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Chronic adolescent stress as a predictive factor for the risk of developing PTSD-like symptoms in adulthood

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A thesis for the partial requirement for a Master of Science degree in the department of Anatomy and Neurobiology at Virginia Commonwealth University

by

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Abstract

Post-traumatic stress disorder is a stress and trauma based psychological disorder that is defined by the DSM-IV as an anxiety disorder that affects approximately 7.8% of people in the United States. PTSD is when those who suffer a traumatic event have intense and distressing feelings, emotions, and memories for a prolonged period of time after the event. A prominent feature of PTSD is the impaired ability to properly extinguish a fear response after a dangerous trigger or stressor is no longer present, also known as safety learning. Stressors are threats perceived within the environment that activate a response within the hypothalamic-pituitary-adrenal (HPA) axis as well as the autonomic nervous system (ANS). During adolescence, the brain is within a critically sensitive period that is susceptible to damage or alterations in cognition or morphology due to stressors. Chronic stress during adolescence alters brain morphology and cognitive function into adulthood, as seen in studies involving laboratory animals. In addition to the effects of chronic adolescent stress, there are also morphological and cognitive differences due to sex caused by differences in sex hormones. Women are disproportionately affected by PTSD and are twice as likely to develop PTSD after a traumatic event. Combining these factors, we hypothesize that the ability to safety learn will be impaired by chronic adolescent stress and further hindered within female wistar rats. A mixed-modality chronic adolescent stress paradigm was used to create social stress, which simulates negative social interaction and aggression, and chronic restraint stress, which simulates a stressful situation that forces immobility. Safety learning ability was assessed using a startle paradigm created based on fear conditioning that has been used previously in multiple studies testing for behavior that is indicative of PTSD-

like behavior. In contradiction to the hypothesis, the females who underwent chronic adolescent stress did extinguish the fear and safety learn successfully better than the nonstress counterparts. In order to look at the predictability of the startle response due to the effects of chronic adolescent stress, multiple linear regression analyses were run. It was found that for the baseline, fear conditioning, and extinction days within the startle response paradigm were able to be predicted significantly, however, the days that were testing the actual fear potentiated startle response and safety learning had no significant predictability. The results of this study found that CAS increased the ability to safety learn as well as sex did not influence the ability to safety learn, which were both not supportive of the hypothesis. In addition, the regression analysis was not a reliable model of predicting startle response within CAS data. This study can be a useful steppingstone in determining the ways that chronic adolescent stress can predict how a stressor can cause an increase in the risk of psychological disorders later in adulthood.

Keywords: Post-traumatic stress disorder (PTSD), anxiety, stress, adolescence, sex differences, acoustic startle response

Introduction

At some point during one's lifetime, everyone will experience stress. While this stress can be helpful, it can cause mental and physical impairments when prolonged and overwhelming and therefore affecting livelihoods. Anxiety disorders affect more

than 40 million people in the United States, around 20% of the population (McLean et al., 2011). Categorized as an anxiety disorder within the DSM-IV and a stress disorder within the DSM-V, post-traumatic stress disorder (PTSD) affects around 7.8% of the US population and nearly 20% of people within the military specifically (Kessler, et al., 1995). One of the characteristics of PTSD is the inability to overcome excessive fear and anxiety which has been theorized to be a result of impaired fear conditioning processes (Jovanovic et al., 2012). When exposed to a severe trauma or stressor only about 7.8% of individuals will develop PTSD suggesting that there may be underlying mechanisms and risk factors that can lead to increased vulnerability (Gillespie et al., 2009).

In addition to stress as a risk factor, age and previous trauma can also contribute to the likelihood of developing PTSD (Kessler et al., 1995). Children from ages 3-17 are within the most important developmental stages of their lives, and almost 7.1% of children and adolescents in this age range develop an anxiety disorder during this time (Ghandor et al., 2019). There are times within childhood where an individual has feelings of anxiety, so determining whether an anxiety disorder is present can be a difficult task (Beesdo et al., 2009). However, due to the sensitivity of many neurological circuits, such as the HPA axis, environmental stressors causing impairments in brain region development can result in an increase in risk of developing psychological disorders (Paus et al. 2008; McCormick et al., 2008).

Another risk factor for the development of PTSD is sex (Neigh and Ali, 2016). In the United States, there are 30.5% of women who have been diagnosed with an anxiety disorder, which is significantly more than the 19.2% of men (McLean et al., 2011). As

seen with general anxiety disorders, women are almost twice as likely to develop PTSD when compared to men, with 6.1% of women and 3.2% of men having PTSD (Kimerling et al., 2018). It has also been found that the age of on set for an anxiety disorder is younger in females than in males (Beesdo et al., 2009). A full understanding of this sexual dimorphism is not fully understood, however sex hormones that differ between sexes has shown to affect cognition via the hypothalamic-pituitary-gonadal (HPG) axis (Heck and Handa, 2019).

A key PTSD feature is the dysregulation of fear due to a traumatic event or stressor that results in a subconscious conditioning of heightened fear associated with a trigger. An abnormal dysregulated fear response is key in the diagnosis of PTSD within the DSM-V and provides a likely mechanism for the development of PTSD. Those who suffer from PTSD have an uncontrollable fear, even when away from the original trigger (Jovanovic et al., 2012). Gaining a better understanding of fear response pathways and neurobiological circuitries can help explain the fear learning impairments that are typically seen within those with PTSD (Jovanovic et al., 2011).

Stress

A stressor is the perceived external or internal stimulus that threatens the safety of an individual by threatening to disrupt homeostasis, which then causes stress. External stimuli come from environments, such as social interaction, whereas an internal stimulus is within the individual itself, such an illness. The function of stress is to proactively deal with an unsafe stimulus, threatening situation, or dangerous environment and then return to homeostasis (Schneiderman et al., 2008). Stressors can

negatively affect health and behavior by being acute or chronic (Schneiderman et al., 2008). Chronic stress is when a stressor is perceived over a long period of time and acute stress is when the stressor is a single isolated event (Schneiderman et al., 2008). In order to maintain homeostasis, humans and many animal species have a stress response activated by the hypothalamic-pituitary-adrenal (HPA) axis (Sheng et al., 2020). The HPA axis starts with the perception of a stressor that activates the paraventricular nucleus of the hypothalamus (PVH) to secrete corticotrophin releasing hormone (CRH) (Kinlein et al., 2020). The CRH then stimulates the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland, which goes through the bloodstream to activate the secretion of glucocorticoids by the cortex of the adrenal glands (Kinlein et al., 2020). Within a healthy individual, the HPA axis is within a negative feedback loop, so as glucocorticoids increase, the activation of the HPA axis at all levels is decreased (Sheng et al., 2020). The glucocorticoids that are released by the HPA axis leads to other physiological effects in the body including an increase in heart rate, suppress the immune system, increase blood pressure, and increase the production of glucose (Schneiderman et al., 2008).

Stress responsivity is also regulated by the limbic system, comprised of the hippocampus, amygdala, and prefrontal cortex (Lopez et al., 1999). The hippocampus is essential in the regulation and inhibition of the hypothalamus in response to stress, as well as being involved in memory, learning, and emotion (Fanselow and Dong, 2010). The amygdala’s function is centered in fear learning, reward systems, and emotion regulation, however, amygdala hyperactivity is seen to interfere with HPA axis feedback (Jovanovic et al., 2010). In addition, the prefrontal cortex is critical in emotional

regulation, executive function, and in processes of fear extinction which all are affected by stress response and can cause the HPA axis to become sensitive (Romeo, 2017). These three structures play a role in the regulation of the HPA axis by inhibiting the hypothalamus with different levels of corticosteroids, which aid in the negative feedback loop that controls the output of the hypothalamus (Lopez et al., 1999). However, with individuals that are exposed to chronic stress, the stress response is prolonged and can lead to a dysregulation of the HPA axis which can lead to high or low glucocorticoid levels (Gaffey et al., 2017). This dysregulation can become permanent and harmful leading to psychological disorders such as PTSD, anxiety, and depression (Gillespie et al., 2009).

