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# Pharmaceutical and Natural (Exercise) Mechanisms to Mitigate the Negative Impact of PTSD and Chronic Stress on Synaptic Plasticity and Memory

Roxanne M. Miller  
*Brigham Young University*

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Pharmaceutical and Natural (Exercise) Mechanisms to Mitigate the  
Negative Impact of PTSD and Chronic Stress on  
Synaptic Plasticity and Memory

Roxanne M. Miller

A dissertation submitted to the faculty of  
Brigham Young University  
in partial fulfilment of the requirements for the degree of  
Doctor of Philosophy

Jeffrey G. Edwards, Chair  
Jonathan J. Wisco  
Michael D. Brown  
David D. Busath  
Christopher B. Kirwan

Department of Physiology and Developmental Biology  
Brigham Young University

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## ABSTRACT

### Pharmaceutical and Natural (Exercise) Mechanisms to Mitigate the Negative Impact of PTSD and Chronic Stress on Synaptic Plasticity and Memory

Roxanne M. Miller

Department of Physiology and Developmental Biology, BYU  
Doctor of Philosophy

Synapses can be altered due to experiences in a process called synaptic plasticity, which causes memory formations. Synapses can be strengthened through methods known as long-term potentiation (LTP) or weakened through long-term depression (LTD). Stresses can cause changes by altering synapses through either LTP or LTD. Rats were used to study the effects of post-traumatic stress disorder (PTSD)-like symptoms and a prophylactic treatment using pharmaceuticals. The first model used was the single prolonged stress (SPS) with two weeks of chronic light, which was not as effective for causing changes in synaptic plasticity. The second model, seven days of social defeat (SD) with two weeks of chronic light was more effective at inducing PTSD-like behavior symptoms and causing changes in LTP levels in the ventral hippocampus, amygdala, and prefrontal cortex between stressed and non-stressed rats. For the prophylactic treatment, propranolol and mifepristone were administered one week prior to and throughout the two weeks of the social defeat protocol. The drugs were able to prevent the changes due to stress on LTP in the three aforementioned brain regions, but did not change the anxious behavior of the rats. An enzyme-linked immunosorbent assay (ELISA) was used to determine corticosterone and norepinephrine levels between the different groups of rats. No significant differences were detected between SD and control rats, but SD injected rats were different from controls indicating that the injections were causing added stress. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was used to detect changes in the adrenergic, corticoid, AMPA, and NMDA receptors. There were a few significant changes to some of the targets indicating that the stress protocol and drugs were having an effect on the mRNA expression. Propranolol and mifepristone could possibly be used as a prophylactic treatment for traumatic stress. In a separate study, techniques were used to determine the negative effects chronic stress (non-PTSD-like) has on synaptic plasticity in the dorsal hippocampus and to show how exercise was able to mitigate some of those negative stress effects. Electrophysiology showed differences in LTP between four groups of mice: sedentary no stress (SNS), sedentary with stress (SWS), exercise with stress (EWS), and exercise no stress (ENS). SWS had the lowest amount of LTP, whereas ENS had the highest. SNS and EWS had similar levels of LTP, which were in between the SWS and ENS groups. Corticosterone blood levels measured by an ELISA showed significant increases in the stressed groups compared to the non-stressed groups. The radial arm maze showed that both groups of exercise mice made fewer reference memory errors the second week of testing compared to the sedentary groups. RT-qPCR determined that brain-derived neurotrophic factor (BDNF) and corticoid and dopamine 5 receptors were likely causing some of the memory changes.

Key words: chronic stress, LTP, LTD, corticoid receptor, dopamine receptor, adrenergic receptor, exercise, propranolol, mifepristone, BDNF, hippocampus, amygdala, prefrontal cortex

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## CHAPTER 1: Introduction

Stress is unavoidable and often a part of everyday life. Whether it is physical or emotional stress, it has an effect on the body, especially the brain. Chronic stress alters synaptic plasticity in the hippocampus, prefrontal cortex (PFC), and amygdala (1). The hippocampus is important for the consolidation of memory and encodes context for fear memories (2). The PFC helps determine how a person feels about a memory, whether or not it is an important memory to keep, and is implicated in other long term memory processes (2, 3). The amygdala is responsible for fear conditioned learning and involved with memory consolidation (2, 3). All three of these brain regions have connections to each other directly or through the temporal lobes (2-4). These three brain regions communicate with the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is responsible for controlling the release of glucocorticoids, norepinephrine, epinephrine, mineralocorticoids, and other stress related hormones. In response to a stressful experience, the hypothalamus releases corticotropin-releasing hormone (CRH). CRH then acts on the anterior pituitary causing it to release adrenocorticotrophic hormone (ACTH). ACTH then travels through the blood and acts on the adrenal cortex causing the production and release of glucocorticoids (mainly cortisol in humans and corticosterone in rodents) and mineralocorticoids (5). The sympathetic nervous system is connected to the HPA axis and the amygdala and can regulate release of stress hormones as well by acting on the adrenal gland medullas, which produce and release epinephrine and norepinephrine into the bloodstream (6). Norepinephrine also acts as a neurotransmitter in the central nervous system. Norepinephrine can alter synaptic plasticity (7) and so can glucocorticoids (8-11). Since synaptic plasticity is the scientific explanation behind learning and memory, understanding how stress affects it could lead to methods by which the deleterious effects of stress on memory can be counteracted.

Synaptic plasticity can be characterized by one of two events: a change in neurotransmitter release at the presynaptic neuron or a change in excitability of the postsynaptic neuron (12). In both instances there are many different receptor types, classified as ionotropic or metabotropic, which are located both pre- and postsynaptically and can be responsible for the alterations at different synapses. If the alteration reduces neurotransmitter release or reduces excitability of a neuron, then it is said to be long term depression (LTD), and if it increases either process then it is said to be long term potentiation (LTP) (13). The two receptor types responsible for the postsynaptic plasticity are most often N-Methyl-D-Aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (13). When glutamate is released from the presynaptic neuron, it binds to both AMPA and NMDA receptors. AMPA receptors allow sodium ions to flow into the cell, which depolarizes the membrane and removes the magnesium block from NMDA receptors. After the magnesium block is removed, the NMDA receptors allow calcium ions to flow into the cell (13). NMDA receptors are often called coincidence detectors, meaning that both pre- and postsynaptic events are required, which is most often the release of glutamate from the presynaptic neuron and depolarization of the postsynaptic neuron by AMPA receptors, for them to let calcium into the cell. Normally the intracellular concentration of calcium is very low allowing for calcium to be an important second messenger in signaling pathways. When NMDA receptors let calcium into the cell, the calcium binds to calcium/calmodulin-dependent protein kinase II (CaMKII). Once CaMKII is activated by calcium, one of its actions leads to the insertion of more AMPA receptors into the cell membrane resulting in LTP, which strengthens the synapse (13). The insertion of more AMPA receptors means that the postsynaptic neuron will be more easily excitable in the future. This is implicated in memory because it means that when this neural pathway is stimulated again, it does

not require as strong of a signal as the first time to bring that memory to the forefront of cognition, which can be called remembering. Metabotropic receptors, which often times are G protein-coupled receptors (GPCRs), when found presynaptically tend to alter neurotransmitter release, but the ones located postsynaptically often work in conjunction with AMPA and NMDA receptors (13). Binding with a ligand activates the GPCR, which leads to the activation of adenylyl cyclase and an increase in intracellular cAMP levels, which leads to an increase in protein kinase A (PKA) activity as well. PKA can phosphorylate the C terminus of the GluA1 subunit of AMPA receptors increasing the likelihood of the open state of the receptor meaning it allows more sodium ions to flow into the cell through the channel, thereby increasing conductance (14). All adrenergic receptors are GPCRs so they could possibly be working through PKA to cause changes to synaptic plasticity after stress has occurred. Corticoid receptors are nuclear receptors and can alter gene transcription, which then could affect the expression levels of AMPA and NMDA receptors or other proteins involved in synaptic plasticity. Understanding how synaptic plasticity is altered due to stress through the activation of various receptors that bind stress hormones will elucidate ways that pharmaceuticals (drugs) and exercise can be used to treat memory alterations related to traumatic and chronic stress.

### Specific Aim 1

After determining the best traumatic stress inducing protocol, it was elucidated that the unique combination of propranolol and mifepristone were a viable prophylactic treatment for preventing changes in synaptic plasticity due to traumatic stress.

- 1) Single prolonged stress (SPS) with chronic light was compared to social defeat (SD) with chronic light and it was determined that SD was better at causing anxiety and depression-like behaviors and was the better model for inducing traumatic stress.

Electrophysiology techniques were used to identify if there were any alterations in synaptic plasticity, such as an enhancement of LTP, in selected regions of the hippocampus, amygdala, and PFC in PTSD rats compared to non-PTSD (control) rats.

- 2) Electrophysiology techniques were used to demonstrate the use of pharmacology, specifically propranolol, a beta adrenergic receptor antagonist, and mifepristone, a glucocorticoid and progesterone receptor antagonist, as an effective prophylactic treatment for traumatic stress.
- 3) Specific receptor mRNA expression patterns in PTSD targeted brain regions were researched using RT-qPCR comparing PTSD rats, controls, and stressed rats injected with drugs.

#### Specific Aim 2

Exercise is an effective way to alter synaptic plasticity and improve memory by recovering the attenuated LTP caused by chronic stress and was able to decrease memory errors through molecular alterations.

- 1) Using electrophysiology, it was demonstrated that exercise can enhance LTP and reverse the negative effects on synaptic plasticity caused by stress.
- 2) Using the radial arm maze, it was shown that the spatial memory was enhanced in mice that exercise when compared to mice that are stressed.
- 3) Using RT-qPCR, it was elucidated which stress and exercise pathway targets were altered due to stress and exercise.

## CHAPTER 2: Determining the Best Traumatic Stress Protocol in Rats and a Possible Prophylactic Treatment

### Abstract

Post-traumatic stress disorder (PTSD) is a complex anxiety and depression disorder that affects about 1 out of 4 individuals that have gone through a very stressful and traumatic experience. The most common people that get PTSD are domestic violence/rape victims and veterans of war. What the majority of PTSD victims have in common are episodes of acute stress on top of chronic stress. Good PTSD models incorporate both components. The two models we looked at were single prolonged stress (SPS) and social defeat (SD) (both used with 2 weeks of chronic light). Electrophysiology data show that the SD protocol is better at causing a more traumatic stress experience than the SPS protocol since SD caused significant physiological changes in the ventral hippocampus and SPS did not. Both the SD and SPS protocols were able to cause more anxious behavior when compared to controls, but SD caused more dramatic changes indicating it is more of a traumatic stress experience than SPS. SD causes a significant increase in (long-term potentiation) LTP in the ventral hippocampus, lateral amygdala, and the prelimbic medial prefrontal cortex. Propranolol and mifepristone are able to significantly decrease LTP in the hippocampus, amygdala, and prefrontal cortex of SD rats when compared to SD rats that received drug. Significant alterations in the mRNA expression levels of glucocorticoid and mineralocorticoid receptors, beta 3 adrenergic receptor, AMPAR subunits 1 and 2, and NMDAR subunits 2A and 2B between the controls, SD, and SD with drug rats were observed in the ventral hippocampus and lateral amygdala. Overall, our data suggest that propranolol and mifepristone together may be a viable prophylactic pharmaceutical treatment for PTSD; when taken during a stressful, traumatic experience they could possibly prevent the onset of PTSD altogether.

## Introduction

The three brain regions that have been implicated in the etiology of post-traumatic stress disorder (PTSD) are the amygdala, prefrontal cortex (PFC), and hippocampus (2). A very complex network that is not fully understood connects these three regions together. These three areas can be directly connected or indirectly through the parahippocampal gyrus, and perirhinal and entorhinal cortices, which are all part of the temporal lobe. The anterior part of the parahippocampal gyrus is the perirhinal and entorhinal cortices. In humans and rodents, the perirhinal and entorhinal (layer III) cortices project to the distal CA1 pyramidal cells in the hippocampus (3). Hippocampal CA1 neurons also project back to layer V of the entorhinal cortex (3). Layer II of the entorhinal cortex projects to the CA3 and dentate gyrus regions of the hippocampus. The perirhinal cortex and layer V of entorhinal cortex also have projections to the PFC (3). The ventral portion of the anterior cingulate cortex of the ventromedial PFC connects to the amygdala (3). The ventromedial PFC normally inhibits the fear responses coming from the amygdala and hippocampus, but somehow in PTSD subjects the control the PFC exhibits over the these areas is altered (4). The ventral hippocampus has been shown to have connections with the amygdala and is important in fear memories. The lateral and anterior basolateral amygdala areas have glutamatergic connections to the ventromedial temporal and frontal lobes (15) and glutamatergic neurons are the primary neurons that undergo synaptic plasticity. All the glutamatergic neurons in this complicated network of neural connections have synaptic plasticity potential, which means they can be modified by stress. Since the hippocampus, amygdala, and prefrontal cortex are all connected, alterations in synaptic plasticity due to stress in one area could affect the other two areas.

Stress can have various effects on neural function, including altering synaptic plasticity. Events that invoke a strong emotional response, like those that cause PTSD, tend to result in abnormally strong memories due to the sympathetic nervous system (SNS). The adrenal glands release both catecholamines and corticosteroids which then act on adrenergic, mineralocorticoid, and glucocorticoid (corticoid will be used to describe both receptor types) receptors, respectively, in the amygdala (16), hippocampus (17), and the PFC (18), which leads to enhanced long-term potentiation (LTP) in all three of those regions. All adrenergic receptor types, which bind norepinephrine and epinephrine, are g-protein coupled receptors (GPCRs) that are important in PTSD because they potentially modulate synaptic plasticity through phosphorylation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by protein kinase-A (PKA), which increases sodium conductance into the cell. The other receptors that are important in the onset of PTSD are corticoid receptors, which bind corticosteroids like cortisol (corticosterone in rodents) and are not GPCRs, but are located in the cytosol and translocate to the nucleus to increase transcription of proteins like AMPA and N-methyl-D-aspartic acid (NMDA) receptors. Chronic stress (the type experienced in everyday life) tends to lead to prolonged corticoid activation, which leads to dorsal hippocampal CA1 LTD and reduced LTP, affecting spatial memory, and is independent of adrenergic receptor activation (19-21). Traumatic events that trigger PTSD tend to be acute episodes on a background of chronic stress, meaning that the sympathetic nervous system has a high baseline of activity, that spikes when an acute traumatic event is occurring, which causes the release of both catecholamines and corticosteroids. Adrenergic and corticoid receptor activity have been shown to be dependent on each other (17, 22, 23). However, over time these memories tend to fade and are harder to retrieve in normal subjects, which is probably due to the inhibition of the excitatory pyramidal



cells in the basolateral amygdala upon second exposure to corticosteroids (24). The first time the basolateral amygdala is exposed to corticosteroids, an enhancement in LTP is observed, but if these enhanced neurons are exposed a second time to corticosteroids, then the LTP enhancement first observed is abolished (24). In patients with PTSD, the memory of the trauma tends to remain just as strong as the first day the memory was formed (25). Stress hormones activate the PFC as well as the amygdala and hippocampus which is an implication that they strengthen traumatic memories (18, 26). The American Psychiatric Society states that PTSD develops after an extremely emotional trauma and is characterized by the recurrence of the hyper-arousing and intrusive memories throughout the lifetime of the patient (25). Furthermore, anytime a memory is retrieved out of long term storage, like during prolonged exposure therapy first introduced by Edna B. Foa, that memory becomes susceptible to strengthening or weakening physiologically; as these memories are retrieved they are susceptible to being modified pharmacologically by adrenergic and corticoid antagonists, which can reduce LTP (25). Understanding how synaptic plasticity is altered due to PTSD will elucidate ways that pharmaceutical agents can be used to prevent it altogether.

Since synapses can be strengthened and weakened pharmacologically, many drugs have been used in research as possible treatments for PTSD. They have also been used to study the specific roles of corticoid and adrenergic receptors in high stress situations and how they modify synaptic plasticity (26, 27). One drug that has been used as a treatment to lessen the symptoms of PTSD is propranolol. Propranolol is a nonselective beta 1 and 2 adrenergic receptor antagonist and has been used for treatment of PTSD in war veterans. Propranolol can also reduce fear conditioning in rats (25). Mifepristone, or RU-486, is a glucocorticoid antagonist that has been used clinically as well on war veterans (28). Mifepristone has been shown to attenuate LTP

individually in the basolateral amygdala in mice in vitro (16). Many studies have shown that activation of beta-adrenergic and corticoid receptors in the hippocampus and amygdala cause LTP by increasing the number of AMPA receptors on a cell's surface (8, 11, 21, 24, 29, 30). It has also been shown that corticosterone, an endogenous corticoid agonist in rodents, can regulate NMDA receptor function as well (9). The utilization of these drugs in combination can target these receptors and provide a potential for preventing PTSD in individuals. The combination of these two drugs has never been used for treatment prior to this study and we believe that this study will show it to be a possible prophylactic treatment for preventing PTSD-like changes in synaptic plasticity.

Currently there is no standardized model for inducing PTSD-like symptoms in rodents. Based on a literature review, it was determined that the single prolonged stress (SPS), first developed by Liberzon et al (31), and social defeat (SD), first developed by Miczek (32), methods were the most common stress protocols used by researchers studying PTSD-like symptoms in rodents. We used these models and combined both with two weeks of chronic light, which has been shown to cause depression-like behavior in rodents (33). We then compared the SPS with chronic light model to the SD with chronic light model to see which would cause more profound behavior and physiological changes before continuing with our drug studies.

## Materials and Methods

### *Screening Protocol*

Two-hundred and twenty Sprague-Dawley rats were screened before being used for either the SPS or SD protocols. The screening process was modeled after a technique performed by Nalloor et al (34). Male and female rats between 40-65 days old were placed in an open field

arena. Rats performed alone in the arena. Multiple rats were never placed in the arena at the same time. One quadrant of the arena had a ball of cat fur with one drop of fox urine (Just Scentsational). The rats were then observed for 5 minutes for anxious behavior. Anxious behavior consisted of immobility (not moving for longer than 10 seconds), avoidance of the quadrant with the cat fur and fox urine (not in the boundaries of the quadrant with fur and urine and not directly touching the fur or urine), any urination and defecation, and lack of rearing (not climbing on the walls or getting on the hind legs at all). Rats that displayed anxious behavior were used for either stress protocol. Non-anxious rats were used as controls since they did not participate in any stress protocols; they were also housed with littermates. Anxious rats were housed together until they were selected for a stress protocol. Once in the stress protocol, they were housed individually.

