and other monoamines, such as serotonin and dopamine, available in synaptic clefts by preventing their metabolism. SNRIs inhibit the reuptake of norepinephrine which increase the extracellular concentrations of the neurotransmitters. The success of these antidepressants has been proposed to be due to the downregulation of the β-ADR as a result of elevated norepinephrine levels. Such downregulations are seen with various receptors in an effort to maintain homeostasis. Moreover, the average period of time required for these antidepressants to effectively alleviate depressive behavior correlates with the time required to downregulate β-ADR from the cell surfaces. Our theory could explain why depressive symptoms get worse following acute treatment with these antidepressants. The hypothesis presented in this study that increase in levels of norepinephrine lead to depression by increased production of cytokines effectively explains the initial worsening of depressive symptoms in patients taking SNRIs or MAOIs. Although these antidepressants initially increase the levels of norepinephrine between the synapses, they are effective nonetheless. The increased levels of the neurotransmitter lead to a downregulation of β-ADR and hence a decreased induction of brain cytokines following chronic antidepressant administration.
HPA axis dysregulation might not be a regulator of depressed mood, as propranolol does not block the production of glucocorticoids. Glucocorticoids play an important role in energy liberation during stressful situations by glucogenesis, gluconeogenesis, and glycolysis. Liberation of energy is the beneficial effect of glucocorticoids but long-term exposure to glucocorticoids in the brain leads to premature apoptosis of neurons, decreased neurogenesis, and decreased synaptic plasticity that many researchers have proposed play a critical role in the etiology of depression (Goshen et al., 2008). This study showed that propranolol did not interfere in the HPA axis response to stress, yet it was successful at preventing the development of depressive symptoms. This suggests that glucocorticoids do not play a role in the etiology of depression. Chronic stress requires a constant availability of glucocorticoids, and to meet this demand the adrenal glands hypertrophy and produce an increased amount of these steroids. Adrenal hypertrophy was observed in this study. Propranolol does not affect the change in body weight as a response of to stressors. Spleen weight was affected as propranolol has been previously shown to block catecholamine mediated stress-induced reduction in spleen weight (Johnson et al., 2005).

The amount of cytokine mRNA present in the tissue sample varied from previous studies. While propranolol is known to block the induction of IL-1 (Johnson et al., 2005), there were no increased levels of cytokines observed in the 0mg stressed group as was predicted. This could be attributed to the mRNA turnover rate in the brain areas. In previous studies, tissues were harvested 24 hours after the last stressor and there were marked increases in cytokine levels hippocampus. In this study, tissues were harvested 48 hours after the last stressor and there were no significant changes in cytokine mRNA transcription. The change in timeline from the previous study is likely responsible for the discrepancy in the results.

**Implications**

The current study has extended past research by assessing the effects of a β-adrenergic receptor blocker on preventing stress-induced depression. The success
of this study was due to the blockade of elevated levels of norepinephrine acting on these receptors. This study contradicts the mechanism of current antidepressant therapies and could be a more effective way to prevent depression. Treatment with propranolol should not have worsening of depressive symptoms as seen with the SNRIs and MAOIs as the behavioral assessments after the osmotic pump implantation showed normal levels of exploration, social interaction, and sucrose preference.

**Limitations of the Study**

The mechanism by which propranolol prevents stress-induced depression could be centrally or peripherally mediated. Although we hypothesized that the mechanism of action of propranolol was by blocking central β-ADR, propranolol is known to block changes in heart rate and hypertension (Vachakis et al., 1980). The role of peripheral afferent signaling was not studied in this project. Also, the study did not assess the effects of propranolol and the unaltered levels of the glucocorticoids on hippocampal neurogenesis. If changes in hippocampal neurogenesis are critical for depressive symptomology, then propranolol should block these changes. This would imply that norepinephrine, in addition to glucocorticoids, plays a role in the suppression of hippocampal neurogenesis.