In conjunction with the HPA axis, the autonomic nervous system (ANS) is seen to cause exaggerated fear responses within individuals suffering with PTSD (Seligowski et al., 2019). The ANS is divided into the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS) that work together to regulate involuntary processes in the response to a stressor to maintain homeostasis (Waxenbaum et al., 2021). The parasympathetic nervous system is known as the “rest and digest” activity within the ANS that contributes to recovery and maintenance of the body (Muller et al., 2017). The sympathetic nervous system is known as the “fight or flight” response that activates to allow the individual to react to their environment when in danger situations or when in a stress is detected (Waxenbaum et al., 2021). When the body needs to manage a stressor, the body has an autonomic stress response that leads to the increase in function of the SNS and decrease in function of the PNS (Muller et al., 2017). Since

both the PNS and SNS are constantly active, they work together to maintain homeostasis within the body (Waxenbaum et al., 2021).

Sex Differences

Sex is a risk factor for developing PTSD and can cause a disproportionate effect on the HPA axis (McLean et al., 2011; Panagiotakopoulos and Neigh, 2014). Sexual dimorphisms seen within the HPA axis can be linked to the interaction of the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis (Handa and Weiser, 2014). During the development of the HPA axis and the HPG axis, the hormonal effects of the sex steroids formed within the HPG axis may lead to the emergence of sexual differences in the HPA axis such as levels of corticosteroids produced or volume of brain regions (McCormick and Mathews, 2007). The effects of the HPG axis on the HPA axis in adolescence is heightened due to the influx of sex hormones, but as the HPG axis and HPA axis both mature, the concentration of sex hormones subsides (Bebbington et al., 2009). The evidence of sex differences within cognition due to the differences in the endocrine system has been highly supported (Stephens et al., 2016). Within rodents, the activity of the HPA axis is enhanced by estradiol, a predominant sex hormone within females (Heck and Handa, 2019)

Although the influx of developmental sex hormones subsides within adulthood, there are also lasting morphological and neurological differences between males and females. Females are more vulnerable to harmful stimuli in comparison to males (Baran et al., 2009). Within the hippocampus, there are size differences between sex, as males tend to have a larger volume compared to females (Ruigrok et al., 2014). In addition, the amygdala of males tends to also be increased in size compared to female

counterparts (Ruigrok et al., 2014). It has been seen in literature that within the hippocampus, amygdala, and prefrontal cortex, there are significant sexual dimorphisms that can lead to onset of behavioral differences including the fear learning mechanisms (Baran et al, 2009).

Adolescence

The brain develops rapidly during the early life of humans and is in a vulnerable state that is easily influenced by life experiences (Heim and Nemeroff, 2001). Childhood to young adulthood has been seen as a critically sensitive period when environmental factors alter the brain's morphological development within neuronal dendritic complexity and size (Romeo, 2017). In addition to the normal growth and development of this stage of life, there is also growing concern for the disruptions that occur during this sensitive period (Giedd and Rapoport, 2010). Adverse life experiences that occur during the adolescent period of life can lead to a cognitive state that is vulnerable to changes that lead to psychological disorders (Heim and Nemeroff, 2001). Adolescents are exposed to a variety of stressors that can affect their biological responses to this stress and how they react to their environment (Corr et al., 2021). The HPA axis during this time is vulnerable to alterations during the transition during adolescence due to prolonged activity from stressors (McCormick and Mathews, 2007). About 50% of mental illness can be identified before age 14 and about 75% before age 24 (Kessler et al., 2005).

Adolescents have a higher risk of anxiety disorders manifesting that can lead to a variety of physiological and psychological disorders within their lives. Research with rodents has found that life adversities and ongoing stressors can either positively or

negatively affect a developing brain (McCormick et al., 2008). It has been found in a variety of studies that chronic adolescent stress is able to cause long-lasting effects that interact with stress within adulthood (Rowson et al., 2019). Corticosterone production during an acute stress event is blunted in adulthood after experiencing chronic adolescent stress (Bekhbat et al., 2019). During adolescence, the alteration of the hippocampus has been seen in changes within the hippocampal transcriptome (Rowson et al., 2019) and within the dendritic complexity found later in adulthood (Romeo, 2017). As stated before, the hippocampus is crucial in the regulation of the HPA axis and of fear learning. Adolescents' ability to recover corticosterone levels after a stressor is less than in adulthood, which may be due to a developing HPA axis that is sensitive (Bourke and Neigh, 2011). Mixed-modality chronic adolescent stress is able to alter the ratio of glucocorticoid receptors within the hippocampus and prefrontal cortex, which implies a decrease in efficiency (Hyer et al., 2021). In one study with Long-Evans rats, chronic social stress was performed within the adolescent period and led to a change in anxiety-like behaviors within the females but not within the males (McCormick et al., 2008). This sexual dimorphism within adolescence can be due to the introduction of hormones that modulate the HPA axis reactivity, as corticosterone levels decrease with testosterone and increase with estradiol (McCormick and Mathews, 2007).

Fear Learning

PTSD is defined as being a disorder with a major symptom of impaired fear response, learning, and memory, and therefore can be further understood by looking at the pathways and circuitry involved in fear (Jovanovic et al., 2012). Fear learning includes the acquisition of a fear, the extinction of fear, and renewal of fear in order to

avoid and defend from a dangerous and threatening stressor (Sah and Westbrook, 2008). Fear acquisition is the first aspect of fear learning as it is where an aversive stressor converges with a contextual trigger to result in the two stimuli being associated and activating a fear response within the amygdala (Sah and Westbrook, 2008). This reaction to fear can lead to behavioral and physiological changes within the body (Keane et al., 1985). This fear acquisition can lead to a fear memory due to the activation of the HPA axis which increases synaptic plasticity aiding in long-term potentiation (LTP), the cellular mechanism for the consolidation of a memory (Myers et al., 2006). Fear can be extinguished by the inhibition of the fear response activated by the prefrontal cortex (Sah and Westbrook, 2008). The inability to extinguish this fear response is a trait that is specific to PTSD and no other psychological disorders such as major depressive disorder (Jovanovic et al., 2012). Although a fear response of a fear memory can be extinguished, the fear memory itself cannot be erased, and therefore the fear response can be renewed (Sah and Westbrook, 2008). The hippocampus is activated when in a learned context or situation and will trigger the inhibition of the extinction pathways within the amygdala, allowing the fear response to be activated once again (Sah and Westbrook, 2008).

Classical Pavlovian conditioning is used to study the neural mechanisms that are involved in fear response and fear learning (Lissek et al., 2005). The conditioning of fear is similar to the acquisition of fear in that the aversive unconditioned stimulus is paired with a neutral conditioned stimulus (Jovanovic et al., 2010). The conditioned stimulus has now become a “danger cue” that triggers a fear due to the aversive stimulus, however this fear response can be suppressed by safety learning which activates the

extinction of a fear (Jovanovic et al., 2010). Safety learning is the ability of an individual to transfer the fear inhibition when in the presence of a safety cue (Jovanovic et al., 2009). Behavioral symptoms of avoidance, another key feature of PTSD, is that it can be reversed through safety learning activated by fear extinction training (Ayash et al., 2019). In order to test whether safety learning ability has also been impaired, a startle paradigm was developed to show that individuals who suffered from PTSD had a hindered ability to transfer the inhibition of fear when the danger cue was paired with the safety cue (Jovanovic et al., 2010). Within rodent models to simulate similar PTSD-like behavior, a conditioned inhibition procedure was implemented in a fear-potentiated startle paradigm measuring the acoustic startle response of rats (Myers and Davis, 2004). In both humans and rodents, the increased magnitude of startle response after transfer of inhibition during fear extinction exemplified an impairment of safety learning (Jovanovic et al., 2010; Myers and Davis, 2004).

Implications

Using a fear conditioning outlook on learning, it may be possible to see why females are more likely to develop PTSD. Adolescence is a critical time for brain development and cognitive function which can lead to damage in the occurrence of chronic stressors. Chronic stress can also lead to a dysregulation and disruption of fear leading to impaired safety learning due to impairments in fear conditioning and fear extinction. In order to look at the possibility of chronic adolescent stress being a predictive factor of developing PTSD, the startle paradigm will be tested to look at impairments within safety learning. I hypothesize that chronic adolescent stress will decrease the ability of the Wistar rats to safety learn and females will have a heightened

impairment of safety learning. In addition, I hypothesize that chronic adolescent stress can be a predictive factor of startle response, and therefore of PTSD-like behavior.