### *Stress Protocols*

Male and female Sprague-Dawley rats were used for the SPS protocol. The SPS model we used was altered from the original to meet the satisfaction of Brigham Young University's (BYU) IACUC. Rats were subjected to 2 hours of restraint in a tube manufactured by the Precision Machining Laboratory at BYU. Immediately after tube restraint, the rats were forced to swim in room temperature water for 20 minutes. After the rats were dried off, they were immediately put in a chamber with 2% isoflurane and remained inside until a loss of consciousness. The rats were then moved back to their home cages, where they were housed individually, to recover. The SPS was performed twice in a two-week period. The SPS occurred on the day 1 and day 8 in chronic light. Their home cages were located in a room with lights (~5 lux) that was on for 24 hours and they lived in this room for 2 weeks. Days 2-7 and 9-14 the rat

was undisturbed living in the light until performing on day 14 in the elevated plus maze and light dark transition and then would be sacrificed on day 15.

Only male Sprague-Dawley rats were used for the SD protocol since it does not work well with females since they are less aggressive than males. The SD model we used was also slightly modified from the original and lasted longer. An older male rat that was allowed to breed with females (in order to increase aggression) would act as the defender and the young, screened male rat would act as the intruder. All females (if applicable, defender rats were not always housed with females, only when they were breeding) would be removed from the defender's cage and then the young intruder rat would be placed in the older defender's cage. The older defender rat was allowed to interact and attack the young intruder rat for 5 minutes and then a wire mesh barrier was placed between in the two rats in the cage for 25 minutes. This barrier allowed the rats to see and smell each other, but prevented them from touching. If the defender broke the skin of the intruder rat, the mesh barrier was immediately put in place to prevent serious injury to the intruder, but this rarely happened. After the 30 minutes of SD was done, the intruder rat was returned to its home cage where it lived alone and the home cage put back into the chronic light room. The SD rats would live in the chronic light for two weeks before being sacrificed. The intruder rat would undergo 7 consecutive days of SD with a different defender rat each time so that the intruder rat never came in contact with the same older rat within the 7 days to avoid familiarity between the defender and intruder to increase the likelihood of attack. The first SD would start on day 1 of the chronic light and would continue once per day until day 7. Days 8-14 the rat was undisturbed living in the light until performing on day 14 in the elevated plus maze and light dark transition and then would be sacrificed on day 15.

### *Drug Preparation and Administration*

Propranolol and mifepristone were ordered from Sigma-Aldrich. Saline and propylene glycol were purchased from MWI. The propranolol was dissolved in sterile saline and the mifepristone was dissolved in sterile propylene glycol. Both drugs were administered at a 10 mg/kg dose by intraperitoneal injection using sterile 25-gauge needles and syringes. The rats were injected every other day for three weeks, receiving a total of 11 injections before being sacrificed for electrophysiology and RT-qPCR. The drugs were injected one week prior to starting the SD and chronic light protocol and continued for the entire two-week duration of stress. Rats were never injected the same day they were sacrificed.

### *Elevated Plus Maze (EPM)*

The elevated plus maze was manufactured by the Precision Machining Laboratory at BYU. It was made out of black plastic and had four arms: two open and two closed. The dimensions were 5-inch-wide arms, 45 inches long from the end of one arm to the other directly across, the closed arms had walls that were 18 inches high, and the open arms had no walls. The maze sat on top of a 47-inch aluminum stool. On day 14 of either SPS or SD with chronic light, the rats were placed in a closed arm of the EPM. Only one rat at a time was placed in the EPM. We then observed them for 5 minutes recording how much time they spent in closed versus open arms and how many times they entered open arms. This is used to test anxiety in rodents since more time spent in the closed arms is considered a more anxious behavior. One-way ANOVAs with Bonferroni post-hoc analysis were used to determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

### *Light Dark Transition (LDT)*

The light dark transition boxes were manufactured by the Precision Machining Laboratory at BYU. The dark side was made out of black plastic and the light side was made out of white plastic. The dimensions of each side were 16 inch x 16 inch x 16 inch. The boxes were put together with a removable black plastic divider separating the two sides. The divider was raised just enough to allow the rat to move between the two sides freely. An electric LED light was placed in the white side. On day 14 of either SPS or SD with chronic light, the rats were placed in the dark side of the LDT boxes. Only one rat at a time was placed in the LDT. We then observed them for 5 minutes recording how much time they spent in dark versus light boxes and how many times they entered the light box. This is also used to test anxiety in rodents since spending more time in the dark is considered higher anxious-like behavior. One-way ANOVAs with Bonferroni post-hoc analysis were used to determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

### *Sucrose Preference Testing*

After performing in the EPM and LDT behavior assays, the rats were given two bottles to drink from: one contained distilled water and the other contained a 10% sucrose solution (30 g of sucrose dissolved in 300 mL of water). They were given access to both bottles for 12 hours. At the end of 12 hours, the volumes were recorded from both bottles and mL consumed was calculated and compared between the groups of rats. This is used to test anhedonia and depression-like behavior in rodents and if an animal drinks less sucrose solution, they are thought to be more depressed. Prior to sucrose testing, the rats were allowed to drink water ad libitum, so it is unknown if all the rats were equally thirsty at the time of testing. One-way

ANOVAs with Bonferroni post-hoc analysis were used to determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

### *Animals and Brain Slice Preparation*

Male (females were only used in the SPS protocol) Sprague-Dawley rats between the ages of 60 and 105 days were used in the electrophysiology, RT-qPCR, and behavioral experiments. Animals were handled in accordance with the Institutional Animal Care and Use Committee (IACAC) standards. All animals were killed by decapitation, following deep isoflurane anesthetization. The brain was rapidly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) (in mM): NaCl, 119; NaHCO<sub>3</sub>, 26; KCl, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 1; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.3; glucose, 10; saturated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> (pH 7.4). Brains were sliced (400 μm thick) transverse manner in ice cold oxygenated ACSF using a Leica vibratome. Slices were placed in a submersion chamber containing oxygenated ASCF at room temperature (ventral hippocampus) or in an incubator at 35°C (lateral amygdala and ventral PFC). Slices were allowed to rest for at least 1 hour before being used for experiments. Slices remained in this solution until they were transferred to a submerged (ventral hippocampus) or interphase (lateral amygdala and ventral PFC) recording chamber. The brain slice preparation was the same for all experiments involving electrophysiology and RT-qPCR.

### *Field Electrophysiology*

Following an interval of at least 1 h, slices were transferred to a submerged or interphase recording chamber. Slices were then perfused with oxygenated regular ACSF (hippocampus) or ACSF with 4 mM picrotoxin and 500 mM ascorbic acid (amygdala and PFC). The chamber bath

temperature at which experiments were conducted were 28-32°C for submerged and 32-34°C for interphase. Slices were continuously perfused with oxygenated ACSF at a flow rate of about 3 mL/min for submerged and 1 mL/min for interphase. A bipolar stainless-steel stimulating electrode was placed in the stratum radiatum CA1 of the ventral hippocampus, external capsule of the lateral amygdala, or the ventral pre-limbic (PrL) area of the medial PFC. Borosilicate glass patch pipettes (2-3 M $\Omega$ ) were filled with 1 M NaCl for field recording electrodes at were placed 400-700  $\mu$ m from the stimulating electrode. Stimulation occurred at 80-500  $\mu$ A for 100  $\mu$ sec at 0.1Hz. Recordings were performed in current clamp mode to measure excitatory postsynaptic potentials (EPSPs) using an Axopatch 200B or 700B amplifier. Stimulation intensity was adjusted to elicit an EPSP of 0.5-1.5 mV at the beginning of each experiment. EPSPs were evoked and monitored for at least 60 minutes post-conditioning at a stimulus of 0.1 Hz. Data was filtered at 4 kHz and acquired with an axon 1440A digitizer (Molecular Devices) and inputted onto a Dell personal computer with pClamp10.4 clampex software (Molecular Devices).

The EPSPs slopes for hippocampus experiments and amplitude of EPSPs for amygdala and PFC experiments were calculated using pClamp10.4 clampfit software (Molecular Devices). EPSPs measured every 10 s were averaged into 1-minute intervals. Values were normalized to pre-theta burst baseline recordings 10-15 minutes immediately prior to high frequency stimulus (HFS). The HFS protocol consisted of two theta bursts. Each individual theta burst consisted of 5 consecutive stimuli at 100 Hz. The time between each repetition of stimuli was 200-ms. A significant increase in EPSP slope or amplitude that persists for longer than 30 min indicated that LTP was induced. EPSP normalized slope and amplitude values were compared at 26-30 min post HFS (unless otherwise stated in the figure caption or results) between the various experimental groups. Only one experiment was performed per slice and the reported “n” is the



number of slices not the number of animals, with 1-3 slices per animal. Microsoft Excel, IBM SPSS and Origin (Natick, MA) software were used to organize, average, graph, and perform statistical analysis on the data. One-way ANOVAs with Bonferroni post-hoc analysis and student's t-tests were used to determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

#### *Tissue Extraction and Reverse Transcription Quantitative PCR (RT-qPCR) Reaction*

Bilateral whole tissue samples of the ventral hippocampus, lateral amygdala, and PrL of PFC were obtained from 400  $\mu\text{m}$  thick brain slices that were stored in filtered ACSF during the extraction. Tissue was homogenized, and the mRNA was extracted using TriZOL (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. After extraction, each mRNA sample was placed into a reverse transcription mixture containing 4  $\mu\text{L}$  iScript reaction mix (BioRad), 1  $\mu\text{L}$  iScript reverse transcriptase, 1000 ng/mL of sample mRNA and ddH<sub>2</sub>O to bring the total volume to 30  $\mu\text{L}$ . This mixture was then cycled in a C1000 Thermocycler (BioRad) according to the iScript reaction protocol, which is: 25.0 Deg C for 8 min, 42.0 Deg C for 60 min, and 70 Deg C for 15 min. After this process, the mRNA was successfully turned into cDNA.

For the quantitative PCR procedure, cDNA from the iScript reverse transcriptase reaction described above was used. Each target was run individually in triplicates (triplicate values were averaged together for analysis). Each sample was run on a CFX96 qPCR machine (BioRad) using Sso Fast EvaGreen Supermix (BioRad) according to the following protocol: 95° C hot start for 3 minutes, followed by 50 cycles of 95° C for 15 seconds, 57° C for 20 seconds, and 72° C for 25 seconds. Amplification was measured using FAM (excitation at 488 nm, absorption at 494 nm, and emission at 518 nm) by detecting increased relative fluorescence during each cycle. A cycle threshold (Ct) value was assigned to each target using BioRad CFX Manager software. The

18S ribosomal gene was the housekeeping control gene used for expression comparison. All the forward and reverse primers besides 18S were purchased from realtimeprimers.com and can be seen in Table 2.1. Relative quantities of gene expression were determined using Microsoft Excel and the Livak and Schmittgen delta delta Ct/Cq method (35). For each individual target, all four rat groups (Control, SD, SD with Drugs, SD with Vehicles) were run on the same plate for more accurate comparison. If a sample failed to have the majority of three replicates show up, then we did not use it and thus there are varying sample sizes for the different targets. Samples were re-run if pipetting errors occurred during the first run. One-way ANOVAs with Bonferroni post-hoc analysis were used to determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

*Competitive Enzyme Linked Immunosorbent Assay (ELISA):*

To determine differences in corticosterone and norepinephrine levels, a corticosterone and norepinephrine ELISA kits were purchased from MyBioSource.com. Whole blood was collected between 9 – 11:30 AM from the electrophysiology and RT-qPCR rats subsequent to isoflurane anesthesia and decapitation. For each sample, 1 mL of blood was added to a plastic tube containing 200  $\mu$ L of heparin to prevent clotting. The blood samples were stored at  $-80^{\circ}$  C until the ELISAs were performed. The blood samples were brought to room temperature and processed according to the manufacturer's specifications. The plate was read at a 450-nm optical density on a BioTek Synergy HT plate reader using the BioTek Gen5 1.11 software. Duplicates were made of all samples, including standards. Standard values were then plotted into a graph using Microsoft Excel. The equation obtained from the best-fit line in the graph was used to determine the corticosterone and norepinephrine concentrations of the whole blood samples from the three rat groups. One-way ANOVAs with Bonferroni post-hoc analysis were used to

determine statistical significant differences. Statistical significance was  $p < 0.05$  and a trend was  $p < 0.15$ . Two-way ANOVAs did not provide any significant data.

## Results

For the SPS we used males and females. One way ANOVAs revealed that both SPS males and SPS females showed significantly ( $p < 0.05$ ) increased anxiety-like behavior by spending less time in the light during the LDT ( $F_{3,127} = 12.324$ ) and entering fewer times into the open arms on the EPM ( $F_{3,127} = 6.052$ ) when compared to controls (Figure 2.1A-B). A 2x2 ANOVA revealed that there were significant difference between genders ( $F_{1,127} = 4.330$ ,  $p < 0.05$ ) performances and SPS versus non SPS ( $F_{1,127} = 33.657$ ,  $p < 0.05$ ) time spent in the light in the LDT; a 2x2 ANOVA also showed that there were significant differences SPS versus non SPS groups in EPM performance (Figure 2.1A-B) with stressed groups entering less into open arms. Neither SPS males nor SPS females showed a significant difference in hippocampal LTP when compared to controls; although there was a trend ( $p < 0.10$ ) in short-term potentiation between male SPS and male controls (Figure 2.1C-D).

We then compared SPS males' EPM, LDT, and hippocampal electrophysiology data to SD males. According to one-way ANOVAs, both male SPS and SD rats had significantly increased ( $p < 0.05$ ) anxiety-like behaviors when compared to controls since both stressed groups had fewer entries into open arms on the EPM ( $F_{2,121} = 32.558$ ) and spent less time in the light during the LDT ( $F_{2,121} = 13.068$ ). When anxiety-like behavior was compared between SD and SPS rats using Bonferroni post hoc analysis, SD rats displayed significantly ( $p < 0.05$ ) more anxious-like behavior by entering fewer times into the open arms in the EPM than SPS rats (Figure 2.2A-B). Comparing the ventral hippocampal LTP between SD, SPS, and control rats, only the SD rats displayed significantly ( $p < 0.05$ ) higher levels of LTP when compared to

controls. However, even though the change was not significant, the SPS rats showed a trend of lower LTP when compared to controls. In summary, the SD rats showed an increase in LTP, whereas the SPS rats almost showed a decrease in LTP when compared to controls, showing that the different stress protocols were not having the same effect on ventral hippocampal LTP (Figure 2.1D and 2.3A). Based on the results from figure 2, we decided that the SD stress protocol was the more effective protocol at inducing physiological and behavioral changes in response to traumatic stress, so we used the SD protocol for the prophylactic drug experiments.

We performed prophylactic drug LTP experiments in three brain regions: the CA1 ventral hippocampus, the lateral amygdala, and the ventral PrL of the medial PFC. The drugs used were propranolol and mifepristone, both at a 10 mg/kg dose through IP injections. The drugs were administered one week before the rats started the SD protocol and during the entire two weeks of the SD and chronic light stressors, with injections occurring every other day for the total three week period. Saline and propylene glycol vehicle IP injections were also administered by the same schedule as the SD drugs rats. One way ANOVAs revealed that there were significant differences ( $p < 0.05$ ) in LTP at 21-25 minutes (Hippocampus:  $F_{3,37} = 3.401$ , PFC:  $F_{2,24} = 3.383$ ), at 46-50 minutes (Hippocampus:  $F_{3,37} = 5.368$ , amygdala:  $F_{2,13} = 5.085$ ) and at 86-90 minutes (Hippocampus:  $F_{3,37} = 3.232$ ) between all the groups of rats. In all three brain regions, the SD rats had significantly higher (T-test  $p < 0.05$  at 21-25, 46-50, 86-90 minutes for hippocampus, 46-50 minutes with trends at 21-25 and 86-90 minutes for amygdala, and 21-25, 40-44, and 76-80 minutes in the PFC) LTP when compared to SD rats injected with drugs and controls; SD rats injected with drugs had LTP levels that were not significantly different from controls (Figure 2.3A-C). In the hippocampus, the SD vehicle rats had LTP levels that were not different from SD

rats, but they were significantly different ( $p < 0.05$ ) from SD drug rats, indicating that the drugs were truly having an effect on LTP and not the injections (Figure 2.3A).

Even though SD rats injected with drugs showed significant differences in electrophysiological changes compared to SD rats with no treatment and SD vehicle injected rats, their anxious-like behavior was not diminished. According to one way ANOVAs, all three groups of SD rats, whether they received no injections, injections with drugs, or injections with vehicles showed significantly more ( $p < 0.05$ ) anxious like behavior in the EPM ( $F_{3,117} = 26.473$ ) and LDT ( $F_{3,117} = 9.610$ ) when compared to controls by spending less time in the light and going into the open arms fewer times (Figure 2.4A-B). Between all four groups of rats, there were no significant differences in sucrose or plain water consumption, indication that our SD protocol was not strong enough to induce depression-like behavior. If any SD groups were depressed, they would have drank significantly less sucrose than control rats (Figure 2.4C).

Blood levels of corticosterone and norepinephrine were measured between controls, not injected SD rats, SD rats with drug injections, and SD rats with vehicle injections. SD with drugs and SD with vehicle injected rats both displayed significantly higher ( $F_{3,32} = 14.066$ ,  $p < 0.05$ ) levels of corticosterone compared to controls and not injected SD rats; SD rats and controls did not significantly differ from each other and neither did SD drugs versus SD vehicles (Figure 2.5A). For blood levels of norepinephrine, the control, SD with no injections, and SD with drug injections groups were not significantly different from each other, but the SD with vehicle injections were significantly different ( $F_{3,32} = 4.488$ ,  $p < 0.05$ ) from the other three groups (Figure 2.5B).