**Future Projects**

In future studies, it will be important to assess the effects of propranolol on the levels of cytokines. A study, similar to the current study, except where the animals are sacrificed 24 hours after the last stressor, will have to be done to assess the levels of IL-1 in the brain regions with pre-treatment with propranolol. The effects of propranolol on hippocampal neurogenesis and the role of hippocampal neurogenesis itself in the etiology of stress-induced depression will have to be examined. Also, examining the effects of blocking peripheral β-ADR will be necessary in identifying the mechanism of action of propranolol.
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Figure 1: Effects of Stress on the HPA Axis. During psychological and physical stressors, neurons from the hypothalamus release CRH which acts on the pituitary and induced the secretion of ACTH. ACTH acts on the adrenal glands and stimulates the secretion of glucocorticoids which have been thought to play a role in the reduction synaptic plasticity, suppression of neurogenesis, and an increase in apoptosis.
**Figure 2:** Results from previous study. Administration of 4 days of chronic mild stress produced depressive symptoms such as decreased sucrose preference, exploration and interaction with juvenile. A significant increase in the level IL-1β was seen in the hippocampus 24 hours after the last stressor.
Figure 3: Chemical Structure of Propranolol.
Figure 4: Timeline of present study (adapted from Dr. J. D. Johnson). Animals were implanted with osmotic mini-pumps and baseline behavior was assessed after four days. Four days of chronic mild stress was administered to the animals as shown above or animals were left undisturbed in home-cages. Post-stress behavior was assessed 24 hours after the last stressor and animals were sacrificed 24 hours later. Trunk blood, hippocampi and hypothalami were collected and processed at a later time. The adrenal glands and the spleen were weighed.
Figure 5A: Effects of β-adrenergic receptor blockade on post-stress exploratory behavior. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg propranolol and were left undisturbed in home cages. Behavior was assessed 24 hours after the last stressor. Chronic mild stress significantly reduced exploration time in stressed groups receiving 0mg, 0.5mg and 10mg doses of propranolol. 2mg dose of propranolol blocked stressed-induced decreases in exploration. * represents significant (p<0.05) difference compared with non-stressed control group.
Figure 5B: Effects of β-adrenergic receptor blockade on post-stress interaction with juvenile. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Behavior was assessed 24 hours after the last stressor. Chronic mild stress significantly reduced interaction time in stressed groups receiving 0mg of propranolol. 0.5mg, 2mg and 10mg doses of propranolol blocked stressed-induced decreases in interaction. * represents significant (p<0.05) difference compared with non-stressed control group.
Figure 5C: Effects of β-adrenergic receptor blockade on post-stress sucrose preference. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Behavior was assessed 24 hours after the last stressor. Chronic mild stress significantly reduced sucrose preference in stressed groups receiving 0mg and 0.5mg doses of propranolol. 2mg and 10mg doses of propranolol blocked stressed-induced decreases in sucrose preference. * represents significant (p<0.05) difference compared with non-stressed control group.
Effects of Propranolol and Chronic Stress on HPA Responses

**Figure 6A:** Effects of β-adrenergic receptor blockade on corticosterone levels following acute stressor. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. One hour restraint stress was administered on day 1 of the chronic mild stress paradigm. Blood samples from tail veins were taken at 0, 30 and 60 minutes. Chronic mild stress significantly increased the amount of corticosterone in blood samples taken at 30 and 60 minutes in all the stressed groups. Propranolol does not affect the corticosterone response upon exposure to acute stressor.
**Figure 6B:** Effects of β-adrenergic receptor blockade on basal corticosterone levels. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Trunk blood samples were collected 48 hours after the last stressor. Chronic mild stress significantly increased the levels of basal corticosterone in trunk blood samples. Propranolol does not block the effect of chronic mild stress on basal corticosterone levels. * represents significant (p<0.05) difference compared with non-stressed control group.
Figure 7A: Effects of β-adrenergic receptor blockade on combined adrenal weights. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Chronic mild stress significantly increased the adrenal weights in all the stressed group. Propranolol does not block the effect of chronic mild stress on adrenal hypertrophy. * represents significant (p<0.05) difference compared with non-stressed control group.
Figure 7B: Effects of β-adrenergic receptor blockade on change in body weight. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Chronic mild stress significantly decreased change in body weight in all stressed groups. * represents significant (p<0.05) difference compared with non-stressed control group.
Figure 7C: Effects of β-adrenergic receptor blockade on change in spleen weight. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Chronic mild stress significantly decreased spleen weight in groups receiving 0mg, 0.5mg and 10mg doses of propranolol. * represents significant (p<0.05) difference compared with non-stressed control group.
Effects of Propranolol and Chronic Stress on Circulating Epinephrine

**Figure 8:** Effects of β-adrenergic receptor blockade on circulating epinephrine levels. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Trunk blood samples were collected 48 hours after the last stressor. Chronic mild stress significantly increased the levels of circulating epinephrine in trunk blood samples in groups receiving 0.5mg and 10mg doses of propranolol. Significant (p<0.05) difference compared with non-stressed control group.
Effects of Propranolol and Chronic Stress on Cytokines in the Hippocampus

A.  

B.  

C.  

D.  

![Graphs showing mRNA fold over for hippocampus IL-1, IL-1R1, IL-1R2, and IL-6]
Figure 9 (A-E): Effects of β-adrenergic receptor blockade on cytokine mRNA in hippocampus. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Hippocampi were collected 48 hours after the last stressor and processed for levels of pro-inflammatory cytokines. There was no effect of stress or propranolol on hippocampal IL-1R1, IL-1R2, IL-6 and COX-2 mRNA. There was an increase in hippocampal IL-1 in the 10mg stressed groups, but all other groups showed no significant differences from the control groups in IL-1 mRNA levels. * represents significant (p<0.05) difference compared with non-stressed control group.
Effects of Propranolol and Chronic Stress on Cytokines in the Hypothalamus

A.  

B.  

Hypothalamus IL-1 mRNA

Hypothalamus IL-1R1

C.  

D.  

Hypothalamus IL-1R2 mRNA

Hypothalamus IL-6 mRNA
Figure 10 (A-E): Effects of β-adrenergic receptor blockade on cytokine mRNA in hypothalamus. Subcutaneous osmotic mini-pumps with different doses (0mg, 0.5mg, 2mg and 10mg) of propranolol were surgically implanted in rats one week prior to administration of chronic mild stress. Control animals received 0mg of propranolol and were left undisturbed in home cages. Hypothalami were collected 48 hours after the last stressor and processed for levels of pro-inflammatory cytokines. There was no effect of stress or propranolol on hypothalamic IL-1, IL-1R1, IL-1R2, IL-6 and COX-2 mRNA. * represents significant (p<0.05) difference compared with non-stressed control group.