Methods

For this experiment, two cohorts of female pregnant Wistar rats were ordered from (Charles River, North Carolina). All animals were kept in a 12:12 light cycle room with temperatures ranging steadily between 20 and 23 degrees Celsius. The Wistar rats used in the procedures were born and the litters were decreased to four of each sex when applicable, as some litters had less than four of each sex. The first cohort had 42 of which 22 were male and 20 were female. The second cohort had 44 of which 25 were male and 19 were female. The experimental Wistar rats were kept with their mothers until postnatal day (PND) 22. All the animals were weaned on PND 22, but they were divided evenly by sex and labeled into a chronic adolescent stress (CAS) group or a non-stress group. On this day, the CAS groups were individually isolated in cages and placed into a separate room and the non-stress groups were pair housed and left in the room. The weight of the animals was recorded each week after weaning (shown in Figure 2). Within the CAS group, there were 27 males and 21 females. In the nonstress group, there were 20 males and 18 females. Between PND 39 to 50, the CAS group underwent the stress paradigm, described in detail below, consisting of isolation, social defeat, and restraint. The duration of this stress paradigm was twelve days of randomized stress, six days of social defeat and six days of restraint. The period of 30 to 60 days of age within rats is seen to be comparable to human adolescence of 10 to 18 years of age (Romeo, 2017). The mixed-modality stress paradigm has been seen to be effective within multiple studies for creating chronic stressor during adolescence

(Bourke and Neigh, 2011; Rowson et al., 2019; Hyer et al., 2021). On PND 75, the non-stressed groups were also isolated in preparation for the startle paradigm. All rats in both groups were undisturbed between PND 75 and PND 85.

Chronic adolescent stress

Social Defeat

Retired breeder Long Evans rats (Charles River, New York and Charles River, California) were used as a dominant aggressor that resulted in stress-induced stress behavior within the Wistar rats. This social defeat paradigm has been previously used within the lab (Bourke and Neigh, 2011).

The Long Evans rats were kept in opposite sex pair-housed cages within the room housing the CAS group cages. Since the females were all retired breeders, they underwent ovariectomy prior to arriving to the lab. For the procedure, one of the Long Evans rats would be removed from the cage and a barrier would be placed within the cage. A wistar rat of the same sex as the remaining Long Evans rat would then be placed within for two minutes. After the two minutes are over, the barrier is lifted, and the two rats interact. This interaction period lasts 5 minutes or until the Long Evans rat pins the Wistar rat on its back three times. Behavior was recorded and the most notable things observed were pinning and kicking. When the timer is over or the Wistar rat has been pinned three times, the barrier is replaced, and the two rats are separated within the cage for another 25 minutes to prolong the stress effects of being in the cage of an aggressor. The Wistar rat is then returned to its own cage. The average number of pins and average number of kicking was recorded and shown in Figure 4.

Restraint

Within the stress paradigm, the hour-long restraint portion is conducted with a narrow plastic restraint tube (Braintree Scientific, Braintree, MA) that does not compress the rats. Within the restraint tube, the rats are not able to freely move or turn around. During the hour, struggling behavior of the Wistar rats was observed every two minutes and recorded as either struggling or not as shown in Figure 3. Struggling behavior was defined as digging, biting, moving, or pushing while within the restraint.

Startle response paradigm

In order to collect and analyze fear extinction and safety learning, a Startle Response System (San Diego Instruments Inc., San Diego, CA) was used to record and measure the Wistar rat's startle response during the paradigm shown in Figure 1. The Startle Response System is known as a "startle chamber" within this study and is a large box with a narrow plastic tube similar to the restraint tube within it. This tube is hooked up to a metal grating that is wired to send the measurements of movements to the system that records the data. This metal grating is also equipped to send an electrical foot shock to the rats during the experiment. Within the chamber, there is also background white noise that is at approximately 55 dB. The startle paradigm begins on PND 86 and runs for a total of 10 days with the first day being only transportation habituation, then 2 days of habituation, then the startle paradigm, and the final day of sacrificing the rats. Each of the days have different tests occurring during the duration of the paradigm described in detail below.

Habituation

Habituating the animals was important in collecting accurate results as to not alter the fear memory pathway with a novel environment that would heighten the learning of fear (Jovanovic et al., 2009). On the first day of the paradigm, the rats stayed on a housing cart within the vivarium and were transported up to the behavior experiment room and closed in a side room where they stayed for 30 minutes. After the time was up, they were placed on the cart and brought into the main behavior room and handled before being returned to their cage and brought back to the housing cart. After all the rats were handled, they were returned to the vivarium on the housing cart.

During the next two days of the paradigm, the animals were put on a cart and wheeled from the vivarium to the behavior experiment room once again. They were left in a closed room within the experiment room for 30 minutes to habituate to the transportation between spaces. Then they were placed on a cart two at a time and wheeled into the actual room that the startle chamber was housed. The rats were then handled and placed into the startle chamber for 5 minutes. The light within the startle chamber was turned on for the habituation process. When the time was up, the rats were returned to their cages and placed back on the housing rack. On the second day of habituation, fecal samples were collected and placed in a labeled tube.

Baseline

For the beginning of the measured tests portion of the paradigm, it begins on the first day with the baseline test. Following the same habituation process, the rats were brought up and left in the side room for 30 minutes to habituate to the room. After 30

minutes, the first round of rats was placed on the transport cart and moved into the main behavior room. They were then placed into the assigned startle chamber and the baseline test began. The programmed software allows for 5 minutes of habituation within the startle box before playing an acoustic startle tone at low 90 dB, medium, 95 dB, and high 105 dB. The tones are randomized between thirty 10 second intervals for a duration of approximately 5 minutes. After the trial is over, the rats are removed from the chamber and returned to the transportation cart. The chambers are then cleaned with ethanol. The rats are returned to the housing cart and the process repeated until all trials are over. The housing cart with the rats was then returned to the vivarium.

Fear Conditioning

The next testing day is fear conditioning. Following the same habituation process, the rats were brought up and left in the side room for 30 minutes to habituate to the room. After 30 minutes, the first round of rats was placed on the transport cart and moved into the main behavior room. They were then placed into the assigned startle chamber and the fear conditioning test began. The programmed software allows for 5 minutes of habituation within the startle box. At the bottom of the tube, is a metal grating that delivers a 0.6 mA footshock to the rats. 3 seconds prior to the randomized footshock, a cue light would turn on. The trial lasts approximately 20 minutes, then the rats are removed from the chamber and returned to the transportation cart. The chambers were then cleaned with ethanol. The rats are returned to the housing cart and the process repeated until all trials are over. The housing cart with the rats was then returned to the vivarium.

Fear Potentiated Startle

The next day tests the rats' fear potentiated startle response. Following the same habituation process, the rats were brought up and left in the side room for 30 minutes to habituate to the room. After 30 minutes, the first round of rats was placed on the transport cart and moved into the main behavior room. They were then placed into the assigned startle chamber and the fear potentiated startle test began. The programmed software allows for 5 minutes of habituation within the startle box. During this trial, the randomized acoustic startle tones are once again played at thirty second intervals, but this time, is preceded by the presence or absence of the cue light used the day before in fear conditioning. This tests the effect of the previous day's test on the startle response of the rats. The trial lasts approximately 25 minutes, then the rats are removed from the chamber and returned to the transportation cart. The chambers were then cleaned with ethanol. The rats are returned to the housing cart and the process repeated until all trials are over. The housing cart with the rats was then returned to the vivarium.

Fear Extinction

The following two days are fear extinction days of the paradigm and are the same processes. Following the same habituation process, the rats were brought up and left in the side room for 30 minutes to habituate to the room. After 30 minutes, the first round of rats was placed on the transport cart and moved into the main behavior room. They were then placed into the assigned startle chamber and the extinction began. The

programmed software allows for 5 minutes of habituation within the startle box. Every thirty seconds, the cue light would turn on with nothing happening to the rats within the chamber. The goal of these two days was to train the rats to learn that the light cue was no longer associated with the footshock. The trial lasts approximately 25 minutes, then the rats are removed from the chamber and returned to the transportation cart. The chambers were then cleaned with ethanol. The rats are then returned to the housing cart and the process repeated until all trials are over. The housing cart with the rats was then returned to the vivarium.

Safety Learning Assessment

The final day of the startle paradigm was the safety learning assessment. Following the same habituation process, the rats were brought up and left in the side room for 30 minutes to habituate to the room. After 30 minutes, the first round of rats was placed on the transport cart and moved into the main behavior room. They were then placed into the assigned startle chamber and the safety learning assessment began. The programmed software allows for 5 minutes of habituation within the startle box. During this trial, the randomized acoustic startle tones are once again played at thirty second intervals, but this time, is preceded by the presence or absence of the cue light, similar to the fear potentiated startle day. This tests the effect of the previous days' extinction and safety learning on the startle response of the rats. The trial lasts approximately 25 minutes, then the rats are removed from the chamber and returned to the transportation cart. The chambers were then cleaned with ethanol. The rats are returned to the housing cart and the process repeated until all trials are over. The housing cart with the rats was then returned to the vivarium.