RT-qPCR was performed on the whole ventral hippocampus, lateral amygdala, and medial PFC to elucidate any differences in mRNA expression between specific targets. The

targets analyzed were the beta 1, beta 2, and beta 3 adrenergic receptors (AR); catechol-O-methyltransferase (COMT; the enzyme that degrades catecholamines), glucocorticoid (GR) and mineralocorticoid (MR) receptors; AMPA receptor subunits 1 and 2; and NMDA receptor subunits 2A and 2B. The ventral hippocampus had four targets that showed significant differences: GR, MR, AMPAR subunit 1, and NMDAR subunit 2A. With the GR expression, SD drugs rats had significantly higher ( $F_{3,28}= 7.351$ ,  $p<0.05$ ) mRNA levels compared to SD and SD vehicles rats; control rats had significantly more mRNA than SD vehicles rats. MR expression was significantly higher ( $F_{3,28}= 8.949$ ,  $p<0.05$ ) in control rats when compared to the three other groups. In AMPAR subunit 1 expression, controls had significantly higher ( $F_{3,31}= 4.191$ ,  $p<0.05$ ) levels when compared to SD drugs rats. NMDA subunit 2A expression levels in controls were significantly higher ( $F_{3,29}= 4.465$ ,  $p<0.05$ ) than SD rats (Figure 2.6A). The targets that showed significant changes between the groups in the lateral amygdala were the beta 2 and 3 adrenergic receptors (AR), GR, AMPAR subunits 1 and 2, and NMDAR subunit 2A. SD drugs rats showed a significant increase ( $F_{3,16}=3.329$ ,  $p<0.05$ ) in beta 2 mRNA expression when compared to SD rats. The beta 3 AR expression showed a significant increase ( $F_{3,15}= 7.865$ ,  $p<0.05$ ) in SD drugs rats when compared to all other three groups. SD drug rats showed a significant increase ( $F_{3,27}= 4.537$ ,  $p<0.05$ ) in GR mRNA expression when compared to SD vehicles rats. For AMPAR subunit 1, there were significant decreases ( $F_{3,27}= 9.744$ ,  $p<0.05$ ) in expression levels when SD, SD drugs, and SD vehicles rats were compared to controls. SD drugs rats had significantly higher ( $F_{3,25}= 6.873$ ,  $p<0.05$ ) mRNA expression levels of AMPAR subunit 2 when compared to SD and SD vehicles rats. Lastly, there was a significant increase ( $F_{3,26}= 5.153$ ,  $p<0.05$ ) in NMDAR subunit 2A expression with controls compared to SD (Figure 2.6B). In the medial PFC, 9 targets showed significant changes: beta 1, 2, and 3 and alpha 1D ARs, GR, AMPAR subunits

1 and 2, and NMDAR subunits 2A and 2B. The control mRNA expression levels were significantly higher in beta 1 AR ( $F_{3,22}= 7.529$ ,  $p<0.05$ ), beta 3 AR ( $F_{3,19}= 13.490$ ,  $p<0.05$ ), and AMPAR subunit 2 ( $F_{3,26}= 17.003$ ,  $p<0.05$ ) when compared to all three SD groups. In beta 2 AR expression, SD drugs rats had significantly higher ( $F_{3,20}= 3.738$ ,  $p<0.05$ ) levels than SD rats. SD and SD vehicles rats had significantly lower expression levels of alpha 1D AR ( $F_{3,26}= 5.908$ ,  $p<0.05$ ) and AMPAR subunit 1 ( $F_{3,31}= 4.881$ ,  $p<0.05$ ) when compared to controls. GR expression was significantly higher ( $F_{3,27}= 4.755$ ,  $p<0.05$ ) in SD drugs rats when compared to SD vehicles rats. NMDAR subunits 2A ( $F_{3,25}= 3.576$ ,  $p<0.05$ ) and 2B ( $F_{3,25}= 4.471$ ,  $p<0.05$ ) expression levels were significantly higher in controls when compared to SD rats; NMDAR subunit 2B control levels were also higher than SD drugs rats.

## Discussion

We determined that SD stress protocol was more effective at inducing physiological and behavioral changes compared to the SPS protocol in male rats. Even though both SPS and SD were able to increase anxious-like behaviors in stressed rats when compared to controls, the behavior changes were more pronounced in the SD rats. Also, the SPS protocol did not induce any significant changes in ventral hippocampal LTP in either male or female rats indicating that perhaps SPS was not a traumatic enough stress to produce all PTSD-like changes in rats. Even if the changes in SPS male hippocampal LTP were significant it would have been the opposite direction, a decrease instead of an increase, which could be an indication that duration of the stress protocol, makes a difference. However, the SD stress protocol showed a significant increase in ventral hippocampal LTP compared to controls, which is more in line with what other researchers have reported regarding chronic stress and its effect on ventral hippocampal LTP (21). The SD protocol was also able to cause significant increases in LTP in the lateral amygdala

and the ventral PrL of medial PFC, like what was observed in the hippocampus. Since we were able to get increased LTP in all three brain regions, we wanted to see if providing propranolol and mifepristone before and during the SD stress protocol could counteract the effects the traumatic stress was having on synaptic plasticity. Others have had success with propranolol (36) and mifepristone (16, 37, 38) counteracting stress changes in the brain, but none have studied these drugs in combination prior and during chronic stress. We indeed saw a significant decrease in LTP back to control levels in SD rats that were injected with drugs in all three brain regions. This indicates to us that the combination of propranolol and mifepristone could possibly be an effective treatment for preventing traumatic stress changes to LTP in these three brain regions if taken before and during a traumatic chronic stress experience. Also, the SD with vehicle injections data in the hippocampus was similar to SD rats with no treatment, which indicates that the vehicles and injection did not interfere with the drugs' abilities to alter synaptic plasticity.

Our RT-qPCR data revealed some possible molecular targets these drugs and the chronic stress could be affecting. In general, in all three brain regions, SD with no injections expression levels were lower in the significant targets, indicating that the SD stress protocol was indeed causing molecular changes in the body of the rat. However, it is important to note that we saw significant decreases in alpha 1D mRNA expression in SD rat groups that did not receive drugs compared to controls, which is the opposite of what another study found in the dentate gyrus of the hippocampus (39), but in line with the decreased alpha 1D mRNA expression they observed in the CA1, CA2, and CA3 regions of the hippocampus (22). This group of researchers also showed evidence that it was corticosterone that was causing the changes in alpha 1D expression in the hippocampus (22), so corticosterone could possibly be the hormone causing the expression changes in the PFC that we observed as well, indicating that the corticoid and adrenergic systems



can alter the function of each other. There were a few targets in which the drugs treatment was able to increase expression levels compared to control or at least match control expression levels; these targets would be the most promising to look at in a future study to try and determine the actual pathways that cause PTSD-like changes in synaptic plasticity. In the all three brain regions, significant increases in expression were present in SD with drug rats when compared to the other SD groups; the drugs were also able to bring the expression levels of GR back to control levels or higher. Mifepristone is an antagonist to the glucocorticoid receptor, so the decreased activation of the receptors could have caused the mRNA expression levels to stay at control levels in the SD with drugs rats. The HPA axis works by negative feedback, so over production of glucocorticoids and mineralocorticoids could have caused the decreased mRNA expression levels of GR and MR in SD rats that received no drugs and SD with vehicle rats. The alterations in expression levels of AMPAR and NMDAR subunits we observed between the three groups in both the hippocampus and amygdala could be related to the ability of glucocorticoids (40) and norepinephrine to alter AMPAR and NMDAR activity (40). In the lateral amygdala and medial PFC, the increase in mRNA expression of the beta 2 AR in the SD with drugs groups compared to SD with no injections could also have been caused by the action of propranolol blocking those receptors since it is a beta adrenergic antagonist and blocking the beta receptors prevent the negative feedback loop in the HPA axis from functioning normally; this could also hold true for why we see a significant increase in beta 3 AR expression in SD with drugs rats compared to the other three groups in the lateral amygdala. Lastly, the drugs were able to increase mRNA expression of the AMPAR subunit 2 in the lateral amygdala compared to the SD no injections group, possibly highlighting this as a direct cause of synaptic plasticity changes in the amygdala.

Even though we saw that the drugs could alter synaptic plasticity and mRNA expression levels in the brain, we did not see those alterations translate into behavioral changes. All SD rats, whether they were not injected or injected with drugs or vehicles, still showed significantly increased anxious-like symptoms when compared to controls as seen in the EPM and LDT assays. Although, the anxious behaviors we tested could also not be affected by the same synaptic plasticity that the drugs were altering, which could explain why no changes in behavior were seen with drug treatment. Another caveat as to why we did not observe behavior changes is because the drugs were injected systemically and not directly into the brain. Also, in humans, most behavior changes do not happen with just drug treatment, but also require talk therapy sessions with a psychiatrist or psychologist (27), but in rodents it is impossible to do talk therapy. Another possibility could be that the doses of the two drugs were high enough to cause cellular changes, but not high enough to cause behavioral changes.

However, we believe that the IP injections were causing added stress, more than what the regular SD rats were experiencing. This is why we think they still showed significant anxiety-like behaviors. We also believe the stress from the injections is what caused the SD with drugs and SD with vehicles rats to have higher blood levels of corticosterone and the SD with vehicles rats to have higher norepinephrine levels. This higher level of corticosterone possibly caused from an injection from the day before could also be causing some changes in mRNA expression therefore affecting expression of the targets we analyzed using RT-qPCR, since alterations can happen within an hour after experiencing stress. It would have been ideal to administer the drugs orally to the rats, which is how humans would consume the drugs, but it is impossible to force a rat to swallow a set amount of drugs with confidence that the entire drug dosage entered its body. It is also peculiar that SD rats with no injections had similar levels of corticosterone and

norepinephrine as controls. This could be because after the seven consecutive days of SD stress, the rats were not handled and left alone for one week in chronic light before being behavior tested and sacrificed for electrophysiology, RT-qPCR, and ELISA experiments. When the traumatic stress events were occurring during the first seven days, it is possible that we would have seen changes in corticosterone and norepinephrine levels in the SD rats. Perhaps blood levels of these hormones only stay elevated in the blood for a short amount of time after a traumatic stressful event, but how they make long lasting changes is by binding to their respective receptors causing alterations in mRNA expression of certain targets and that is why we were still able to see synaptic plasticity and behavior changes in the SD compared to controls despite no changes in blood hormone levels.

## Conclusion

Overall, our data provides evidence that the SD model of stress with chronic light is better at inducing anxious behavior in rats and physiological changes in synaptic plasticity when compared to the SPS with chronic light model. Our data also elucidate that propranolol and mifepristone together may be a viable prophylactic pharmaceutical treatment for PTSD in humans since it was able to alter LTP and mRNA expression in rats. Also, it is important to note that the time periods in which our drugs were administered were truly prophylactic since they were administered before stress even occurred and during the whole stress protocol. Often in the literature, prophylactic treatment is referred to as drug intervention immediately after a trauma occurs, even though a true prophylactic treatment should be administered before a disease or disorder develops to prevent the onset all together. However, for our true prophylactic treatment presented in this study, the most effective dose would have to be determined that could cause all the desired molecular, physiological, and behavioral changes. These drugs, in this unique

combination, when taken together before and during a stressful, traumatic experience have the capacity to prevent the physiological changes that happen during synaptic plasticity in the brain, possibly preventing the onset of PTSD altogether. This is an especially useful finding for military personnel that know they will be deployed into a war zone since they can take these drugs to theoretically prevent PTSD. Policemen and women could also take these drugs regularly since it is very likely that they will encounter traumatic stress on duty and these drugs could prevent those traumatic stresses from developing into PTSD.

#### Acknowledgements

I would like to thank all the undergraduates in Jeff Edward's lab that worked on this project. This research would not have been accomplished in a timely matter without all of you. I would also like to thank our animal care facility personnel at BYU for helping accommodate my needs concerning the behavioral components of this project. I would also like to thank Therin Garrett and the people he works with at the precision machining lab for making much of the equipment needed to carry out this study.

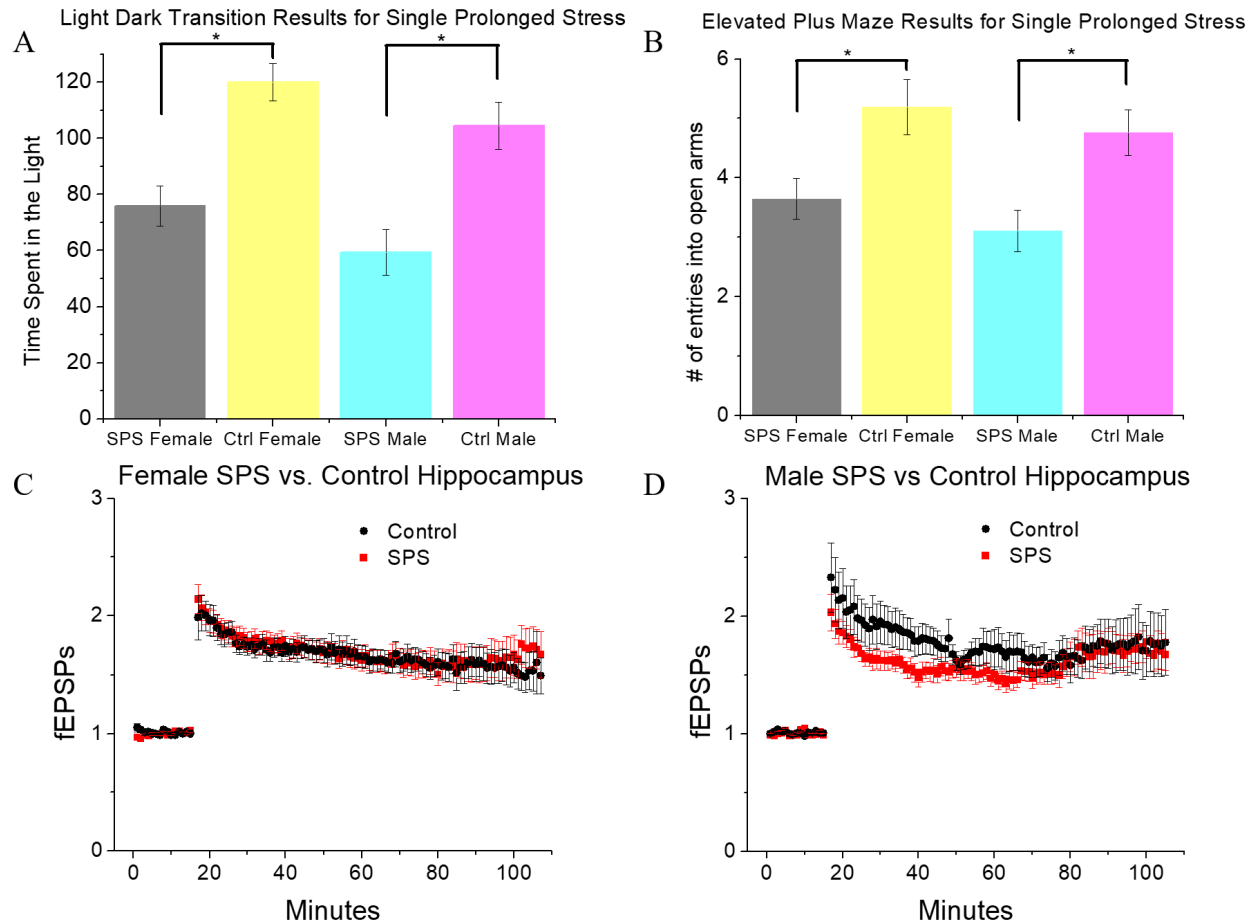


Figure 2.1: Male and Female Singled Prolonged Stress (SPS) Data. Comparison of Light Dark Transition (LDT), Elevated Plus Maze (EPM), and Hippocampal Long-term Potentiation (LTP) of Males and Females that went through the SPS Protocol to Controls. A) Results of the LDT showing that SPS males and females significantly ( $p < 0.05$ ) spent less time in the light when compared to controls, which indicates that the SPS rats were more anxious. B) Results from the EPM showing that SPS males and females entered into open arms significantly ( $p < 0.05$ ) fewer times than controls, indicating the SPS was increasing anxious behavior. SPS female  $n=40$ , ctrl female  $n=27$ , SPS male  $n=35$ , ctrl male  $n=29$  for A and B. C) Hippocampal LTP levels were not different between SPS females ( $n=28$ ) and controls ( $n=18$ ). D) Hippocampal LTP levels were not different between SPS males ( $n=19$ ) or controls ( $n=25$ ). The  $n$  values vary between behavior and electrophysiology experiments in all figures because not all LTP experiments are usable due to experimental error and failures, which are more common in electrophysiology than behavior assays.

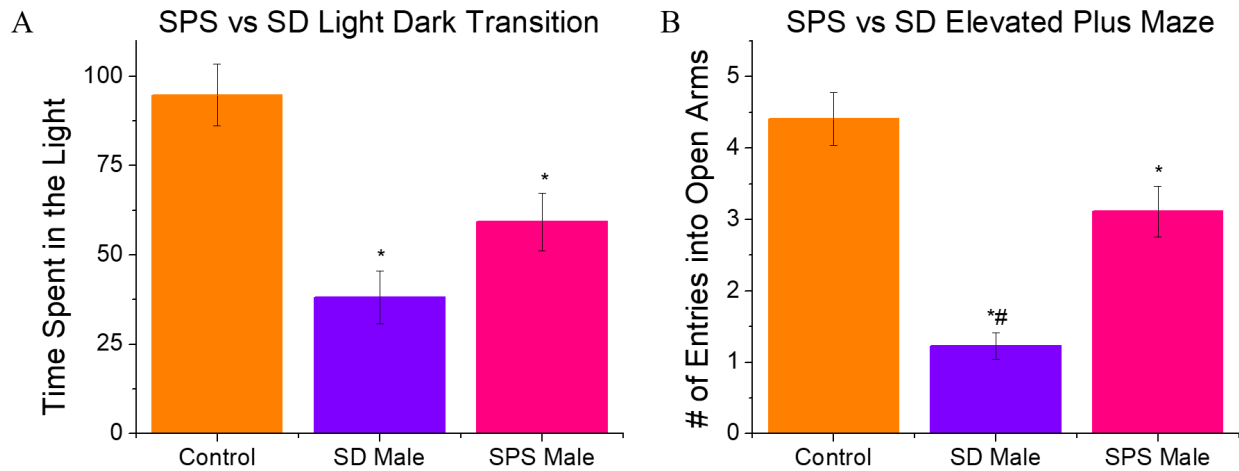


Figure 2.2: A Comparison of SPS and Social Defeat (SD) Males and Controls on LDT and EPM. Male SPS EPM and LDT data is the same as figure 2.1, but shown here for comparison to SD males. A) Both SD and SPS males spent significantly ( $p < 0.05$ ) less time in the light compared to controls indicating greater anxiety. \*=significant difference compared to control. B) In the EPM, both SD and SPS males entered into the open arms significantly fewer ( $p < 0.05$ ) times compared to controls indicating anxiety. The SD males entered into the open arms significantly fewer ( $p < 0.05$ ) times compared to SPS males, indicating that the SD protocol was causing more anxiety than SPS. # = significant difference compared to SPS, \* = significant difference compared to control. Control  $n = 37$ , SD male  $n = 52$ , SPS male  $n = 35$  for both A and B.

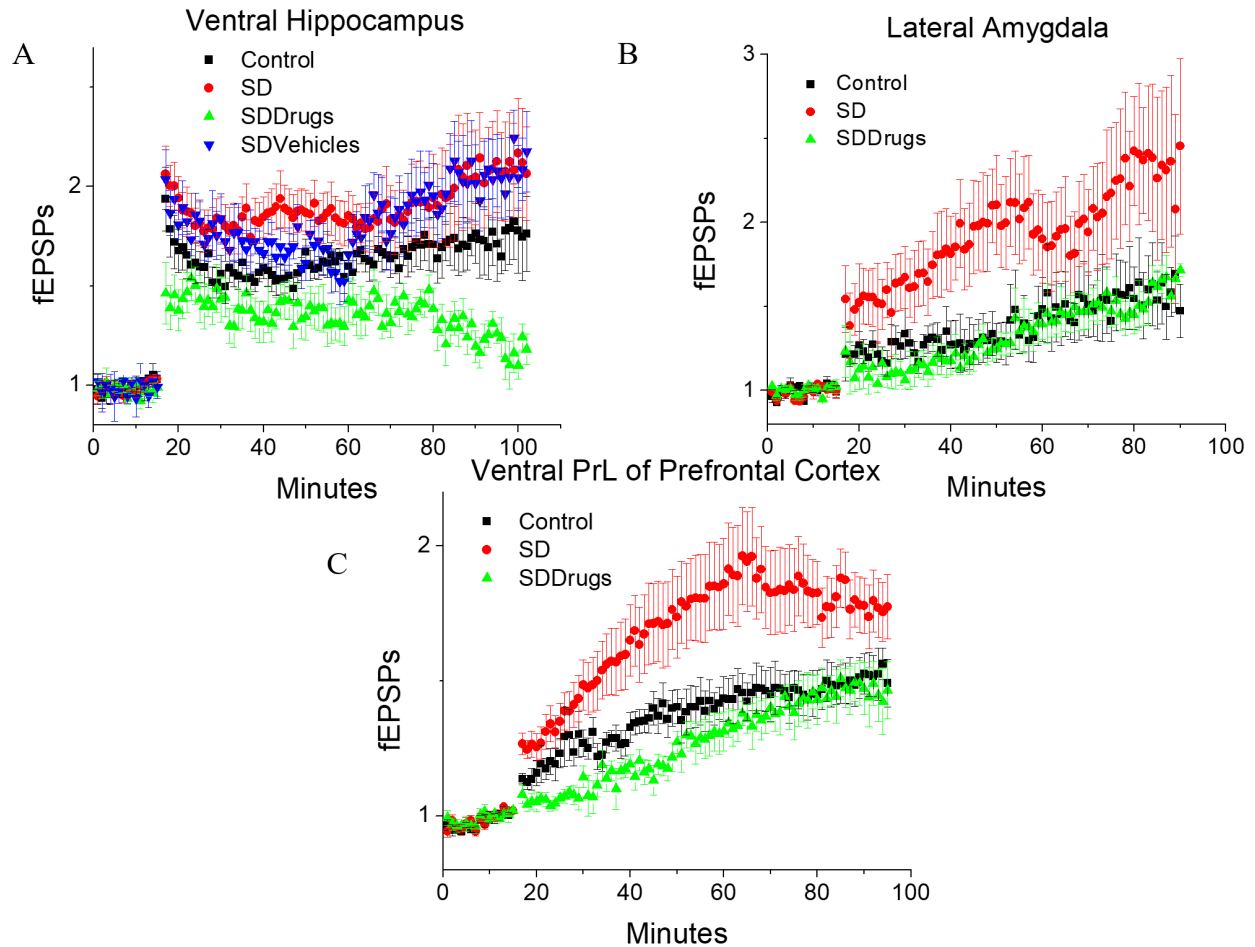


Figure 2.3: Differences in LTP Between All Rat Groups in Three Brain Regions. The three brain regions are ventral hippocampus, lateral amygdala, and medial prefrontal cortex. A) SD rats had significantly greater ( $p < 0.05$ ) LTP in the hippocampus compared to controls rats, which shows that it was the better model for causing changes in LTP when compared to the SPS model. SD with vehicles rats also had significantly greater ( $p < 0.05$ ) LTP compared to SD with drugs, but were not different from SD rats. Controls  $n=9$ , SD  $n=14$ , SD Drugs  $n=13$ , SD Vehicles  $n=5$ . B) SD rats had significantly greater ( $p < 0.05$ ) LTP in the amygdala compared to controls and SD with drug rats. Controls  $n=6$ , SD  $n=6$ , SD Drugs  $n=4$ . C) SD rats had significantly greater ( $p < 0.05$ ) LTP in the prefrontal cortex compared to controls and SD with drug rats. Controls  $n=6$ , SD  $n=16$ , SD Drugs  $n=5$ . Since the LTP for the SD with drug rats was not different from controls in all three brain regions, this indicates that propranolol and mifepristone were able to counteract the stress related changes.

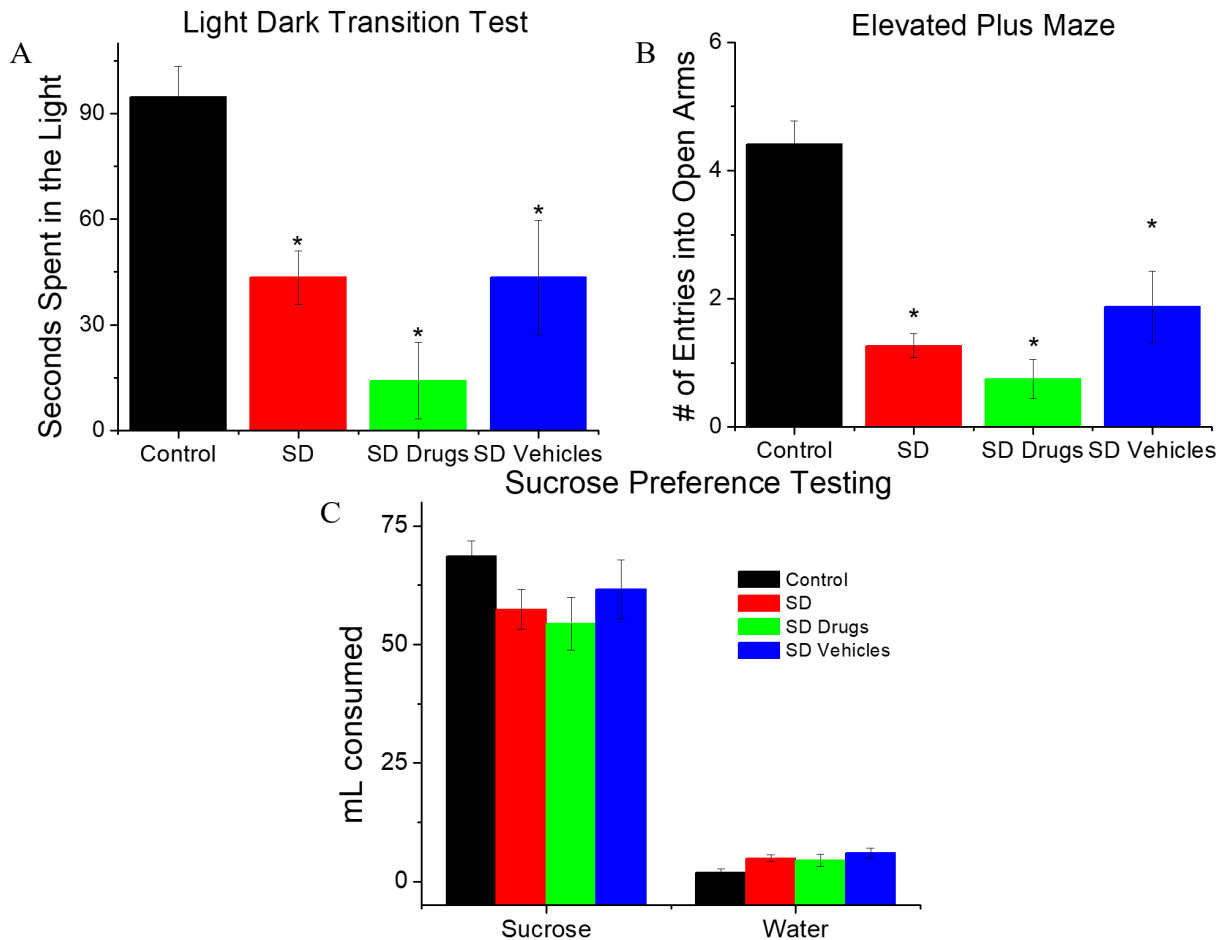


Figure 2.4: A Comparison of LDT, EPM, and Sucrose Preference Testing Between All Four Groups. Male SD data was also used in figure 2.2 A) The SD groups spent significantly less ( $p < 0.05$ ) time in the light when compared to controls indicating that they were more anxious. There were no differences within the SD groups. B) The SD groups entered significantly fewer ( $p < 0.05$ ) times into the open arms when compared to controls indicating that they were more anxious. There were no differences within the SD groups. Control  $n=37$ , SD  $n=56$ , SD Drugs  $n=12$ , SD Vehicles  $n=16$  for both A and B. C) There was no difference in sucrose or plain water consumption between the groups indicating that the SD protocol was not strong enough to induce depression-like symptoms in the rats. Control  $n=11$ , SD  $n=44$ , SD Drugs  $n=9$ , SD Vehicles  $n=16$ . Overall, this figure shows that any rat that experiences SD, regardless of treatment, will display anxious-like behavior.



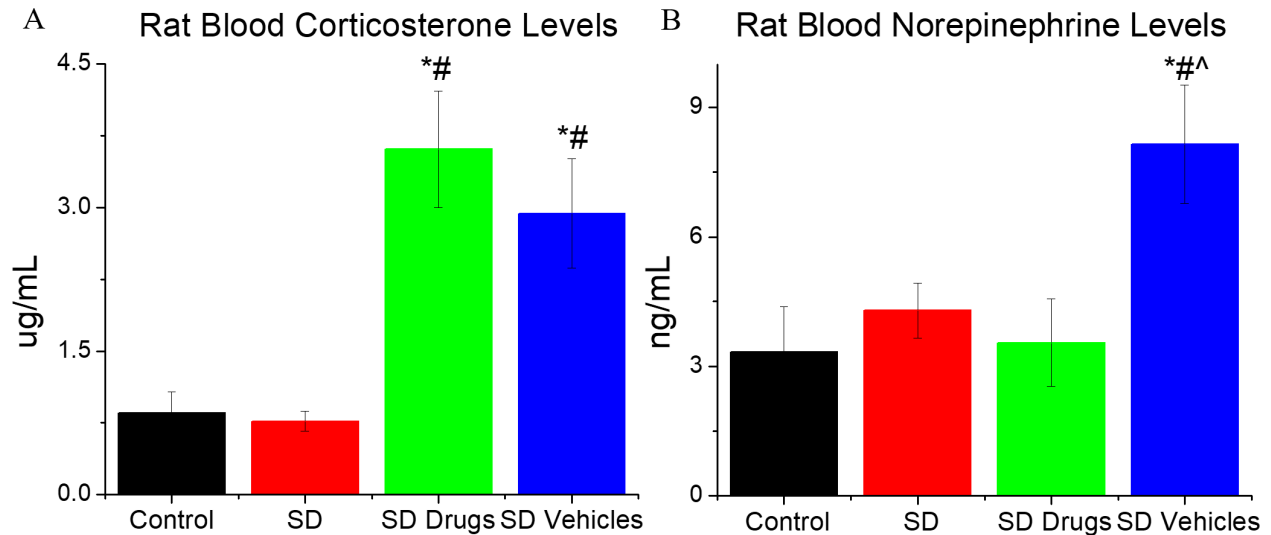


Figure 2.5: Blood Levels of Corticosterone and Norepinephrine Compared Between All Four Groups. A) SD with drugs and SD with vehicles rats had significantly greater ( $p < 0.05$ ) corticosterone concentrations than SD and control rats; SD and control rats did not differ. \*=significant compared to control; #=significant compared to SD. Control  $n=8$ , SD  $n=12$ , SD Drugs  $n=9$ , SD Vehicles  $n=7$ . B) SD with vehicles rats had significantly increased ( $p < 0.05$ ) levels of norepinephrine compared to the other three groups. \*=significant compared to control; #=significant compared to SD; ^=significant compared to SD Drugs. Controls  $n=7$ , SD  $n=13$ , SD Drugs  $n=9$ , SD Vehicles  $n=7$ . For both A and B, the SD hormone levels could be similar to controls because of the 8 days of rest that occurred from the last SD performed to the time the blood was collected in the morning. Hormone levels in the blood might only stay elevated for a short amount of time after a stress event occurs which could explain why SD with drugs and SD with vehicles rats had elevated corticosterone and norepinephrine (only SD vehicles) levels since an injection occurred just the day before.

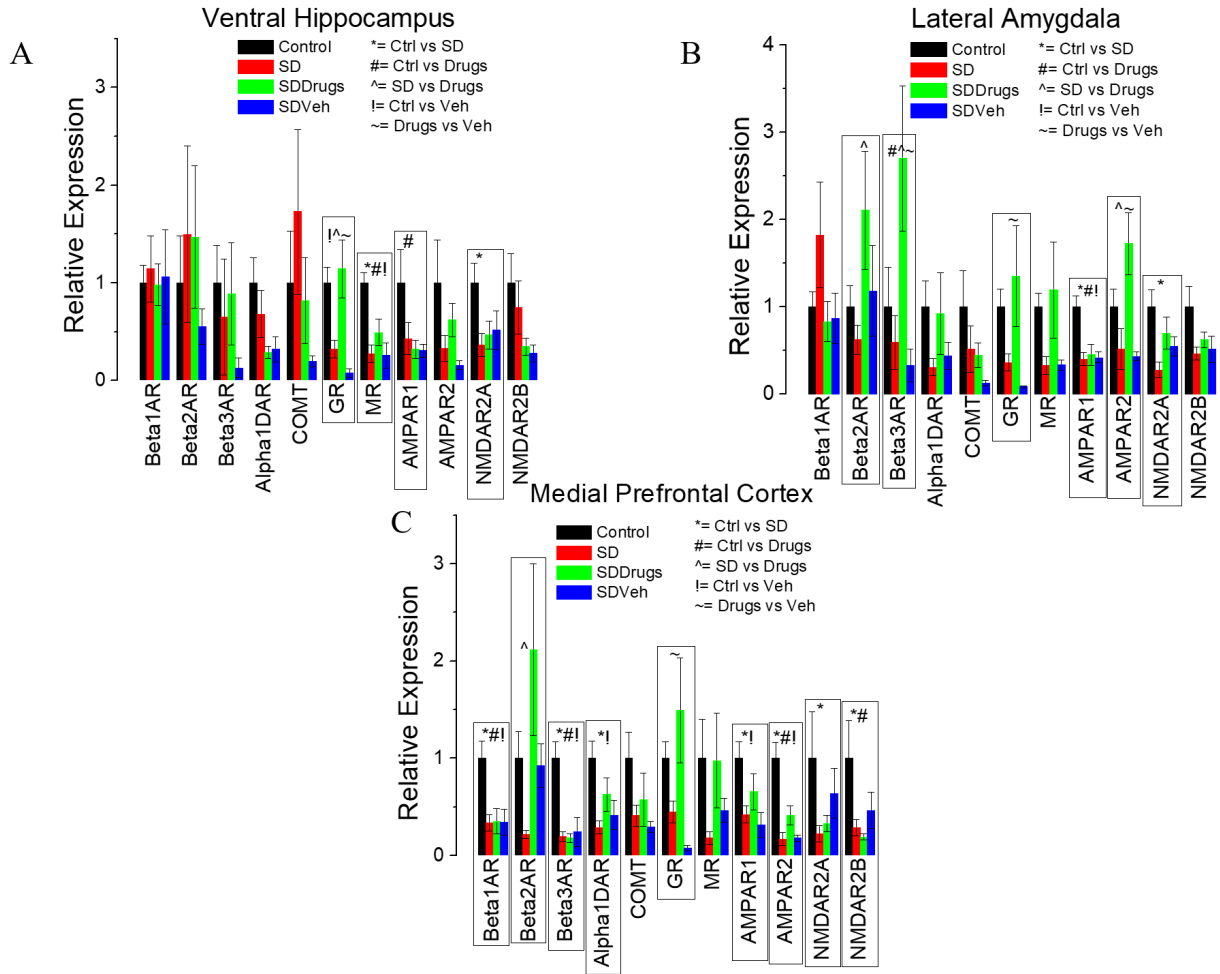


Figure 2.6: RT-qPCR Results from the Ventral Hippocampus, Lateral Amygdala, and Medial PFC. Target expression levels were compared to 18S levels to determine actual increases in mRNA. A black box outlining the target means it had a significant change in mRNA expression. The symbol legends on the graph detail which groups significantly differed from each other. A) Four targets had a significant change ( $p < 0.05$ ) in mRNA expression levels in the ventral hippocampus. SD  $n = 8-12$ , controls  $n = 7-11$ , SD with drugs  $n = 4-9$ , SD with vehicles  $n = 4-6$ . B) Six targets had a significant change ( $p < 0.05$ ) in mRNA expression levels in the lateral amygdala. SD  $n = 8-12$ , controls  $n = 7-11$ , SD with drugs  $n = 4-9$ , SD with vehicles  $n = 4-6$ . C) Nine targets had a significant change ( $p < 0.05$ ) in mRNA expression levels in the lateral amygdala. SD  $n = 8-12$ , controls  $n = 7-11$ , SD with drugs  $n = 4-9$ , SD with vehicles  $n = 4-6$ .

Table 2.1: Sequences for the Primers Used to Target Specific PTSD-related Genes. Forward and reverse primer sequences, accession file, amplicon length in base pairs, and protein name provided in the table.

Species	Gene	Protein Name	Accession	Amplicon	Region	Forward Sequence	Reverse Sequence
Rat	Adrb1	adrenoceptor beta 1	NM_012701	219	202-420	ATCGTGCTGCTCATCGTAGT	TAGCAGTCTACCGAAGTCC
Rat	Adrb2	adrenoceptor beta 2	NM_012492	209	1001-1209	GAGCACAAAGCCCTCAAGAC	TGGAAGGCAATCCTGAAATC
Rat	Adrb3	adrenoceptor beta 3	NM_013108	281	1808-2088	TCCCTTCCTACTGCTTTCCT	CTGGAATTTCTCCCAAACCT
Rat	Adra1d	adrenoceptor alpha 1D	NM_024483	135	2525-2659	GGAAAAGATCCGTGGACAGT	AGCGGAAGAGCAACAGATTT
Rat	Comt	catechol-O-methyltransferase	NM_012531	145	1349-1493	AATGTCCAGACGCCAAATAA	CTGGATACTGGGGATGACAG
Rat	Nr3c1	nuclear receptor subfamily 3, group C, member 1	NM_012576	192	1407-1598	GCTTCAGGATGCATTACGG	TCGAGCTCAAGGTTTCATTC
Rat	Nr3c2	nuclear receptor subfamily 3, group C, member 2	NM_013131	109	5530-5638	ACGCTGTGAGACTGGATTTC	AGTTACCCGGAGACACATGA
Rat	Gria1	glutamate receptor, subunit AMPA 1	NM_031608	157	81-237	CAAGGAACTGCAGGAAGAAA	CTAGAAAACCGGTGCAGAAA
Rat	Gria2	glutamate receptor, subunit AMPA 2	NM_017261	109	377-485	GAGGAAGAAAGGGAAACGAG	TCAGTCCCATAAAACAGGA
Rat	Grin2a	glutamate receptor, subunit NMDA 2A	NM_012573	278	3217-3494	GCTGTCAGCACTGAATCCAA	GCCATTGACCGTTTGAAGTT
Rat	Grin2b	glutamate receptor, subunit NMDA 2B	NM_012574	194	860-1053	CATCGTCACCACCTACTTCC	CCTTCGTGCAATAAAGGAGA

## CHAPTER 3: Running Exercise Mitigates the Negative Consequences of Chronic Stress on Dorsal Hippocampal Long-Term Potentiation in Male Mice

### Abstract

In the hippocampus, learning and memory are likely mediated by synaptic plasticity, known as long-term potentiation (LTP). While chronic intermittent stress is negatively correlated, and exercise positively correlated to LTP induction, we examined whether exercise could mitigate the negative consequences of stress on LTP when co-occurring with stress. Mice were divided into four groups: sedentary no stress, exercise no stress, exercise with stress, and sedentary with stress. Field electrophysiology performed on brain slices confirmed that stress alone significantly reduced dorsal CA1 hippocampal LTP and exercise alone increased LTP compared to controls. Exercise with stress mice exhibited LTP that was significantly greater than mice undergoing stress alone but were not different from sedentary no stress mice. An ELISA illustrated increased corticosterone in stressed mice compared to no stress mice. In addition, a radial arm maze was used to examine behavioral changes in memory during 6 weeks of stress and/or exercise. Exercised mice groups made fewer errors in week 2. RT-qPCR was used to examine the mRNA expression of components in the stress and exercise pathways in the four groups. Significant changes in the expression of the following targets were detected: BDNF, TrkB, glucocorticoid, mineralocorticoid, and dopamine 5 receptors. Collectively, exercise can mitigate some of the negative impact stress has on hippocampal function when both occur concurrently.