Statistics

The data was analyzed within Microsoft Excel and GraphPad 9.3 Prism Software (San Diego, CA). Using Microsoft Excel, the startle response for each of the test days was corrected for weight to be proportionate in order for the responses to be comparable between subjects. Using GraphPad Prism, a multiple regression analysis was also performed to analyze the predictability of each startle paradigm day using the chronic adolescent stress results. Within the GraphPad Prism Software, a two-way Analysis of variance (ANOVA) was run for comparison of the weights between sex and the CAS data between sex. Within the GraphPad Prism Software, a three-way and two-way Analysis of variance (ANOVA) was run for the startle paradigm test data to analyze the startle responses. Significance was set at alpha level 0.05. Bonferroni's multiple comparisons test was used for all post-hoc analyses within the GraphPad Prism software using simpler and multiple linear regression.

Results

Weight and Stress

Looking at physiology in Figure 2A, the weight of the males was significantly greater than the females ($F(1, 82) = 1005$; $P<0.0001$). Between non-stress (NS) and chronic adolescent stress (CAS), there was no significant difference ($F(1, 82) = 1.345$; $P=0.2495$). There was not an interaction between stress group and sex at an alpha level of 0.05 ($F(1, 82) = 3.942$; $P=0.0504$). Within the males, there was a significant difference between the NS group and the CAS group ($F(1, 45) = 4.460$; $P=0.0403$), as

seen in Figure 2B. Within the females there were no significant differences between the NS group and the CAS group ($F (1, 37) = 0.4283$; $P=0.5169$), as seen in Figure 2C.

The average number of times struggling within the restraint days of the CAS paradigm is recorded and shown in Figure 3. There was no significant difference between males and females ($F (1, 44) = 1.120$; $P=0.2956$). There was a significant difference in the session restraint occurred between sexes ($F (5, 220) = 17.11$; $P<0.0001$). There was no interaction of session and sex ($F (5, 220) = 1.88$; $P=0.0975$)

The average number of pins during the social defeat days of the CAS paradigm is recorded and shown in Figure 4. In Figure 4A, there was a significant difference between male and females ($F (1, 44) = 26.18$; $P<0.0001$). There was also an interaction between session and sex ($F (5, 220) = 3.193$; $P=0.0084$). Bonferroni's multiple comparisons test was run as a post-hoc test and there were significant effects on session 2 ($P= 0.0463$) and session 5 ($P<0.0001$). In Figure 4B, the average number of kicking were recorded and there was a significant difference between male and females ($F (1, 44) = 25.85$; $P<0.0001$). There was also no interaction between session and sex ($F (5, 220) = 0.9322$; $P=0.4609$).

Startle paradigm

The startle responses that were recorded were taken from the max mV of the session for that subject and weight corrected. Each session was averaged and divided by the weight of each subject. On the fear potentiated startle and safety learning assessment days, a formula was used to create a proportional startle response as a result of the conditioned stimulus being present. To do this, the CS- and Cue level

response was subtracted from the CS+ and Cue level response and then divided by the CS- and Cue level response. Also, for the days of extinction and safety learning, the portion of the cohorts that received D-cycloserine as an injection were removed and included in a separate part of the study that is not covered here.

Baseline

After analyzing the baseline startle responses with a three-way ANOVA, there was a significant difference between stress ($F(1, 78) = 2.529$; $P=0.1158$), but no significant difference between sex ($F(1, 78) = 0.4038$; $P=0.5270$). There was a significant difference in cue level ($F(1.415, 110.4) = 331.1$; $P<0.0001$). However, withing the three-way ANOVA, there was an interaction of cue and stress ($F(2, 156) = 3.289$; $P=0.0398$) as well as an interaction between cue and sex ($F(2, 156) = 8.880$; $P=0.0002$). A two-way ANOVA was run for each of the parameters, and within the CAS group, there was an interaction of cue and sex ($F(2, 80) = 3.176$; $P=0.0471$). Each of the two-way ANOVAs for all parameters had a significant difference in cue level and subject ($P<0.05$).

Fear conditioning

For the fear conditioning day, a three-way ANOVA was run which found that there was a significantly different startle response between male and female, as females had a higher startle response than the males ($F(1, 82) = 238.5$; $P<0.0001$) (Figure 6). There were no significant differences within shock cue ($F(7.174, 588.2) = 1.256$; $P=0.2689$) or stress group ($F(1, 82) = 0.06000$; $P=0.8071$). There were also no

interactions between sex and stress (F (1, 82) = 0.05412; P=0.8166), cue and stress (F (9, 738) = 0.9355; P=0.4933) or cue and sex (F (9, 738) = 1.251; P=0.2602).

Fear potentiated startle

The fear potentiated startle day has a proportioned ratio to see the effect of the conditioned stimulus on the startle response by finding the change in response of the conditioned stimulus being present or absent. Pictured in Figure 7, using a three-way ANOVA, there is a significant effect of cue level (F (1.638, 132.7) = 16.24; P<0.0001), but no significant difference in sex (F (1, 81) = 0.02921; P=0.8647) or stress (F (1, 81) = 0.04824; P=0.8267). There were no interactions between cue and stress (F (2, 162) = 1.442; P=0.2395). There was an interaction of cue and sex (F (2, 162) = 4.318; P=0.0149) and sex and stress (F (1, 81) = 4.034; P=0.0479). Due to the visual differences of the stress groups within the males, an A priori test was run that found the power analysis that the sample size needed for significance is 14, which was met due to the fact there were 25 in the CAS group and 22 in the NS group.

Fear extinction

For the first extinction day seen in Figure 8A, using a three-way ANOVA, females' startle response was significantly higher than the males (F (1, 82) = 150.1; P<0.0001). Stress (F (1, 82) = 1.986; P=0.1626) and cue level (F (4.233, 347.1) = 2.251; P=0.0596) were not significantly different. There were no significant interactions

between cue and sex (F (5, 410) = 1.024; P=0.4032) cue and stress (F (5, 410) = 0.07890; P=0.9954) or stress and sex (F (1, 82) = 1.816; P=0.1815).

In Figure 8B, the second extinction day was analyzed by using a three-way ANOVA, females' startle response was again significantly higher than the males (F (1, 39) = 45.22.1; P<0.0001). Stress (F (1, 39) = 0.03303; P=0.8567) and cue level (F (3.911, 152.5) = 1.557; P=0.1897) were not significantly different. There were no significant interactions between cue and sex (F (5, 195) = 0.5867; P=0.7102), cue and stress (F (5, 195) = 0.6481; P=0.6632), or stress and sex (F (1, 39) = 1.595; P=0.2141).

Safety learning assessment

For the final day of the startle paradigm, the startle response of the safety learning assessment day was also a ratio similar to the fear potentiated startle day to also test for the association of fear with the conditioned stimulus. As seen in Figure 9, by using a three-way ANOVA, there was a significant difference in stress as the CAS group was lower than the NS (F (1, 38) = 4.273; P=0.0456), but no difference in cue level (F (1.732, 65.81) = 3.013; P=0.0631) or sex (F (1, 38) = 0.1021; P=0.7511). There were no significant interactions of cue and sex (F (2, 78) = 0.1128; P=0.8935), cue level and stress (F (2, 78) = 0.3582; P=0.7001), or sex and stress (F (1, 39) = 0.2713; P=0.6055).

Regression analysis

Following the ANOVA analysis, regression analysis was done in order to look at the possibility of predicting the outcomes of startle response based on the explanatory variables of the CAS paradigm. Multiple linear regression and simple regression were both used to look at the predictive effects of chronic adolescent stress on the startle response and safety learning.

Simple linear regression

Simple linear regression for each day was run using the explanatory variable of the total average number of times struggling during restraint to predict the startle response of each day. Within the males, none of the days had a significant P-values. Within the females, for the baseline day shown in Figure 10, there was significance of the regression (P-value=0.0259) however the goodness of fit was weak (R^2=0.2351).

Simple linear regression for each day was run using the explanatory variable of the total average number of times pinned during defeat to predict the startle response of each day. Within the males, for the fear conditioning day shown in Figure 11. There was significance in the regression (P-value=0.0380) however the goodness of fit was weak (R^2=0.1740). Within the females, none of the days had significant P-values.