### Introduction

Plasticity is a unique characteristic of the nervous system. Following environmental stimuli or experiences, neuronal synaptic connections in the brain are modified. The most

common form of synaptic modification observed *ex vivo* is known as long-term potentiation (LTP) and is one phenomenon used to quantify learning and memory. Synaptic plasticity occurring in the hippocampus has become the leading theory of the mechanism for memory formation and recall (13).

One factor that has a dramatic impact on hippocampal learning and memory in rodents is stress (41). There are various types of stress induction techniques (42). Regarding acute stress, it is a single stress incident that can be adaptive in rodents and enhance memory behavioral performance (43, 44) as well as synaptic activity and LTP (45), or alternatively decrease LTP (46, 47). Acute stress particularly affects LTP in the dorsal hippocampus, which is thought to be mediated by the glucocorticoid receptors (48, 49), and has been reviewed previously (Howland and Wang, 2008). The effects of acute stress on LTP can be reversed over time (47). However, chronic/chronic intermittent stress are ongoing stress incidents that are more maladaptive and their effects are harder to reverse over time (20, 50). In behavioral studies, chronic intermittent stress decreases the ability of rodents to form and recall spatial memories (51) and hinders performance in the Morris water maze (52) and novel object recognition (53). Chronic stress also decreases neurogenesis and can induce neuronal cell death (51). As our study employed various chronic stress methods, the factor most pertinent to this study is that chronic stress reduces CA1 hippocampal LTP in rodents (50).

The connection between hippocampal plasticity and the aforementioned behavioral deficits have been reviewed and discussed extensively (41, 48, 51, 54-56). The glucocorticoids are important hormones released during stress. In rodents, corticosterone is a glucocorticoid that is released. Corticosterone binds to both glucocorticoid and mineralocorticoid receptors in the central nervous system. Glucocorticoid and mineralocorticoid release are increased during

chronic stress and have been implicated in causing changes in hippocampal plasticity (1, 57). Studies have shown that corticosterone acting on glucocorticoid and mineralocorticoid receptors alter alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor function and trafficking, as well as induces changes in synaptic plasticity (29, 58). While many things remain unclear, it is clear that chronic stress impedes the ability for neurons to experience LTP and has profound effects on memory.

Conversely, studies have found that mice performing voluntary physical exercise has the opposite effect on the mechanisms that are impaired by stress (59). Rodents that exercise show robust performance in maze navigation and have increased hippocampal LTP in the dentate gyrus (60). Exercise also has anxiolytic and antidepressant effects by increasing brain-derived neurotrophic factor (BDNF) levels (61). BDNF is a protein that promotes neural survival, growth, and differentiation of new neurons and synapses. It is clear that voluntary exercise increases BDNF (52, 62, 63). BDNF and other proteins in the BDNF pathway are thought to be the major contributors for enhancing memory (64) and increasing LTP (65, 66). Recent research illustrates that BDNF activates mTOR, which regulates the expression of AMPA receptors to increase memory and LTP (67). Studies showed chronic stress downregulated BDNF (68) and upregulated interneuron activity (69), while exercise prevented these changes. However, these studies were performed in the ventral hippocampus and not the dorsal hippocampus, which is an important distinction since the different hippocampal subfields have different neural projections and functions (70). The dorsal hippocampus has been studied less in regard to stress and exercise compared to the ventral hippocampus.

While many of the molecular, physiological, and behavioral effects of stress and exercise on rodents have been studied in isolation, they are rarely studied concurrently. Despite the

evidence that exercise and stress influence brain health and plasticity in opposite ways, there is a paucity of data that connects the effects these two factors might have in the dorsal hippocampus when experienced by the same animal. Additionally, the mechanism by which exercise could potentially reduce the negative effects of stress is not completely understood (59). Therefore, we examined whether exercise occurring concurrently with stress could alleviate the negative impact of stress on dorsal hippocampal plasticity. Using behavioral interventions, such as exercise, to combat learning deficits due to chronic stress could be a safe, cost-effective treatment that could improve cognitive function and quality of life for many individuals. Furthermore, considering the neurotoxic effects of chronic stress and the shown benefits of exercise, our results could add to the body of literature seeking to understand and prevent neurodegenerative disorders associated with chronic stress.

## Materials and Methods

### *Treatment Groups*

Adult male C57BL/6 mice were used in this study. Mice were housed in approved conditions with a 12-hour light-dark cycle. The experiments had ethical approval and were conducted in accordance with the Brigham Young University Institutional Animal Care and Use Committee standards and National Institute of Health guidelines to minimize pain and suffering of the mice. The four treatment groups utilized in this study were sedentary no stress (control; SNS), sedentary with stress (SWS), exercise with stress (EWS), and exercise no stress (ENS). The average ages of the mice used were 87 days for the electrophysiology experiments and 145 days for RT-qPCR and behavioral experiments. The difference in ages between these groups is due to the radial arm maze assay lasting 6 weeks. The mice continued to run during the duration

of behavioral testing, and were sacrificed at the conclusion of this memory assay. Their brains were extracted and hippocampi then isolated for RT-qPCR testing as described below. All four groups of mice used in electrophysiology experiments were of similar age. The RT-qPCR and behavioral experiments also used age-matched mice from all four groups.

### *Exercise and Stress Protocols*

Mice from all four groups were housed solitarily with the same type of bedding to ensure that those mice with access to running wheels had accurate distance measurements as well as to maintain a consistent social environment to prevent uncontrolled variables. SNS and SWS mice did not have locked running wheels in their cages and were housed in slightly smaller cages than the exercise cages, and therefore were under slightly more impoverished conditions. No enrichment was provided to the SNS and SWS mice. This is a potential limitation in our methods, though we still note differences in SNS and SWS mice in LTP studies, which were housed identically. ENS and EWS mice were allowed to run ad libitum in a cage with a running wheel purchased from Lafayette Instrument Co and the distance was tracked by software provided by the same company on a portable computer. The average distance run by all exercise mice (stress and no stress) was  $5.42 \pm 0.32$  kilometers per day, which was slightly higher, but still comparable to the average of approximately 4.5 kilometers per day others saw using the same strain of mice (60, 71). ENS and EWS mice ran at least an average of 2 kilometers per day; no mice ran less than this, so no exercise mice were excluded from our study. Mice were exercised for a minimum of 4 weeks before being used for any experimentation (electrophysiology, PCR, and behavior). The mice also were at least 30 days old before being moved into running cages. Surprisingly, there were differences in average daily running distance between ENS and EWS mice used for electrophysiology (ENS =  $6.31 \pm 0.52$  km, EWS =  $4.40 \pm 0.53$  km, t test  $p < 0.05$ ).



This is surprising as running occurred for one month while stress was only the last three days and running distances were not significantly changed after stress. This difference appears to be random based on which mice were selected for entry into stress procedures or not. However, this caveat could influence differences in LTP noted between the two in the results section. No differences in running distance were noted between ENS and EWS for behavioral/PCR experiments ( $5.18 \pm 0.75$  km and  $4.70 \pm 0.62$  km day;  $p > 0.5$ ).

Electrophysiology SWS and EWS mice experienced three consecutive days of stressors to create chronic intermittent/variable stress. We used similar variable stressors from a prior report (72), with some slight modifications to the stressors and shorter stress duration. Another group (73) also modified the original Katz et al protocol to a shorter duration of 5 days. We shortened the stress protocol because we wanted chronic stress physiological changes in the shortest amount of time. The stressors included: a 5-minute cold ( $2-8^{\circ}$  C) water swim on day one, a 30-minute elevated platform stress on day two, and a 60-minute restraint with a 1 second 10 mA tail shock once per minute on day three. During shock stressing, mice were put in restraining plastic tubes. Copper electrodes, in the form of toothless alligator clips, were clamped on the taped down mice tails. Electrode gel was applied before electrode attachment. Mice were sacrificed on the third day of stress 1-2 hours following the completion of the last stress. Each stressor was only performed once.

SWS and EWS mice used for behavioral and RT-qPCR experiments were stressed once per day beginning at the start of behavioral testing, which occurred immediately before performing in the radial arm maze and continued with one daily stress to create chronic intermittent stress until they were sacrificed for RT-qPCR experiments six weeks later. For ENS/EWS groups, exercise began 30 days prior to use for memory behavior testing. It is

important to note that tail shock stress was not used with these mice, instead a 30-minute tube restraint with no shock was used. Stressing started on day 1 of the radial arm maze assay. During the radial arm maze, stressors were alternated Monday through Friday between the 5-minute cold-water swim, 30-minute elevated platform stress, or 30-minute restraining stress in a tube for the entire 6 weeks of the behavioral experiments. Mice were not stressed on the weekends during the duration of the radial arm maze assay.

### *Field Slice Electrophysiology*

The physiology methods used were similar to those described previously by our lab (74). All mice were anesthetized with isoflurane using a vapomatic chamber and decapitated. After decapitation, the brains were removed rapidly and placed in ice-cold, oxygenated artificial cerebral spinal fluid (ACSF). Next, 400  $\mu\text{m}$  coronal slices were cut using a vibratome and then transferred to a holding chamber containing oxygenated ACSF at room temperature.

Following an interval of at least 1 hour, slices were transferred to a submerged recording chamber and perfused with oxygenated ACSF at a temperature on average of 30° C. Slices were continuously perfused with ACSF at a flow rate of 2-3 ml/min. A bipolar stainless-steel stimulating electrode was placed in the stratum radiatum in the CA1 to stimulate the Schaffer Collateral pathway at 8-50  $\mu\text{A}$  for 100  $\mu\text{sec}$  once every ten seconds in order to sample at 0.1Hz. Stimulation intensity was adjusted to elicit an excitatory post-synaptic potential (EPSP) of ~0.8 mV at the beginning of each experiment. Recordings were performed in current clamp mode to measure excitatory postsynaptic potentials using an Axopatch 200B or MultiClamp 700B amplifier (Molecular Devices). Field recording electrodes were borosilicate glass patch pipettes (2-3  $\text{M}\Omega$ ) filled with 1 M NaCl. Theta burst stimulation was used to invoke LTP, which consisted of two bursts with each burst consisting of 10 sets of 5 pulses, each pulse lasting 100

$\mu$ sec and applied at 100 Hz with 200 ms between each set. There was a 20-sec delay between the two bursts. (S)-3,5-dihydroxyphenylglycine (DHPG; Tocris), was used to induce long-term depression (LTD).

For analysis, the value of the EPSPs slopes was calculated using pClamp10.4 Clampfit software (Molecular Devices). EPSPs initially measured every 10 seconds were averaged into 1-minute intervals. EPSP normalized slope values were compared for significance 20-25 minutes post-theta burst stimulus. The time points of acute depression at 26-30 minutes as well as long-term depression at 41-45 minutes and 81-85 minutes were analyzed from the DHPG experiments for statistical significance between the groups. For the paired pulse ratios, the last 5 minutes of baseline and 30-35 minutes for post-conditioning were used. Only one experiment was performed per slice, with the reported n-value being the number of slices not the number of animals and one to three slices were used per mouse. Microsoft Excel and Origin (North Hampton, MA) software were used to organize, average, graph, and perform statistical analysis on the data.

### *Solutions and Chemicals*

Artificial cerebrospinal fluid (ACSF; in mM): NaCl, 119; NaHCO<sub>3</sub>, 26; KCl, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 0.6; glucose, 11; saturated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> (pH 7.4). Salts were purchased from Sigma-Aldrich, Mallinkrodt-Baker, or Fisher Scientific and dissolved in double distilled water.

### *Radial Arm Maze*

An eight-arm radial maze was constructed out of 7 mm thick opaque, white, plastic. The dimensions of the maze were a 27-inch (685.8 mm) diameter, 3.5 inch (88.9 mm) arm width, 6.5-

inch (165.1 mm) arm height, 9-inch (228.6 mm) arm length, and 1-inch (25.4 mm) hole diameter at the end of each arm for food baiting.

Our protocol was designed similarly to previous researchers that used the radial arm maze, showing that food deprivation was one of the most common methods for encouraging rodents to explore the maze (75). Mice were given food ad libitum until testing was initiated and then food was restricted to 4 hours a day starting at approximately 6 pm Monday through Thursday. Feeding restrictions ensured that the mice would search the maze for food. The mice had ad libitum access to food Friday night through Sunday night since no testing was done over the weekends. Mice were weighed at the beginning and end of each week during the testing period to verify they maintained a healthy body weight. If mice lost more than 15% of their body weight or seemed lethargic, they were allotted additional time to feed. Only a few mice needed extra time to feed, which would only occur within the first two weeks.

Testing consisted of 5 trials per day, 5 days a week for 6 weeks. Week 1 was an acclimation week where all eight arms were baited with small pieces of cheese that the mice could eat quickly without biasing the time each trial took to complete. For weeks 2-6, only four arms were baited instead of eight and those same four arms were baited for every trial. A trial was considered complete after the mouse found all four bates or if the timer reached 5 minutes.

The data recorded by researchers from each trial were the time, the number of reference (long-term) memory errors, and the working (short-term) memory errors. A reference memory error was recorded when the mouse would go down an arm that never had food in it. A working memory error was recorded when the mouse would go down an arm it had already been down during the same trial. Videos of each trial were recorded using a GoPro Hero 3 camera and analyzed using ANY-maze software (Stoelting Co.; Wood Dale, IL; version 4.99m).

### *Reverse Transcriptase Quantitative PCR (RT-qPCR)*

DNA and RNA sequences for each gene were downloaded from PubMed and used for primer design. Exon sequences from each gene were selected and compared to the RNA sequence to ensure that the intended sequence did not undergo alternative splicing. Primers were designed using Primer Express (Applied BioSystems), such that when possible primer sets (forward and reverse primers) would lie on either side of an exon-intron boundary. This prevented amplification of genomic DNA. However, there were five targets whose forward and reverse primers were on the same exon: BDNF, EIF4BP1, DRD1, DRD5, and 18S. Table 1 contains all the sequences and provides information about which exons the primers bind to, along with where they are located in the RNA sequence. We used ensembl.org and Primer Express to determine the exons where the primers bound. The BDNF primers were both located on exon 9, which is the common coding exon (76). This is because exon 9 is included in all BDNF transcripts while other exons may or may not be included. This was done to ensure we were examining all potential BDNF transcripts and not missing some BDNF variants. DRD5 has only one exon, and DRD1 and EIF4BP1 have one major exon and other smaller exons that proved more difficult for good functioning primers across exon-intron boundaries. 18S was designed for a site that is specific for both rat and mouse, and keeps our control gene consistent in both. The design parameters were defined with an optimal annealing temperature range from 55-59° C. The range of GC content was set for 40-60%, with primer lengths ranging from 18-30 base pairs. The range for amplicon length was set for 100-150 base pairs. ThermoFisher Scientific/Life Technologies manufactured final primer set sequences. All primers, including 18s, were efficiency tested using serial dilutions of whole mouse brain cDNA template, and

adjusted to be at 90-95% efficiency. The 18S primers used had been previously designed by our laboratory (77). See Table 3.1 for the list of primer sequences used.

The entire hippocampi from both hemispheres were removed from the brain and the dorsal portions were separated from the rest of the hippocampi. Dorsal hippocampal tissue was homogenized, and mRNA extracted using TriZOL (Invitrogen; Carlsbad, CA) as per manufacturer instructions. After extraction, samples were placed into a reverse transcription mixture containing iScript reaction mix and reverse transcriptase (BioRad). This mixture was cycled in a C1000 Thermocycler (BioRad) according to the iScript reaction protocol, which was 25° C for 8 minutes, 42° C for 60 minutes, and then 70° C for 15 minutes. The cDNA was stored at 4° C.

For the quantitative PCR procedure, cDNA from the iScript reverse transcriptase reaction described above was used. Each target was run individually in triplicates (triplicate values were averaged together for analysis). Each sample was run on a CFX96 qPCR machine (BioRad) using Sso Fast EvaGreen Supermix (BioRad) according to the following protocol: 95° C hot start for 3 minutes, followed by 50 cycles of 95° C for 15 seconds, 57° C for 20 seconds, and 72° C for 25 seconds. Amplification was measured using FAM (excitation at 488 nm, absorption at 494 nm, and emission at 518 nm) by detecting increased relative fluorescence during each cycle. A cycle threshold (Ct) value was assigned to each target using BioRad CFX Manager software. The 18S ribosomal gene was the housekeeping control gene used for expression comparison. Samples from each target were also examined using 4% agarose gel electrophoresis to verify amplicon size (Figure 3.5B). Relative quantities of gene expression were determined using Microsoft Excel and the Livak and Schmittgen delta delta Ct/Cq method (35). For each individual target, all four mice groups were run on the same plate for more accurate comparison.

If a sample failed to have the majority of three replicates show up, then we did not use it and thus there are varying sample sizes for the different targets. Samples were re-run if pipetting errors occurred during the first run.