Simple linear regression for each day was run using the explanatory variable of the total average number of times kicked during defeat to predict the startle response of each day. Within both the males and the females, none of the days had a significant P-values.

Multiple linear regression

Multiple linear regression was run for both sexes using the CAS measurements of average times struggling during restraint, average times pinned during defeat, and average times kicked during defeat as explanatory variables to predict the startle responses for each day. Within the males, there was only a significant P-value (F (1, 11) = 6.668; P-value=0.0255) and medium goodness of fit of (R^2=0.3774) for the regression correlation of actual and predicted startle responses for the safety learning assessment day using the explanatory variables shown in Figure 12. Within the females, there was only a significant P-value (F (1, 9) = 17.45; P-value=0.0024) and an almost strong goodness of fit of (R^2=0.6597) for the regression correlation of actual and predicted startle responses for the second extinction day using the explanatory variables shown in Figure 13.

Figures

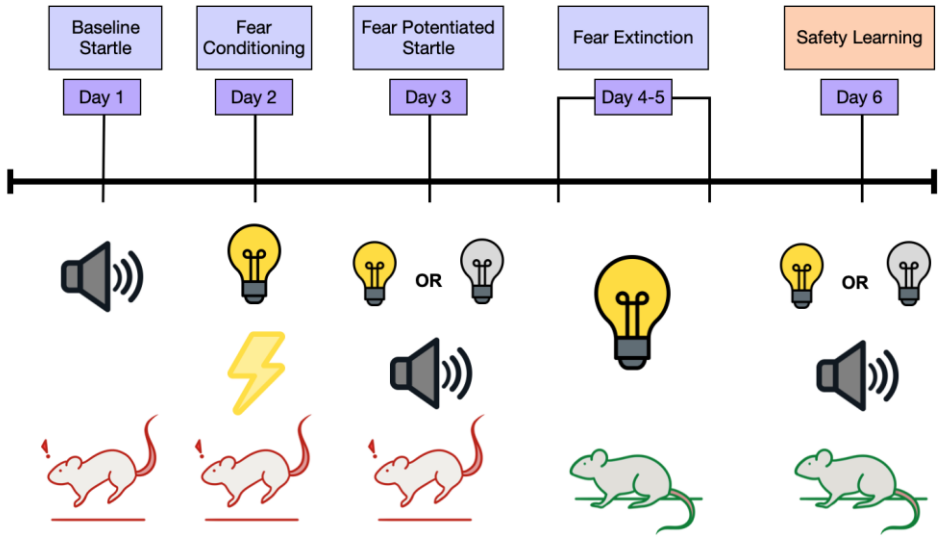

Figure 1: Above shows the procedure that was used for each day of the startle paradigm including showing the pairings of the unconditioned and conditioned stimuli used for the fear conditions; in addition, the expectation of the control rats' behavior is shown on the bottom as well.

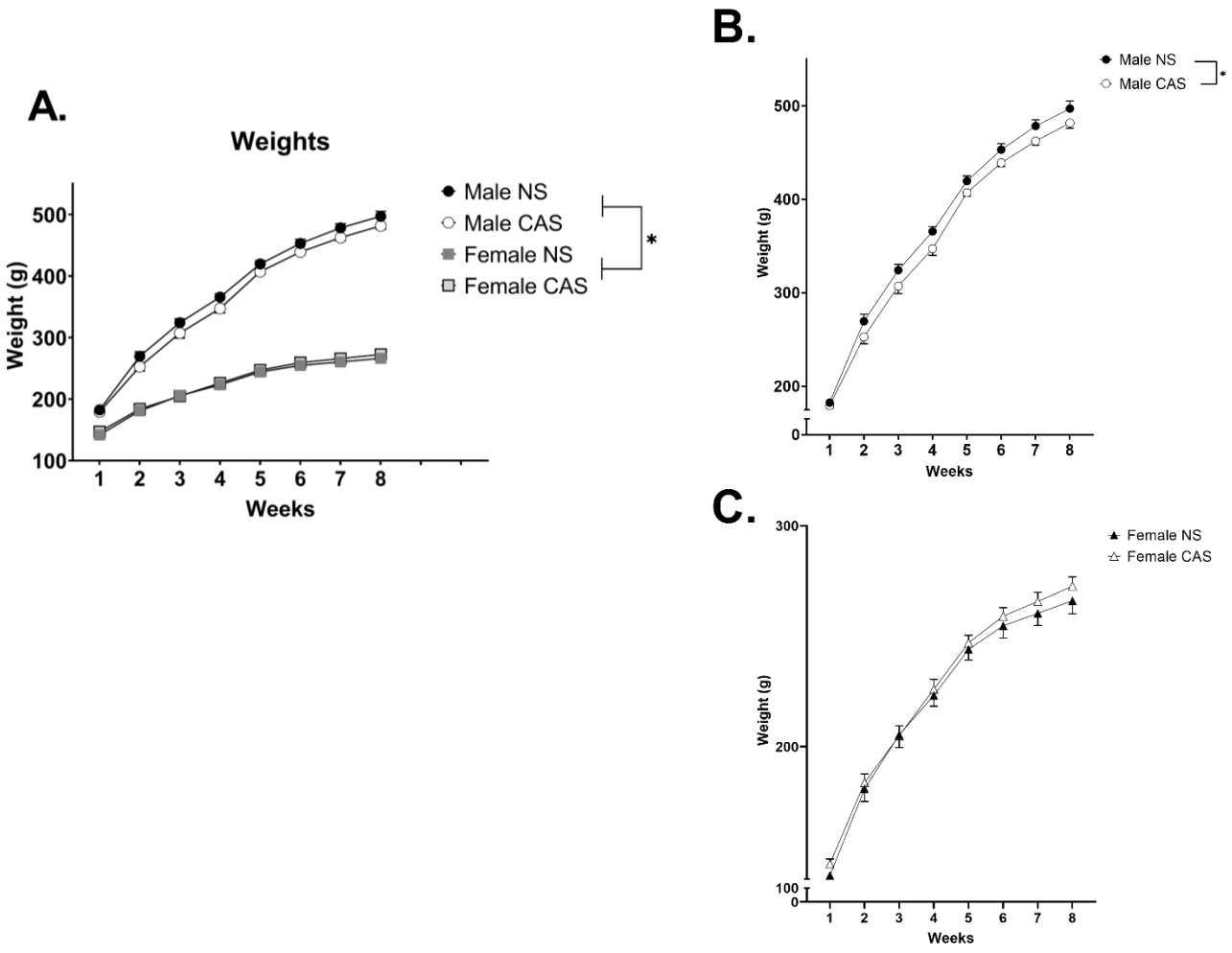

Figure 2A-C: (A) Above shows the recorded averaged weights of the course of 8 weeks divided by sex and stress group, non-stress (NS) vs chronic adolescent stress (CAS). (B) Above shows the recorded average weights over the course of 10 weeks within the Males (M). There was a significant difference between the NS and CAS group within the males ($p < 0.05$). (C) Above shows the recorded average weights over the course of 8 weeks within the Females (F). There were no significant differences found between the female NS and CAS groups ($p > 0.05$). An * represents where there is a significant effect at alpha level 0.05. Data is shown as mean ± SEM.

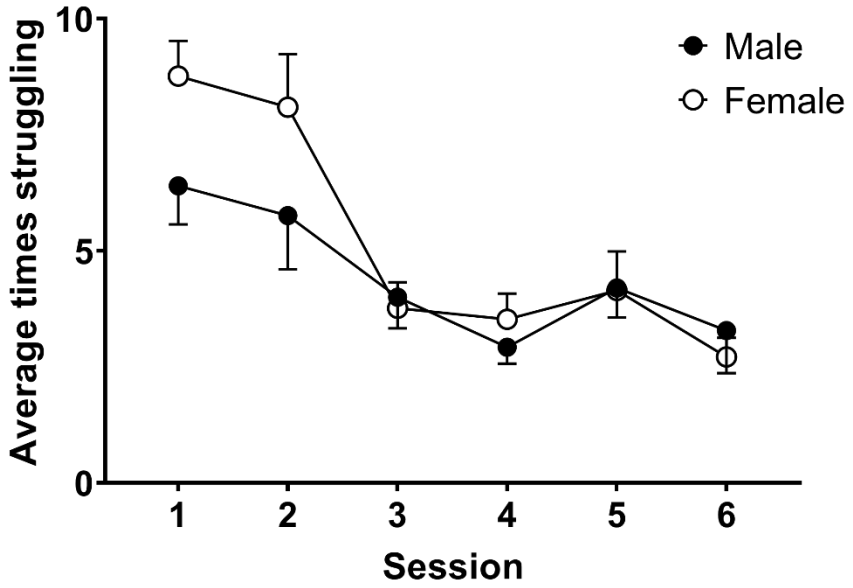

Figure 3: Above shows the average times recorded struggling during the 6 restraint days of the chronic adolescent stress paradigm within the CAS group including the males and females. There were no significant differences between sex ($p > 0.05$). An * represents where there is a significant effect of sex at alpha level 0.05. Data is shown as mean ± SEM.