### *Corticosterone Competitive ELISA*

To determine differences in corticosterone levels, a corticosterone ELISA kit was purchased from Enzo Life Sciences. Whole blood was collected between 8 – 11 AM from the electrophysiology mice subsequent to isoflurane anesthesia and decapitation. Decapitation could alter corticosterone levels, but since all four groups underwent decapitation, the data comparing the groups should still be valid. For each sample, 1 mL of blood was added to a plastic tube containing 100  $\mu$ L of heparin to prevent clotting. The blood samples were stored at  $-80^{\circ}$  C until the ELISA was performed. Blood samples were brought to room temperature and processed according to the manufacturers specifications. The plate was read at a 405-nm optical density on a BioTek Synergy HT plate reader using the BioTek Gen5 1.11 software. Triplicates were made of all samples, including controls. Control values were then plotted into a logarithmic graph using Microsoft Excel. The equation obtained from the best-fit line in the graph was used to determine the corticosterone concentrations of the whole blood samples from the four treatment groups.

### *Statistical Analysis*

The results in the graphs are presented as mean  $\pm$  S.E. The n values for all the experiments are reported in the figure captions. The LTP, RT-qPCR, and ELISA data were analyzed using a two-way (2x2) ANOVA and one-way ANOVA with Bonferroni's post-hoc test. For the LTP data, since our major comparison was between the EWS group to all other 3 groups,

it was appropriate to also use a two-tailed unequal variance Student's T-test for pairwise comparisons between groups to compare if the means between EWS and all other groups was significantly different. The radial arm maze data as a whole were analyzed using a mixed model that is similar to a regression, but incorporated a random effect since not all observations were independent since there were multiple data points for each mouse and post-hoc Tukey's tests were also used after confirmation of normal distribution. The 2x2 ANOVAs were also performed. When analyzing just the second week of the radial arm maze data, one-way ANOVAs were used for a comparison of the means between the test groups. Paired pulse ratios were analyzed with Wilcoxon rank sum tests to compare baseline or post-conditioning within a group. A one-way ANOVA was used to compare the baseline paired pulse ratios between all of the groups. A p value of  $<0.05$  was considered statistically significant for all tests performed. A p value between 0.06 and 0.15 was considered a trend.

## Results

First, it was necessary to demonstrate that our stress and exercise models could effectively alter LTP as noted by others. We used a form of chronic stress, lasting for 3 days with a different stress each day. We waited at least an hour after stress before sacrificing the mouse to perform the electrophysiology experiments. As others have shown differences in LTP between control, exercise, and stress, our primary goal was to note whether exercise could mitigate the negative effects of stress (i.e. is the exercise with stress LTP significantly bigger than stress alone). The sedentary with stress (SWS) group experienced smaller ( $144.3 \pm 4.9\%$ ) LTP compared to the sedentary no stress group (SNS;  $171.6 \pm 4.7\%$ ; Figure 3.1A). In addition, as expected, the exercise no stress (ENS) group experienced LTP ( $192 \pm 7.3\%$ ) that was larger than the SWS group, demonstrating that our exercise protocol was sufficient to induce changes in LTP



mechanisms. Important to this study, exercise with stress (EWS) mice, the novel group in this experiment, had significantly greater ( $p < 0.05$ ;  $165.8 \pm 3.7\%$ ) LTP than SWS mice and was extremely similar to SNS mice, but still significantly smaller ( $p < 0.05$ ) than ENS LTP (Figure 3.1A). A one-way ANOVA with a Bonferroni post hoc test was performed and showed significant differences between the groups ( $F_{3, 56} = 5.471$ ,  $p = 0.002$ ). To further examine interactions between stress and exercise, we also performed analysis using a two-way ( $2 \times 2$ ) ANOVA and demonstrated a significant effect of exercised and non-exercised groups ( $F_{1, 56} = 3.804$ ,  $p = 0.05$ ) and between stressed and non-stressed groups ( $F_{1, 56} = 9.403$ ,  $p = 0.003$ ), again confirming that stress and/or exercise have a significant effect on LTP. The average daily running distances between ENS ( $6.31 \pm 0.52$  km) and EWS ( $4.40 \pm 0.53$ ) mice was significant (T-test  $p = 0.01$ ). Collectively, this demonstrates that not only do our exercise and stress methodology have effects on plasticity, but also that exercise significantly mitigated the negative effect of stress on LTP.

We used the regression function in Microsoft Excel to examine whether there was a relationship between distance mice ran and percentage of LTP achieved in both the EWS and ENS groups (i.e. does the amount of exercise correlate to the amount of LTP), but there was not a correlation. This suggests that there is a ceiling on the effect exercise can have on LTP after a minimum amount of exercise.

Considering that stress may also alter another form of synaptic plasticity, long-term depression (LTD), we examined whether there was stress and exercise induced changes in metabotropic glutamate receptor (mGluR)-dependent LTD (Figure 3.1B). DHPG, a type 1 mGluR agonist, was applied to the bath to induce LTD. No significant changes to mGluR-LTD were mediated by the stress or exercise protocols used in this study.

Paired pulse ratios from LTP experiments were examined to determine if exercise or stress were having a pre-synaptic effect on plasticity (Figure 3.2). Wilcoxon rank sum tests showed no significant difference between baseline and post-conditioning ratios within the groups. A one-way ANOVA showed no significant differences between the paired pulse ratios of the baselines or post-conditioning between the four groups.

A competitive ELISA was used to determine whole blood concentrations of the stress hormone corticosterone among the groups after three days of stress. Significant differences were observed in corticosterone concentrations among the four groups (Figure 3.3). The ENS group had significantly less ( $p < 0.05$ ) corticosterone in their whole blood compared to the SWS group. The SWS group also had significantly more ( $p < 0.05$ ) corticosterone than the SNS group. The 2x2 ANOVA ( $F_{1, 27} = 15.797$ ,  $p = 0.0004$ ) also revealed that there was a significant difference in corticosterone between stressed and not stressed groups. These data demonstrate our stress methods were successful in evoking physiological changes in corticosterone, and interestingly it is suggestive that corticosterone alone cannot account for differences in plasticity.

Since a difference in plasticity was noted, we wanted to examine whether behavioral changes could be seen in these four groups using the memory assay, the radial arm maze. However, one issue was that in order accomplish this we would have to increase the duration of our stress model to the 6 weeks required to perform the radial arm maze in mice. While this is a different stress and thus not comparable to plasticity data, we still thought it important to examine potential changes between all the groups behaviorally as no one had compared exercise with stress alone in the radial arm maze to our knowledge. Therefore, the physiology and behavioral data are not directly comparable, but provide mutual support for one another by

examining the behavioral effect of stressed mice that concurrently exercise versus those that do not.

The radial arm maze tests the spatial short-term and long-term memory between the treatment groups. Reference (long-term) memory errors, working memory (short-term) errors, total distance traveled per trial, and total time to complete each trial were measured (Figure 3.4). The chronic stress protocol for these mice lasted the duration of the behavioral experiments as well as the running. The behavioral ENS and EWS data were extremely close to each other and the SNS and SWS data were very similar to each other as well. The exercise groups made significantly fewer ( $F_{1, 40}=2.901$ ,  $p=0.04$ ) reference memory errors than the sedentary groups during the second week of the maze. Trends ( $p=0.06-0.15$ ) were also observed in the working memory errors between the exercise and sedentary groups, with exercise groups making less errors than sedentary groups. In conclusion, exercise increased learning and memory capabilities in the observed mice during the second week of testing. A 2x2 ANOVA did not show any significance between the relation of stress, exercise, or stress and exercise between the groups.

Next, we examined molecular adaptations that could potentially be involved in the molecular mechanisms of stress and exercise effects on the brain. To do this, RT-qPCR was used to study specific mRNA expression levels in the dorsal hippocampus. Primers were designed for fifteen different targets. Targets included elements involved in the exercise/BDNF pathway, which were BDNF, mTOR, TrkB receptor, EIF4EBP1, and p70s6K. TrkB is the receptor that binds BDNF. EIF4EBP1, mTOR, and p70s6K are downstream targets of the BDNF pathway. Stress targets included glucocorticoid and mineralocorticoid receptors, which bind corticosterone. Dopamine 1 and dopamine 5 receptors were also examined since other researchers have demonstrated that these play a role in the BDNF pathway in the pre frontal

cortex (78) and can modulate LTP and LTD (79). The N-methyl-D-aspartate (NMDA) receptor plays a vital role in synaptic plasticity, especially in LTP, so the expression levels of the different NMDA receptor subunits and the NMDA anchoring protein postsynaptic density protein 95 (PSD-95) were examined. Lastly, the A1 and A2 subunits of 5' adenosine monophosphate-activated protein kinase (AMPK) were inspected to discover whether AMPK expression in the hippocampus increased with voluntary running exercise. The reason why we chose to look at AMPK is because other researchers have discovered increases in AMPK expression in skeletal muscle and the brain due to exercise and AMPK is thought to be crucial to energy metabolism (80); AMPK is also thought to affect the BDNF pathway in the hippocampus (81).

The dorsal portion of the hippocampus was separated from the rest of the brain before isolating mRNA. The mice used for RT-qPCR were the same ones that performed in the radial arm maze in order to reduce animal numbers used. The whole hippocampus was not used because it is known that the dorsal and ventral portions of the hippocampus have differing response to stress (70). As a result, the PCR data cannot be directly correlated to the behavioral data of intact mice. As an important note, parallel processing of all four groups avoided aberrant variations between mRNA isolation or PCR runs. The dorsal hippocampus had several targets that showed significant differences from one-way ANOVAs ( $p < 0.05$ ) in expression level among the treatment groups. The targets that had significant differences in expression levels were BDNF ( $F_{3,24} = 4.918$ ,  $p = 0.008$ ), TrkB receptor ( $F_{3,27} = 3.319$ ,  $p = 0.035$ ), glucocorticoid receptor ( $F_{3,22} = 6.404$ ,  $p = 0.003$ ), mineralocorticoid receptor ( $F_{3,29} = 7.542$ ,  $p = 0.001$ ), and dopamine 5 receptor ( $F_{3,26} = 14.527$ ,  $p = 0.0004$ ) (Figure 3.5A). In general, the ENS mice displayed trends of increased expression of almost all the targets examined with some being significant when

compared to one or more of the other three groups, indicating that exercise alone has the greatest effect for increasing mRNA expression levels of the targets we studied.

Two-way (2x2) ANOVAs were also performed on each of the targets. This analysis demonstrated that other targets besides the ones that were significant in the one-way ANOVAs had differences in expression levels among the treatments. The three targets that showed the most significant differences between the treatments in the 2x2 ANOVA were the dopamine 5 receptor, glucocorticoid receptor, and mineralocorticoid receptor. Dopamine 5 receptor showed a significant difference between stress ( $F_{1,30}=35.955$ ,  $p=0.0004$ ), exercise ( $F_{1,30}=5.316$ ,  $p=0.029$ ), and a trend ( $F_{1,30}=3.669$ ,  $p=0.066$ ) for the exercise and stress interaction. Glucocorticoid receptor had a significant difference between stress ( $F_{1,26}=14.739$ ,  $p=0.001$ ) and stress and exercise combined ( $F_{1,26}=5.347$ ,  $p=0.03$ ). Mineralocorticoid receptor showed a significant change between exercise ( $F_{1,33}=5.263$ ,  $p=0.029$ ), stress ( $F_{1,33}=13.635$ ,  $p=0.001$ ), and exercise and stress combined ( $F_{1,33}=4.023$ ,  $p=0.05$ ). Other targets also had some significant differences in the 2x2 ANOVA, which was due to stress groups having decreased expression: BDNF ( $F_{1,28}=8.177$ ,  $p=0.009$ ), EIF4EBP1 ( $F_{1,24}=6.345$ ,  $p=0.02$ ), NMDA1 ( $F_{1,29}=4.863$ ,  $p=0.037$ ), NMDA2A ( $F_{1,33}=6.890$ ,  $p=0.014$ ), p70s6K ( $F_{1,28}=7.727$ ,  $p=0.01$ ), and PSD95 ( $F_{1,29}=6.566$ ,  $p=0.017$ ). In the 2x2 ANOVA, TrkB was the only target besides the dopamine 5 receptor and mineralocorticoid receptor that showed significant changes ( $F_{1,31}=5.978$ ,  $p=0.021$ ) due to exercise, because both exercise groups had greater expression than both sedentary groups.

## Discussion

Overall, the data demonstrate that exercise mitigates some of the negative effects chronic stress has on LTP and memory. The novel part of our electrophysiology data revealed that if exercise and stress occur concurrently, the exercise is able to combat the stress so that the dorsal

hippocampus can experience normal levels of LTP. Our ELISA data illustrate that corticosterone is less likely playing a role in the effects on LTP that we observed among the four treatment groups and suggest a neuroprotective effect from exercise by another target. Our radial arm maze data showed that exercise was having positive effects on spatial memory when initially learning a new task. The RT-qPCR data identified some potential targets for future examination that exercise, stress or a combination of the two can modify. While the stress paradigms differed between our LTP, behavior and PCR experiments (i.e. due to different stress duration, variation in animal age upon experimentation, etc.), they collectively demonstrate that exercise when occurring concurrently with stress can alter the outcomes of stress alone.

The novel finding of our study is the fact that exercise was able to mitigate the negative effects stress has on dorsal CA1 LTP, bringing the animal back to normal (control) levels of LTP. In a somewhat related study, another group examining ventral hippocampus demonstrated that exercise alleviated chronic sleep deprivation decreases in LTP (68). Our data in combination with their data show that exercise is able to make positive changes in multiple portions of the hippocampus even while experiencing stress. Our SWS electrophysiology data support that chronic stress reduces CA1 LTP particularly in the dorsal portion, which adds to LTP data obtained by other groups in the CA1 (50, 68) and dentate gyrus (82) showing that stress alone reduces LTP levels. We also saw that exercise enhances dorsal CA1 LTP, supporting what others have shown indicating that exercise alone increases LTP in the hippocampus, as occurs in the dentate gyrus (60). Based on the previous published literature and our electrophysiology data, the ideal situation for improving hippocampal LTP and thereby learning and memory would be to experience no stress and to exercise. However, since stress is unavoidable throughout life, knowing that exercise can likely combat the deleterious effects stress has on dorsal CA1

hippocampal LTP is a significant finding. It could also be said that perhaps chronic stress eliminates the boost to LTP caused by exercise. Since none of the paired pulse data had any significant changes, this suggests that the differences we observe in LTP due to exercise and/or stress are more than likely being caused by an alteration to a standard postsynaptic CA1 LTP mechanism. If we did see changes, it would have suggested an alteration in a presynaptic mechanism, but we did not.

Our ELISA data demonstrate that exercise could be having a neuroprotective effect on the dorsal hippocampus, which has been suggested by others (8, 17, 58, 83, 84), despite increased levels of corticosterone. Exercise mitigation of stress effect is a mechanism independent of corticosterone as there were no differences in corticosterone between EWS and SWS mice even though there was a difference between their LTP. Not seeing a difference between EWS and SWS corticosterone levels supports what has been reported by Campeau et al. where exercise does not have an effect on the HPA response if severe stressors are used (85). Our ELISA data are in line with other studies that show that chronic stress increases glucocorticoid release and suppresses LTP in the hippocampus (8, 17, 41, 86) and that chronic increases of glucocorticoids can damage the hippocampus and hinder its functionality (57, 84), supporting our methodology. The lower levels of corticosterone in ENS mice could be why we saw elevated mRNA expression of mineralocorticoid and glucocorticoid receptors in this group, but there are also other glucocorticoids and stress hormones that we did not measure that could be causing the increased stress receptor mRNA expression levels observed in the ENS mice.

While no changes in LTD were observed among the groups, other studies found alterations in hippocampal LTD between acute stressed rats and control rats using DHPG, and that higher levels of corticosterone contributed to the change in mGluR-dependent LTD (87-89).

The difference between our study and theirs could be due to differences in stress techniques because our model was chronic stress and not acute stress like the Chaouloff group. Furthermore, the same pathways that are altered by exercise may not affect this type of LTD. Therefore, it cannot be ruled out that exercise and the type of stressors we used could have an effect on other forms of LTD, such as NMDA-dependent LTD, since other groups (8, 50) have shown that chronic stress can alter AMPA and NMDA dependent LTD.

To examine the effects exercise and stress have on spatial memory, we used the radial arm maze since it is commonly used in the literature to test spatial memory. While these experiments are not directly comparable to the LTP experiments, our behavior data support the notion that exercise rescues some of the changes caused by chronic stress. In relation to reference memory errors, the EWS mice performed as well as the ENS mice, and both the exercise groups performed better than the sedentary groups of mice showing that exercise improves learning and memory of a new task. Others have shown that just being sedentary reduces brain functionality (90), which is probably why both sedentary groups performed similarly in our maze. Exercise enhanced the ability of the mice to learn the maze, since exercise mice made significantly fewer reference memory errors in their second week in the radial arm maze. This is similar to data obtained by some researchers who noted memory assay changes using the Morris water maze in either stress or exercise models (60, 71, 91), however in contrast, others noted no changes in Morris water maze performance in exercised, stressed, and control female mice (71). Kim et al compared male chronic stressed with exercise mice to sedentary chronic stressed mice using the Morris water maze and saw that the exercise with stress mice traveled less distance and found the platform faster than the sedentary with stress mice. Though most publications used the Morris water maze to test spatial memory, we chose not to because of the forced swim aspect of this



assay, which is why we chose the radial arm maze instead. Also, while researchers have demonstrated stress (92) or exercise (93) effects using the radial arm maze, as far as we know, we are the only group that has performed the radial arm maze on male mice that were stressed and exercised concurrently. Particularly important to note in this study is that exercise, when occurring with stress, eliminated the increased reference memory errors at the beginning of the assay associated with stress alone. This data indicate that exercise can help expedite learning new tasks, regardless of being stressed and fasted. It is important to note that while we had to house mice individually in order to track the exercise amount of each animal accurately, and therefore mice needed to be housed individually to reduce variables in our study, individual housing can create stress. Also, it could be suggested that the exercised mice had enrichment with the running cage. Therefore, our data must be considered with these caveats and that control mice may have had added stress, which may have reduced our overall stress effect compared to others. Despite this added stress to all the groups of mice, we still saw differences in the radial arm maze, though not as dramatic as other studies that used Morris water maze.

In addition, it was anticipated that RT-qPCR would identify definitive targets that were altered in exercise, stress, or the combination of the two. Some potential candidates that may play significant roles in molecular changes in the brain due to exercise and stress are dopamine 5, glucocorticoid, and mineralocorticoid receptors since they showed the most significant changes between ENS and the other treatment groups in one-way ANOVAs, and all showed differences when stress and exercise were both examined using 2x2 ANOVAs. The dopamine 5 receptor was the only target that showed a significant difference between the SNS and SWS groups, with the SWS group having lower mRNA levels. Dopamine 5 receptors are coupled to adenylyl cyclase and alter the BDNF pathway in the prefrontal cortex (78) and could be doing

something similar in the dorsal hippocampus. As exercise increases dopamine 5 receptor mRNA expression and stress decreases it, dopamine 5 receptor is an interesting target to examine the changes due to exercise. Regarding dopamine 1 receptor mRNA levels, we did not see significant changes in expression, which correlates to another study examining the effects of exercise in the caudate putamen of rats (94). In summary, as far as we know, the discoveries we have shown on how the dopamine 5 receptor dorsal hippocampal mRNA expression changes due to exercise and stress are novel and have not been shown before, which adds to the possibility of dopamine 5 receptor playing a role in memory changes.

In general, ENS mice had enhanced expression of several targets that were not elevated in the other three groups. The BDNF pathway was indeed more activated in the dorsal hippocampus in the ENS group, which is similar to the western blot data obtained by another group (95). The BDNF pathway is a likely candidate for the positive hippocampal changes in all the targets examined due to exercise since BDNF was expressed more in our RT-qPCR data. The TrkB receptor was the only target that had higher expression levels in our data in both exercise groups compared to both sedentary groups, which indicates that the BDNF pathway activity could be helping exercise mitigate the negative consequences of stress. These observations support what has been shown by others, which is that the BDNF pathway is more activated due to exercise and can have many positive effects on the brain by acting on other pathways and receptors (95, 96) and that BDNF expression levels are elevated (61, 90) due to exercise. Specifically one group showed that the BDNF pathway was interacting with increased AMPK levels to cause the positive changes to learning and memory (71) and we also saw a trend ( $p < 0.15$ ) of increased AMPK A1 subunit expression in our ENS mice compared to the stressed and sedentary groups. It could also be presumed that the NMDA receptor subunits 2A or 1, and

the NMDA receptor anchoring protein PSD-95 could also be involved in the changes in spatial memory caused by ENS, since we did show a trend ( $p < 0.15$ ) of increased expression in those plasticity elements in our ENS mice. Two other groups of researchers also showed increases in PSD-95 protein in exercised rodent hippocampi compared to sedentary (91, 95). The increased expression levels of these proteins involved in synaptic plasticity could be affected by the increased activation of the BDNF pathway, which has been suggested by other researchers (62, 97-99).

As a note, while it has been shown that EWS mice voluntarily run less than ENS mice (73), we did not have a significant difference ( $p = 0.21$ ) in our mice used for behavior/PCR, only in our electrophysiology exercise mice ( $p = 0.01$ ). Therefore, decreased exercise may not account for mRNA expression differences between EWS and ENS. A possible reason why we did not see a significant difference, only a partial difference could be because all the behavior/PCR mice underwent food deprivation, which is a stress, and could have caused the ENS mice to not run as much. Collectively, the data still suggest there is probably more than one molecular pathway, and/or many receptors involved in any effect exercise has on mitigating stress changes. However, it is still uncertain how many molecular targets are specifically interacting between exercise and stress when stress and exercise are experienced concurrently in the same animal. One last caveat to consider is that mRNA levels do not always correlate to protein expression or activation of signaling pathways involved; mRNA expression means that a certain gene was transcribed more, but this does not indicate simultaneous increased translation. Therefore, we cannot exclude any of the pathways we examined based on PCR data for their potential involvement.

## Conclusion

Overall, our data does suggest, even with the differing stress protocols, that exercise may be a legitimate and cost-effective treatment or adjunctive therapy for mitigating the negative effects stress has on dorsal CA1 hippocampal LTP and spatial memory function. Exercise can improve spatial memory and hippocampal LTP when compared to being sedentary. Therefore, our study suggests understanding the interactions between exercise and stress when co-occurring is an important consideration.

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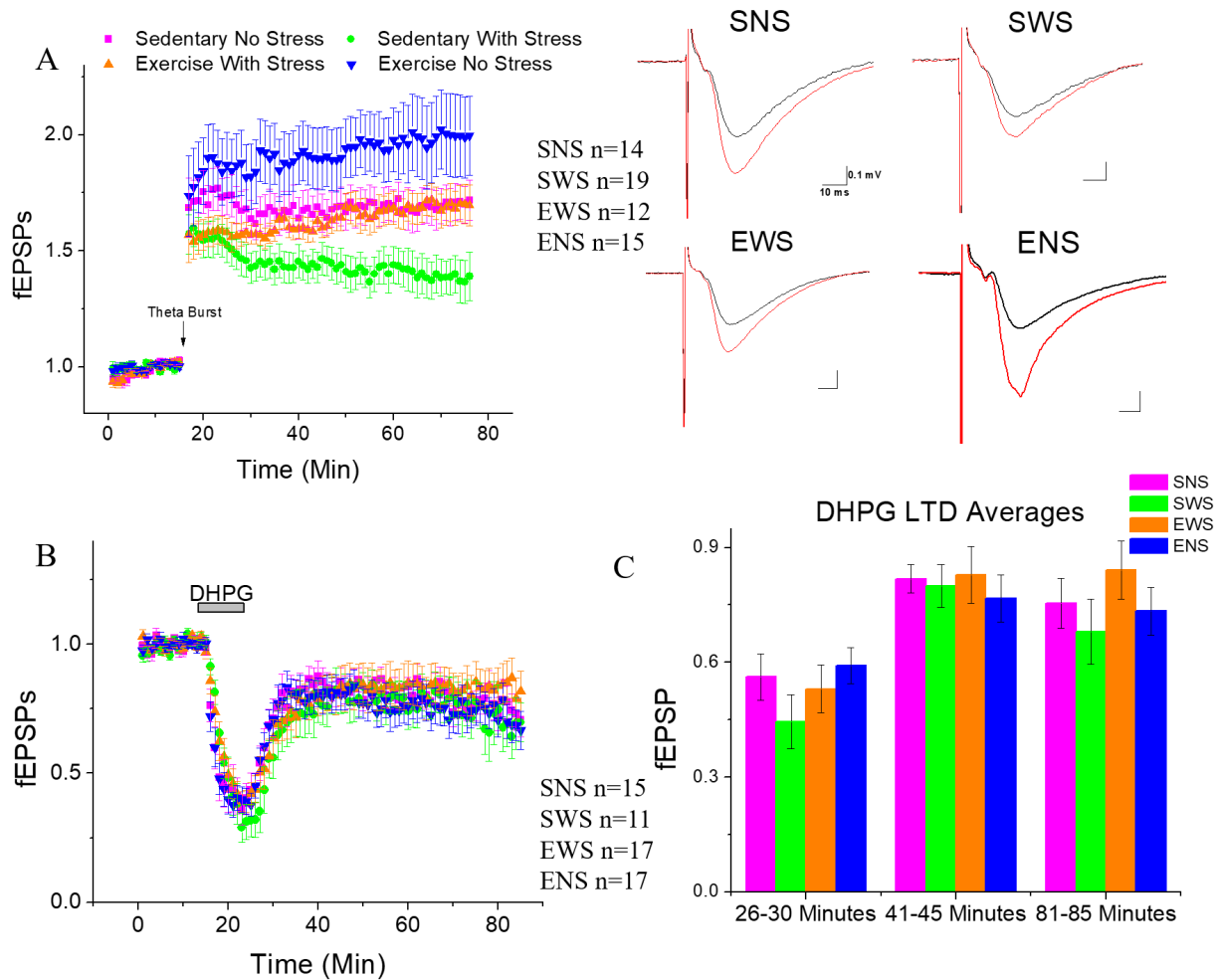


Figure 3.1: Exercise and Stress Significantly Alter Theta Burst Induced LTP, but Not DHPG-mediated LTD. A) The important comparison was that exercise with stress mice had greater theta burst-induced (arrow) LTP as measured using fEPSPs than SWS mice ( $p < 0.05$ ). ENS mice exhibited the largest levels of LTP that were greater than EWS, whereas SWS significantly decreases LTP compared to SNS ( $p < 0.05$ ). EWS mice have similar levels of LTP as SNS mice ( $p > 0.05$ ). The biggest difference in LTP was between the ENS and SWS groups ( $p=0.004$ ). Sedentary No Stress (SNS,  $n=14$ ); Sedentary With Stress (SWS,  $n=19$ ); Exercise With Stress (EWS,  $n=12$ ); Exercise No Stress (ENS,  $n=15$ ). Percent changes for LTP when compared to pre-conditioning baseline: SNS =  $171.6 \pm 4.7\%$ , SWS =  $144.3 \pm 4.9\%$ , EWS =  $165.8 \pm 3.7\%$ , ENS =  $192 \pm 7.3\%$ . Inset: average of 10-15 traces taken just before (black) and 28-30 minutes after (red) the conditioning theta burst stimulation. B) This dot graph represents the average slopes throughout the DHPG experiments. There were no significant differences in metabotropic glutamate receptor (mGluR)-dependent LTD induced by 10 minutes of DHPG ( $100 \mu\text{M}$ ) application among the four groups as analyzed by one-way ANOVAs (SNS  $n=15$ , SWS  $n=11$ , EWS  $n=17$ , ENS  $n=17$ ). C) This bar graph compares the average slopes of all the groups at multiple time points during the DHPG experiments and one-way ANOVAs determined that there were no significant differences at any of the three-time points measured.

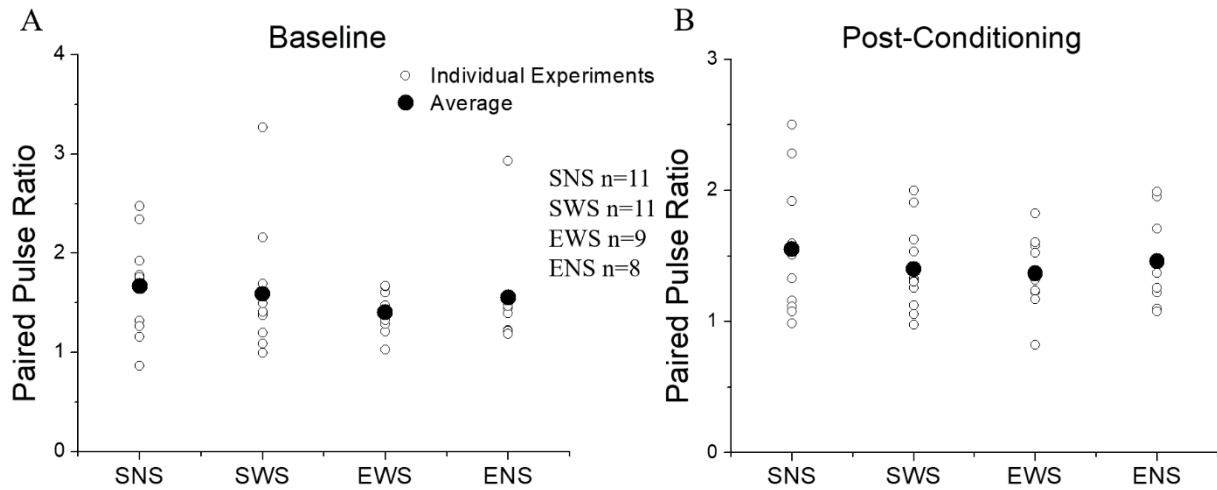


Figure 3.2: Paired Pulse Ratios of Electrophysiology Experiments for All Four Groups. ENS n=8, EWS n=9, SWS n=11, SNS n=11. A) A plot of all the baseline ratios for all four groups. A one-way ANOVA showed no significant differences between the paired pulse ratios of the baselines between the four groups. B) A plot of all the post-conditioning ratios for all four groups. A one-way ANOVA showed no significant differences between the paired pulse ratios between the four groups. This indicates that stress and exercise were not likely having a presynaptic effect on LTP.

### Levels of Whole Blood Corticosterone

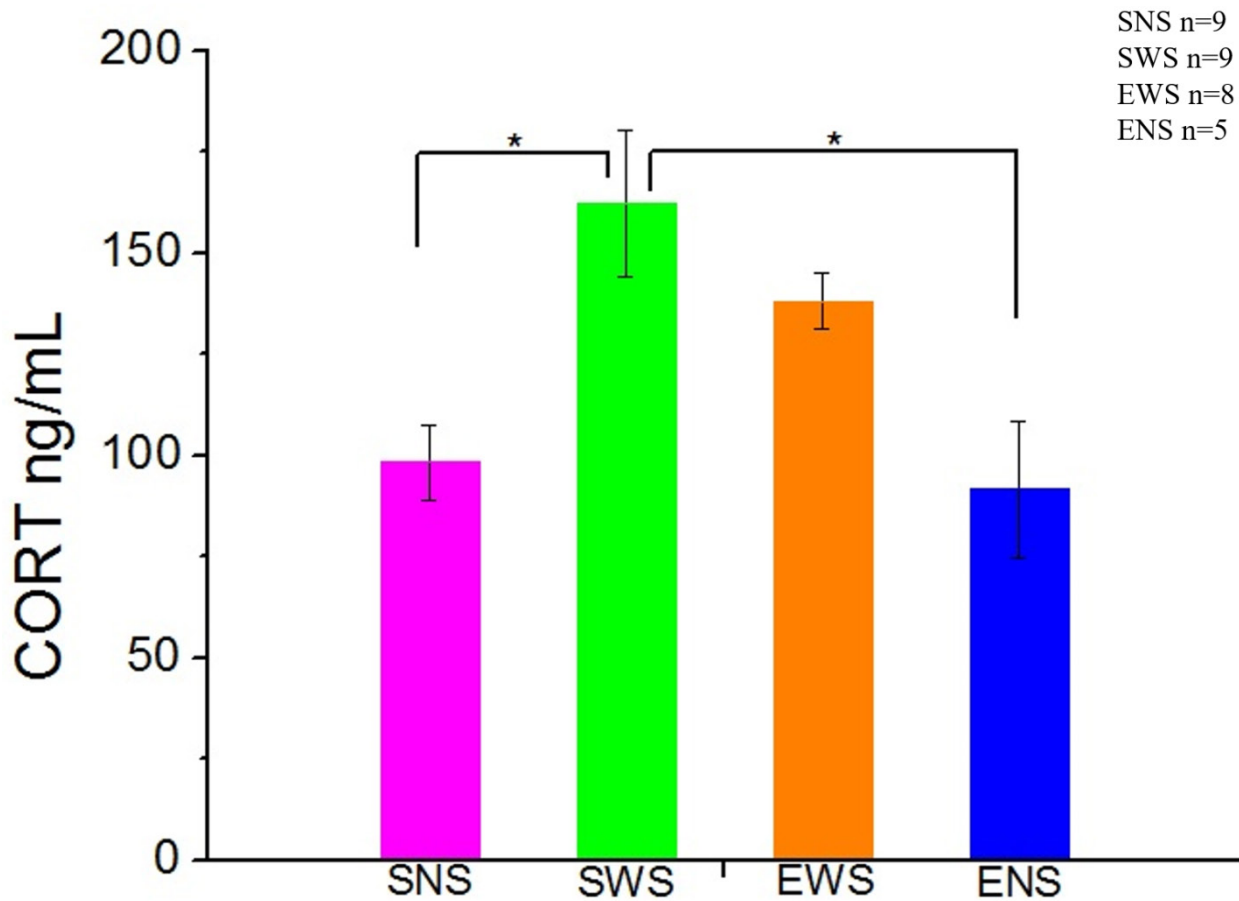


Figure 3.3: Corticosterone Blood Concentrations for All Four Groups Measured by an ELISA. A one-way ANOVA ( $F_{3,27}=6.098$ ,  $p=0.003$ ) showed significant differences in corticosterone concentrations with a Bonferroni post-hoc test. SWS mice had significantly more corticosterone compared to SNS mice ( $p < 0.05$ ). ENS mice had significantly less corticosterone than SWS ( $p < 0.05$ ). There were trends ( $p < 0.15$ ) in differences of corticosterone levels for EWS vs SNS and EWS vs ENS, with EWS having increased levels. A 2x2 ANOVA indicate that corticosterone levels were significantly different ( $F_{1,27}=15.797$ ,  $p=0.0004$ ) between stressed groups and not stressed groups. (SNS n=9, SWS n=9, EWS n=8, ENS n=5). An asterisk (\*) here denotes  $p < 0.05$ .

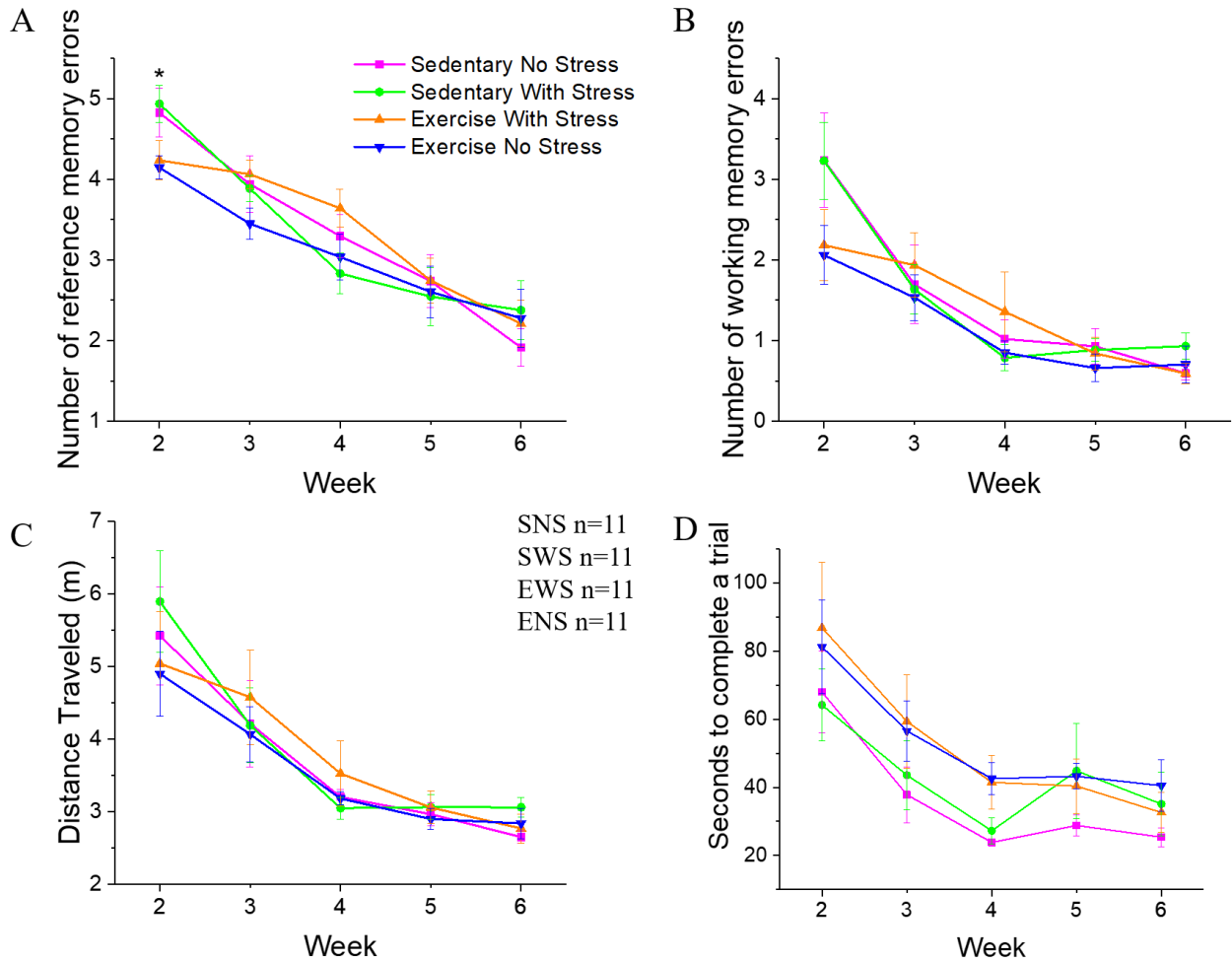


Figure 3.4: Radial Arm Maze (RAM) Results. RAM results indicate learning and memory differences between exercise and stressed mice during the second week of testing. In all the graphs, there was a significant difference ( $p < 0.05$ ) between weeks for all groups showing that all groups improved in maze performance from one week to the next ( $n = 11$  for all groups and graphs). A) Average reference (long-term) memory errors per trial for all groups over the course of 5 weeks. A one-way ANOVA revealed significant differences ( $F_{3,40} = 2.901$ ,  $p = 0.04$ ) in week 2 between exercised and sedentary mice, with the exercise mice making fewer errors (SNS vs SWS  $p > 0.5$ ; EWS vs ENS  $p > 0.5$ ), but significant ( $p < 0.05$ ) differences were importantly noted between SWS vs EWS as well as SNS vs ENS, and SWS vs ENS. B) Average working (short-term) memory errors. Week 2 differences between exercised and sedentary mice were not significant according to a one-way ANOVA ( $F_{3,40} = 1.857$ ,  $p < 0.15$ ), but instead illustrate a trend of exercise mice making fewer errors. Several trends were noted between specific groups (SNS vs EWS  $p = 0.15$ , SNS vs ENS  $p = 0.10$ , SWS vs EWS  $p = 0.12$ , SWS vs ENS  $p = 0.06$ ), with no differences noted between stress or exercise groups (SNS vs SWS  $p = 0.99$ , EWS vs ENS  $p = 0.82$ ). C) There was no significant ( $p > 0.05$ ) difference between the distances traveled in each trial between the four groups. D) There were no significant ( $p > 0.05$ ) difference in time to complete the trial between the four groups.