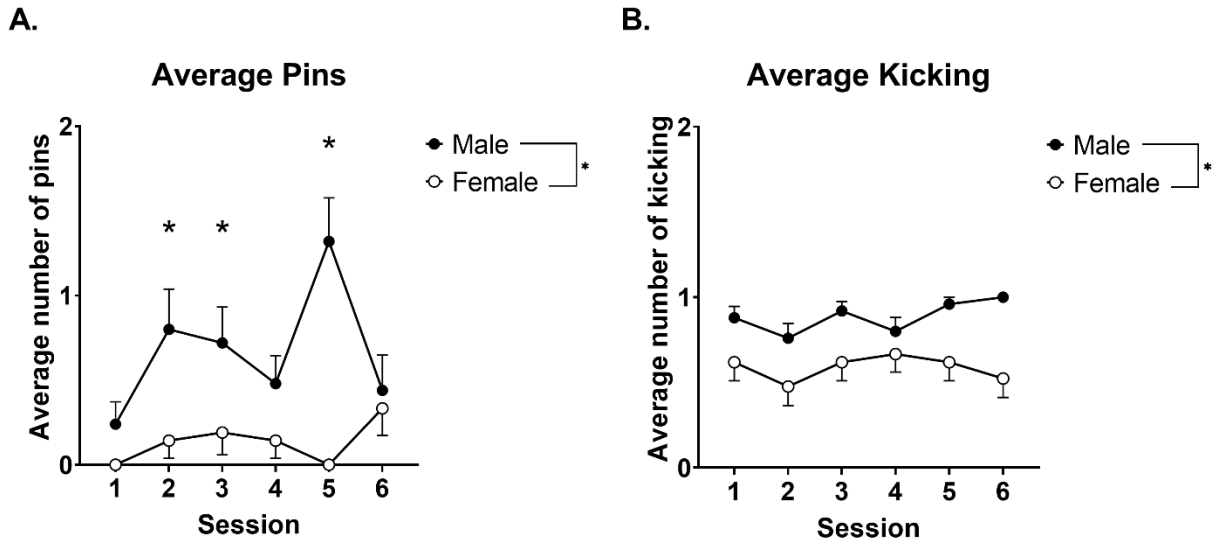

Figure 4A and 4B: Above shows the average number of pins (A) and kicking (B) during the 6 defeat days of the chronic adolescent stress paradigm within the CAS group including the males and females. There were significant differences between sex for both pins and kicking in which males had significantly more pins and kicking than females ($p < 0.05$). An * represents where there is a significant effect of sex at alpha level 0.05. Data is shown as mean ± SEM.

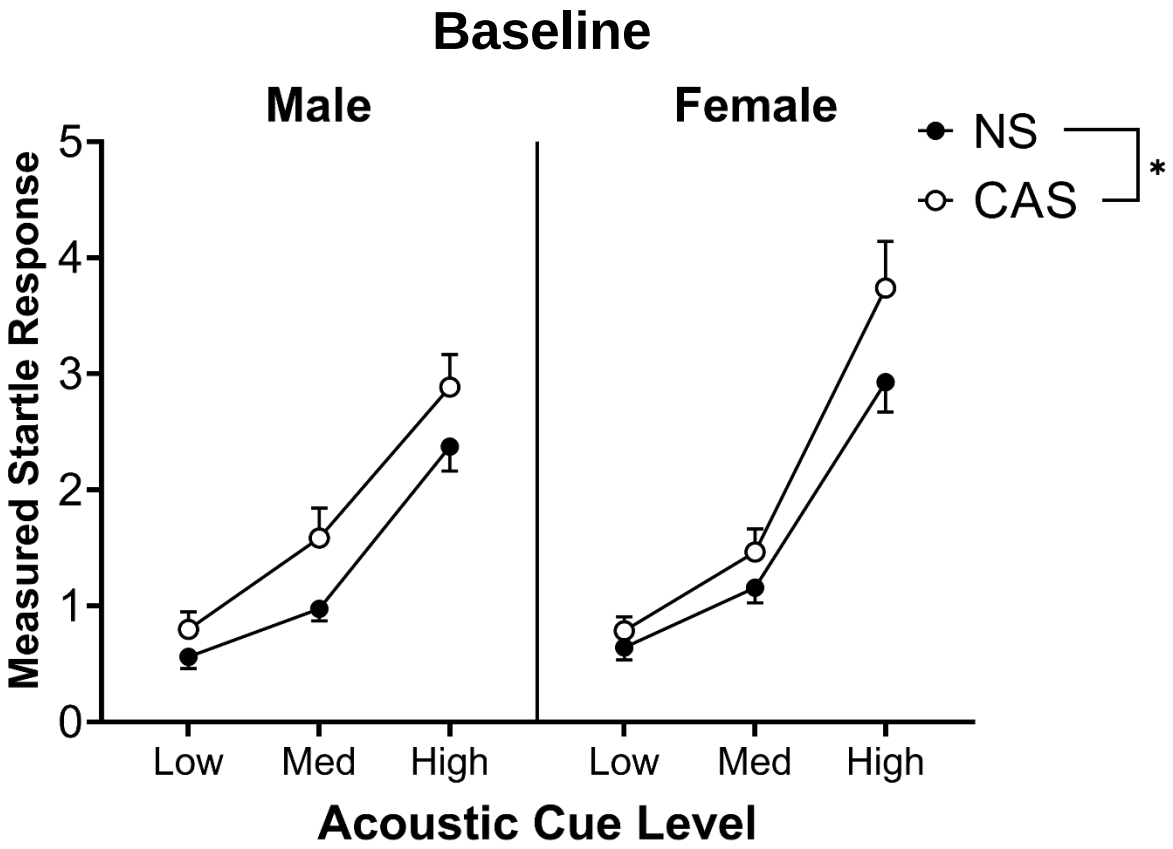

Figure 5: Above is the baseline weight corrected startle response. There were no significant differences between sexes or stress groups ($p > 0.05$). There was a significant interaction of cue level x sex. An * represents where there is a significant stress effect at alpha level 0.05. Data is shown as mean ± SEM.