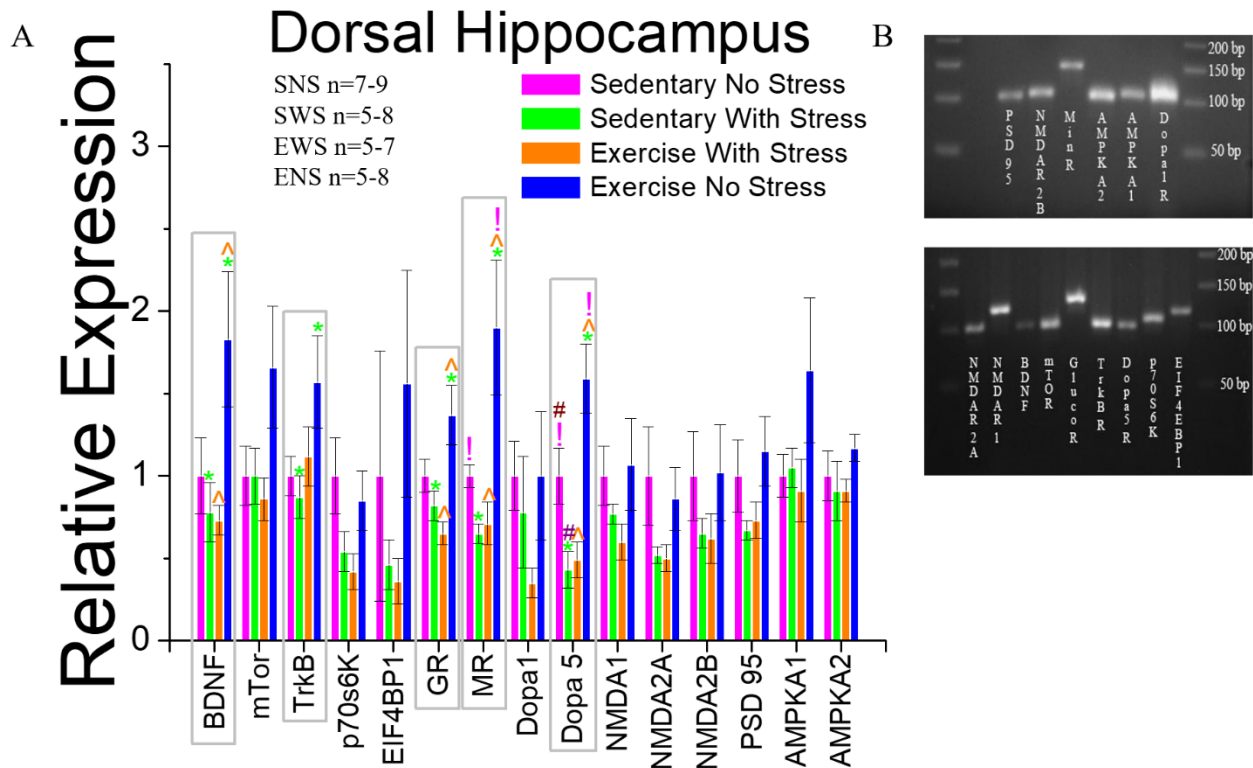


Figure 3.5: Dorsal Hippocampus RT-qPCR Results. The expression of various mRNA targets involved in the exercise and stress pathways, as well as synaptic plasticity. A) Dorsal hippocampus RT-qPCR results: one-way ANOVAs showed significant difference in expression levels for brain-derived neurotropic factor (BDNF), TrkB, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and dopamine 5 receptor. SNS n=7-9, SWS n=5-8, EWS n=5-7, ENS n=5-8; Boxes highlight targets with significant changes in mRNA expression: \* indicates  $p < 0.05$  between ENS and SWS groups; ^ indicates  $p < 0.05$  between ENS and EWS; the ! indicates  $p < 0.05$  between ENS and SNS; # indicates  $p < 0.05$  between SNS and SWS. B) Gels illustrating that the primers used were specific for the designed target as indicated by the appearance of single bands at the correct base pair amplicon length. For specific amplicon base pair lengths and primer sequences, refer to table 1. The gel illustrating 18S was noted in a prior publication (77).

Table 3.1: Primer Sequences Designed for the Genes of Interest. The table includes what part of the nucleic acid sequence the nucleotides were modeled from, the forward and reverse primer sequences, and the amplicon length in base pairs.

Mouse Gene/Target	RNA Sequence (F,R)	Forward (F) sequence	Reverse (R) Sequence	Amplicon	Exon
mTor	3376-3393, 3456-3475	AACAGCCAAGGCCGAATC	CAATCGGAGGCAACAACAAG	100 bp	F=exon 19, R=exon 20
Ntrk2 (TrkB Receptor)	2186-2210, 2266-2286	GTTATTGAAAACCCAGTACTTTG	TCCCTTCTCAGAACGATGTTG	101 bp	F=exon 22, R=exon 23
BDNF (transcript: bdnf-211, exon 2)	534-556, 615-643	GACATCACTGGCTGACACTTTTG	CAAGTCCGCTCCTTATGG	110 bp	F=exon 9, R=exon 9
EIF4BP1	623-646, 717-737	TCTGTCTATACCGAGCTGCTACTG	GCTAGGATTGCCAGATCATTC	115 bp	F=exon 6, R=exon 6
Rps6kb1 (p70s6K)	628-654, 714-733	GTGGAGGAGAACTATTATGCAGTTAG	TGATGTAAATGCCCAAAGC	106 bp	F=exon 8, R=exon 12
NR3C1 (Glucocorticoid Receptor)	2193-2212, 2300-2322	GGCAGCGGTTTTATCAACTG	TCAGCTAACATCTCTGGGAATTC	130 bp	F=exon 11, R=exon 12
NR3C2 (Mineralocorticoid Receptor)	2543-2564, 2667-2686	GCATCAGTCTGCCATGTATGAG	CTCTTGAGGCCATCTTTTGG	144 bp	F=exon 13, R=exon 14
Grin1 (NMDAR Subunit 1)	1240-1259, 1337-1359	CCACGAGCTCTAGAAAAGG	TCTGCATACTTGGGAAGACATCAG	120 bp	F=exon 5, R=exon 6
Grin2A (NMDAR Subunit 2A)	2331-2350, 2409-2430	CGCTCTGCTCCAGTTTGTTG	GCTGCTCATCACCTCATTCTTC	100 bp	F=exon 11, R=exon 12
Grin2B (NMDAR Subunit 2B)	2373-91, 2453-2474	TGGTATCACGCAGCAATGG	CAGAGACAATGAGCAGCATCAC	102 bp	F=exon 15, R=exon 16
DLG4 (PSD-95)	1864-1886, 1945-1963	AGAAAGACTCGGTTCTGAGCTATG	ATCGTTGGCACGGTCTTTG	100 bp	F=exon 20, R=exon 21
PRKAA1 (AMPK Subunit A1)	326-348, 410-429	TGTCTCTGGAGGAGACTATTTG	AATAATCCACCCGAAAGG	104 bp	F=exon 3, R=exon 4
PRKAA2 (AMPK Subunit A2)	350-373, 431-449	AGCACTCCGACAGACTTTTTATG	GCGTCCACCTCTTCAAC	100 bp	F=exon 3, R=exon 4
DRD1 (Dopamine Receptor 1A)	2836-2868, 2916-2936	TGACTGTCCAGGATTAAGATGTG	AGATGAAGAACCAGCGATGAG	101 bp	F=exon 3, R=exon 3
DRD5 (Dopamine Receptor 1B/5)	2416-2439, 2494-2515	ACAATGGTCTGCTGCTAGATAG	TCGCTCTGAAACACCTAGATG	100 bp	F=exon 1, R=exon 1
18S	1331-1351, 1440-1463	GTGCATGGCCGTCTTAGTTG	GCCACTTGCCCTTAAGAAGTTG	133 bp	F=exon 2, R=exon 2

## CHAPTER 4: Conclusion

Even though stress is a part of everyday life, several methods are possible to manage it and prevent its negative effects on the brain (97, 98). Whether it is done by natural means like exercising or eating healthy or by exogenous means like the use of pharmaceutical drugs, the effects stress has on synaptic plasticity can be reversed (27, 59, 100, 101) or prevented. By learning how to better manage stress and using treatments that promote neurogenesis and prevent deleterious changes to synaptic plasticity, there can be a decline in the number of instances in which neurodegenerative diseases occur (59) like PTSD.

Exercise has also been shown in some cases to treat anxiety, depression, and other psychological disorders (59, 102), and exercise maybe be an effective treatment for PTSD. The completion of this research indicates that prescription drugs could possibly be used to prevent PTSD in humans, and that exercise can be a factor in decreasing the effects of chronic stress or PTSD as well. It is a sincere desire that through this electrophysiology, RT-qPCR, and behavioral data, the scientific community will have a better understanding on how different types of stress affect the animal brain and how to better treat disorders and diseases related to stress, improving the quality of life for many individuals. This research as a whole could be applied to many public service personnel in uniform, such as police and military, that know they will encounter traumatic and chronic stress regularly. Knowing that they could possibly take propranolol and mifepristone as a prophylactic treatment to possibly prevent PTSD is life-changing. Also, police, military and any other stressed out professionals can use exercise to mitigate and treat the negative consequences of stress after the stressful event has already occurred.

## Future Directions

Higher dosages of propranolol and mifepristone could be studied to try and alter anxious behavior in rats. Also, a subdermal pump could be inserted into the stressed rats to more accurately deliver the drugs. This would also make sure that the rats do not experience the reoccurring injection stress, just a one-time surgery stress. Also, we could add cannabidiol treatment to the existing drug combinations since it has been shown that the endocannabinoid system is altered due to stress (*103-106*) by interacting with glucocorticoids and their receptors in the hippocampus (*107*) and amygdala (*106, 108*). We could also study the effects of the drugs individually to see if the drugs are truly have a synergistic effect and to what extent.

Since we studied the effects of running exercise on mitigating the effects of chronic stress, it would also be interesting to see if weight lifting or resistance exercise could also have positive effects on LTP and memory behavior. This would also be interesting because the effects of weight and resistance training are hardly ever studied for their possible effects on the brain.

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105. D. Hauer *et al.*, Plasma Concentrations of Endocannabinoids and Related Primary Fatty Acid Amides in Patients with Post-Traumatic Stress Disorder. *PLoS ONE* 8, (2013).
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## CURRICULUM VITAE

Roxanne Miller

1436 S State St Provo, UT 84606 · (760) 793-6211 · roxannemm87@gmail.com

### Education

Ph.D. Physiology and Developmental Biology, Dec 2017

Brigham Young University

Studying Neurophysiology under the guidance of Jeffrey Edwards, PhD

Dissertation Title: Pharmaceutical and Natural (Exercise) Mechanisms to Mitigate the Negative Impact of PTSD and Chronic Stress on Synaptic Plasticity and Memory

B.S. Pre-professional Biology, June 2009

Brigham Young University Hawaii

Minor in Biochemistry

A.A. General Education, Dec 2007

Cerro Coso Community College

### Employment

Lead Phlebotomist, Donor Center Technician, Trainer Feb 2016 – Apr 2017

Talecris Plasma Resources owned by Grifols

I inserted needles into veins of donors to collect plasma from their blood. I also tested their vitals in a screening process to ensure the donor was healthy enough to donate. I worked with Haemonetics plasma collection machines, refractometers, centrifuges, blood pressure monitors, and other laboratory equipment. As the lead phlebotomist, I would supervise 3 to 8 employees at a time directing them in their duties on the phlebotomy floor and would coordinate with other area supervisors to help the center run more efficiently. I was also the person employees would come to if they needed a problem resolved.

Protein Purification Technician I, Mar 2011 – Aug 2012

Aalto Scientific, Ltd.

I used column chromatography and organic precipitation to extract proteins of interest from human organs. Practiced cGMP and followed FDA regulations with manufacturing and paperwork.

Quality Control Analyst I, May 2010 – Mar 2011

Aalto Scientific, Ltd.

I used hospital analyzers from manufacturers like Roche, Abbott, Siemens, Haemonetics, etc. to test in vitro diagnostic controls and calibrators. Practiced cGMP and followed FDA regulations with manufacturing and paperwork.

Taxonomist, Apr 2007 – Jun 2009  
Brigham Young University Hawaii Museum of Natural History  
I did classification of marine invertebrates from around the world.

### Awards and Memberships

Academic Scholarships, Aug 2012 – Dec 2017  
Full tuition paid for by Brigham Young University Department of Physiology and  
Developmental Biology

Teaching and Research Assistantships, Aug 2012 – Dec 2017, funding received from the  
Physiology and Developmental Biology department

Research Presentation Award, Oct 2015, travel funds to present research at a conference  
Brigham Young University Graduate Student Society

Society for Neuroscience, Jan 2015-Present, Member

American Association of Clinical Anatomists, 2013-2014, Member

Academic Scholarship, Jan – Dec 2008  
Brigham Young University Hawaii Department of Biology

Who's Who Among American High School Students, 2003  
Sherman E. Burroughs High School in Ridgecrest, CA

### Teaching Experience

Teaching Assistant, Aug 2012 – Dec 2017  
Brigham Young University  
Tissue Biology and Advanced Physiology Laboratory

Substitute Teacher, Sept 2009 - May 2010  
Muroc Joint Unified School District and Sierra Sands Unified School District  
Grades Kindergarten through High School Seniors, all subjects

Teaching Assistant, Jan – Dec 2008  
Brigham Young University Hawaii  
Zoology

### Research Experience

Research Assistant, 2007-2009, 2012-Current

Brigham Young University  
Jeffrey Edwards, PhD - Neurophysiology  
Jonathan Wisco, PhD - Clinical Anatomy  
David Busath, PhD - Influenza Treatments  
Roger Goodwill, PhD - Marine Ecology of Saipan

Sole Researcher and Author, 2008-2009  
Brigham Young University Hawaii Senior Project  
“The Anti-Microbial Properties of *Euterpe oleracea*”

## Publications

### Poster and Abstract

“High Resolution Magnetic Resonance Imaging (MRI) of the larynx in human and pig cadaveric specimens” MILLER, Roxanne<sup>1</sup>, Neal BANGERTER<sup>1</sup>, Danny PARK<sup>1</sup>, Kim STEVENS<sup>1</sup>, Shelby WARD<sup>1</sup>, and Jonathan J. WISCO<sup>1,2</sup> <sup>1</sup>Brigham Young University, Provo, UT 84602; <sup>2</sup>David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

Abstract published and poster presented at the July 2013 American Association of Clinical Anatomists Conference in Denver, CO. In the process of being submitted to a peer-reviewed journal.

### Poster and Abstract

“Running Exercise Mitigates the Negative Consequences of Stress on Hippocampal LTP”  
RM. Miller, D. Marriot, J. Trotter, R. De Roque, D. Lyman, T. Hammond, J. Welch, A. Field, B. Walker, N. Christensen, D. Haynie, M. Lewis, Z. Badura, JG. Edwards Brigham Young University Provo, UT 84602

Abstract published and poster presented at the October 2015 and November 2016 Society for Neuroscience Conference in Chicago, IL and San Diego, CA. This was also chosen as a hot topic and was published in a book given to the press.

### Manuscript

“Running Exercise Mitigates the Negative Consequences of Chronic Stress on Dorsal Hippocampal Long-Term Potentiation in Male Mice”

Roxanne Miller; David Marriott; Jacob Trotter; Tyler Hammond; Dane Lyman; Timothy Call; Bethany Walker; Nathanael Christensen; Deson Haynie; Zoie Badura; Morgan Homan; Jeffrey Edwards. Brigham Young University Provo, UT 84602

Revisions submitted and under review for publication in the journal of Neurobiology of Learning and Memory

## Research Skills and Interests

- Electrophysiology techniques, applications, and theories
- Polymerase Chain Reaction (PCR) techniques, applications, and theories
- Pharmaceutical medical applications and development



- Behavioral modifications due to environmental factors and pharmaceuticals
- Preventative medicine
- Nutrition and metabolism mechanisms
- Exercise Physiology

### Extracurricular Service

Mentor for Anatomy Academy, 2012

4<sup>th</sup> grade students at Freedom Academy in Provo, UT were taught about the basics of the human body and healthy living.

Shadowed an ENT specialist physician and observed three thyroidectomy surgeries in Ridgecrest, CA, 2009

President of Brigham Young University Hawaii Pre-Medical Society, 2008

Organized and coordinated meetings, activities, workshops, fundraising, and guest lectures.

Shadowed a family medicine physician in Hauula, HI, 2008

Presidency Member of Key Club (high school chapter of Kiwanis Club), 2003-2005