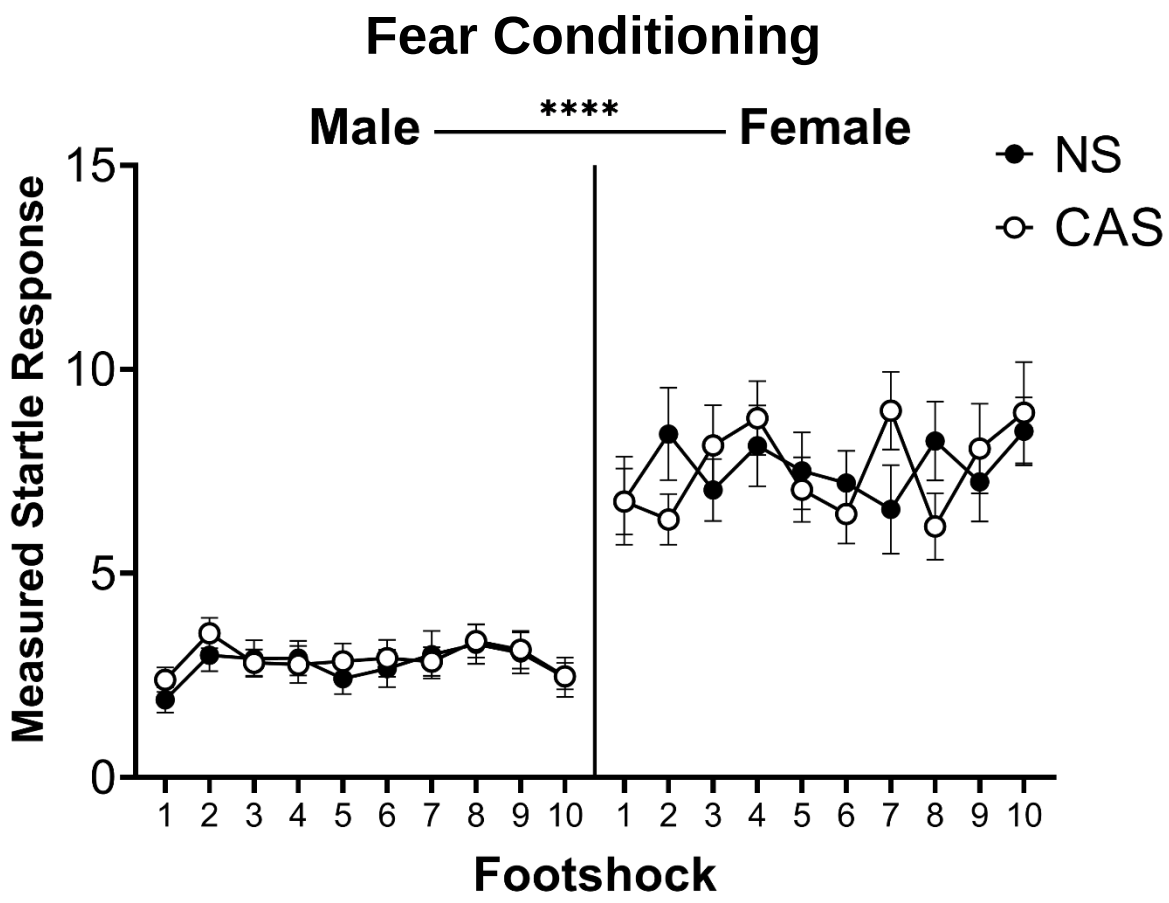

Figure 6: Above is fear conditioning weight corrected startle response. There was a significant difference in startle response between males and females, as females had a significant increase compared to males ($p < 0.05$). An * represents where there is a significant effect of sex at alpha level 0.05. Data is shown as mean ± SEM.

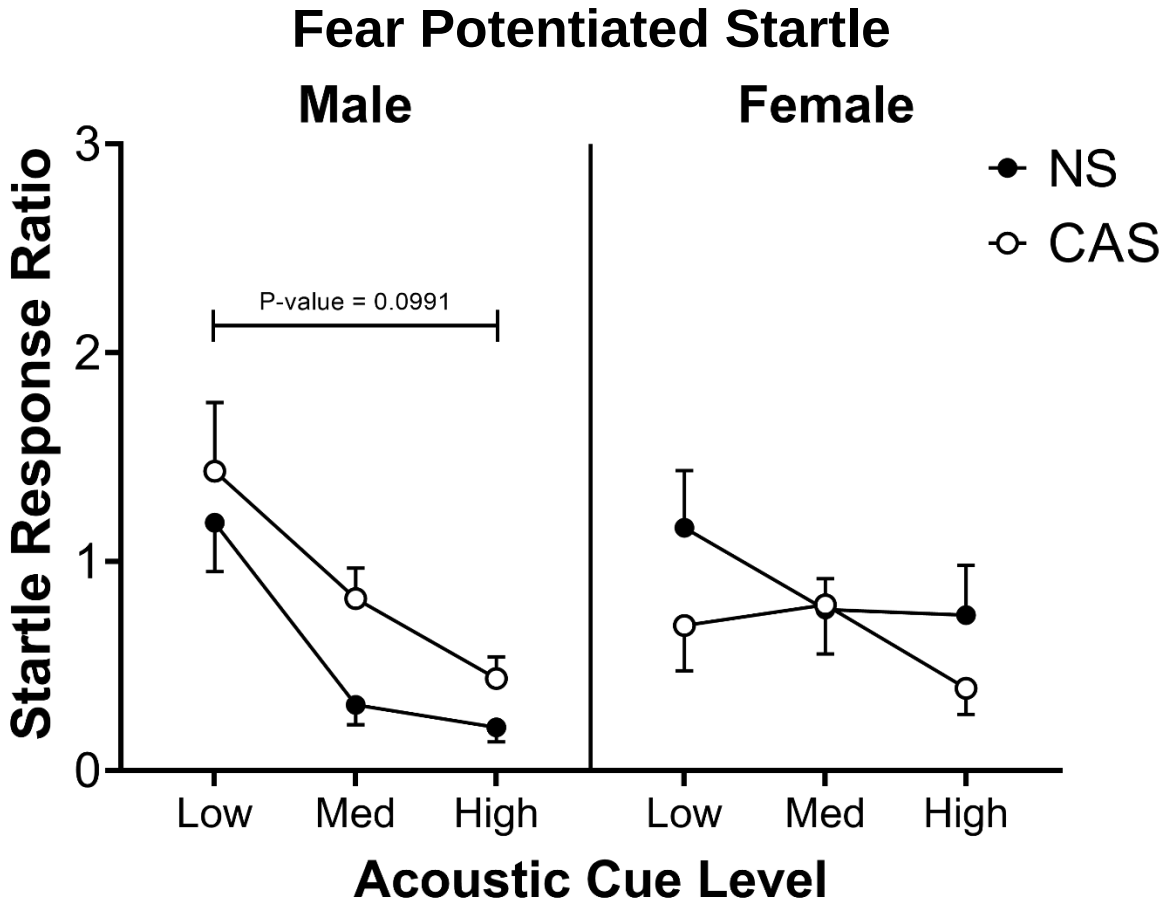

Figure 7: Above is the weight corrected proportional fear potentiated startle response. There were no significant differences between sexes or stress groups ($p > 0.05$). There was a significant interaction of cue level and sex as well as stress and sex. After looking at the Bonferroni multiple comparisons post-hoc test, there was no significant effect at the medium within the males ($p < 0.05$) (not shown on graph). An * represents where there is a significant effect of stress at alpha level 0.05. Data is shown as mean ± SEM.

Extinction Days

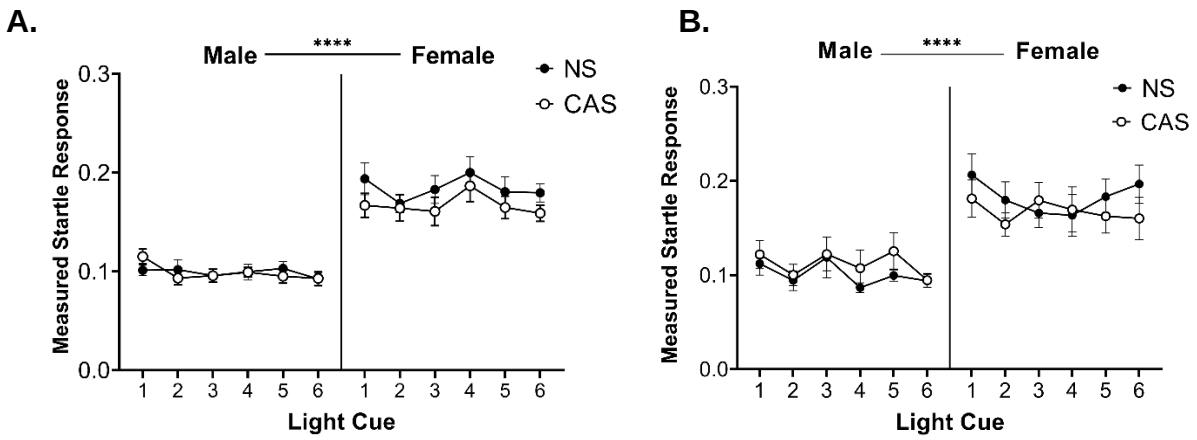

Figure 8A and 8B: Above are the weight corrected startle responses for the extinction (Day 1) and the extinction (Day 2) (A) There was a significant difference in startle response between males and females ($p < 0.05$). An * represents where there is a significant effect of sex at alpha level 0.05. Data is shown as mean ± SEM. (B) There was a significant difference in startle response between males and females ($p < 0.05$). An * represents where there is a significant effect of sex at alpha level 0.05. Data is shown as mean ± SEM.

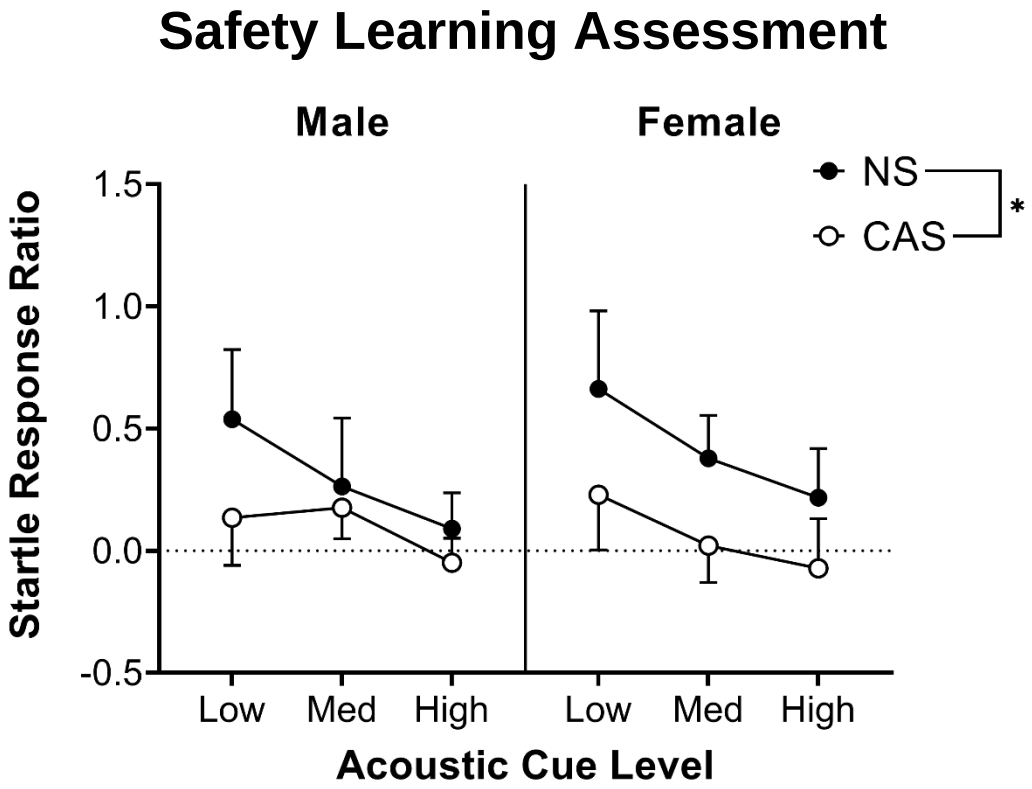

Figure 9: Above is the baseline weight corrected startle response. There was a significant difference between NS and CAS ($p < 0.05$). An * represents where there is a significant effect of stress at alpha level 0.05. Data is shown as mean ± SEM.

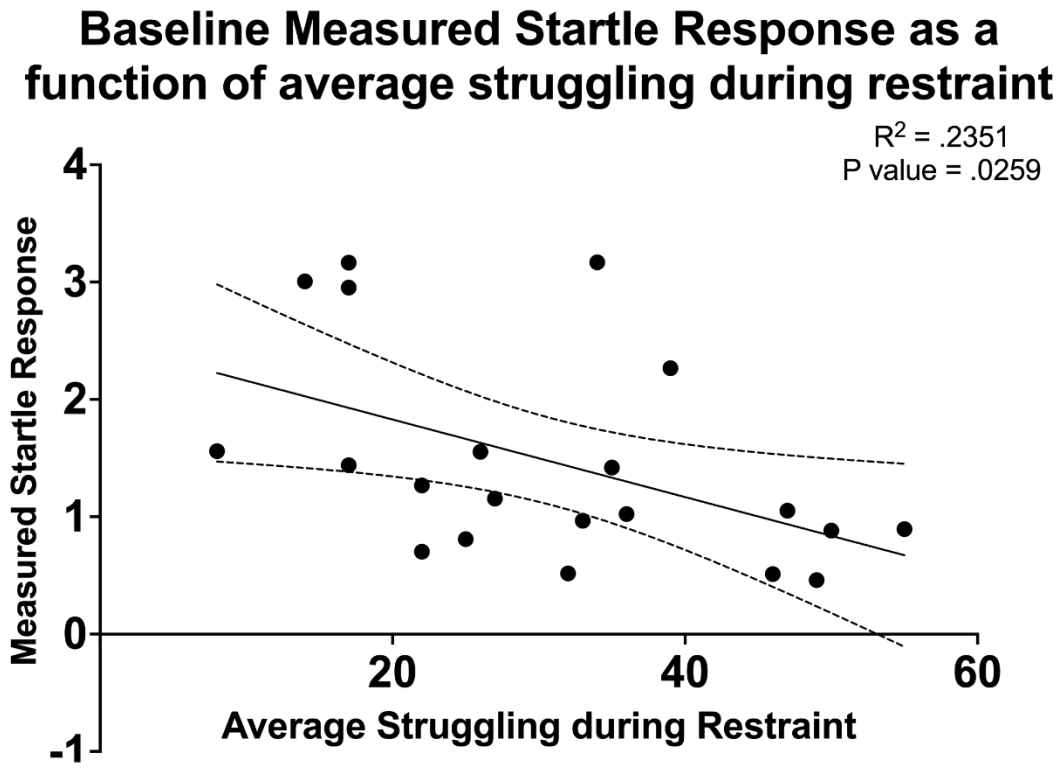

Figure 10: Above is the simple linear regression for the baseline day within the females using average times of struggling during restraint as an explanatory variable. There was a significant regression (P-value=0.0259) and a weak goodness of fit of (R^2=0.2351).

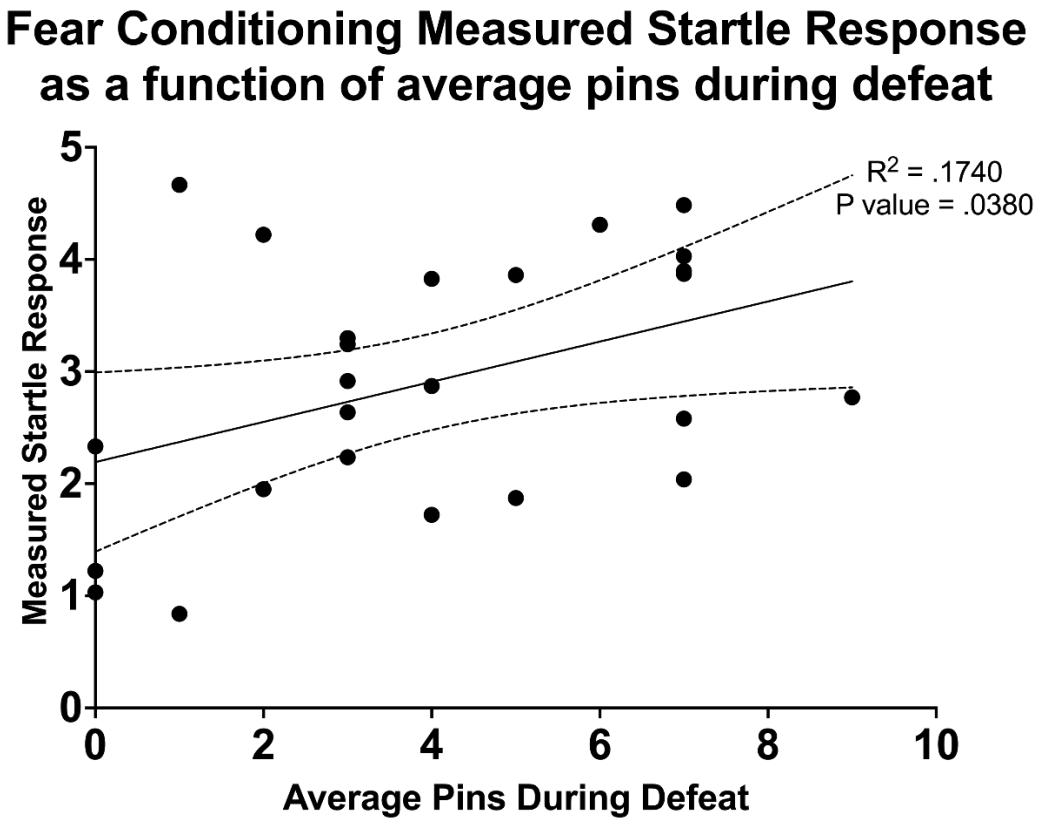

Figure 11: Above is the simple linear regression for the fear conditioning day within the males using average times of struggling during restraint as an explanatory variable. There was a significant regression (P-value=0.0380) and a weak goodness of fit of (R^2=0.1740).

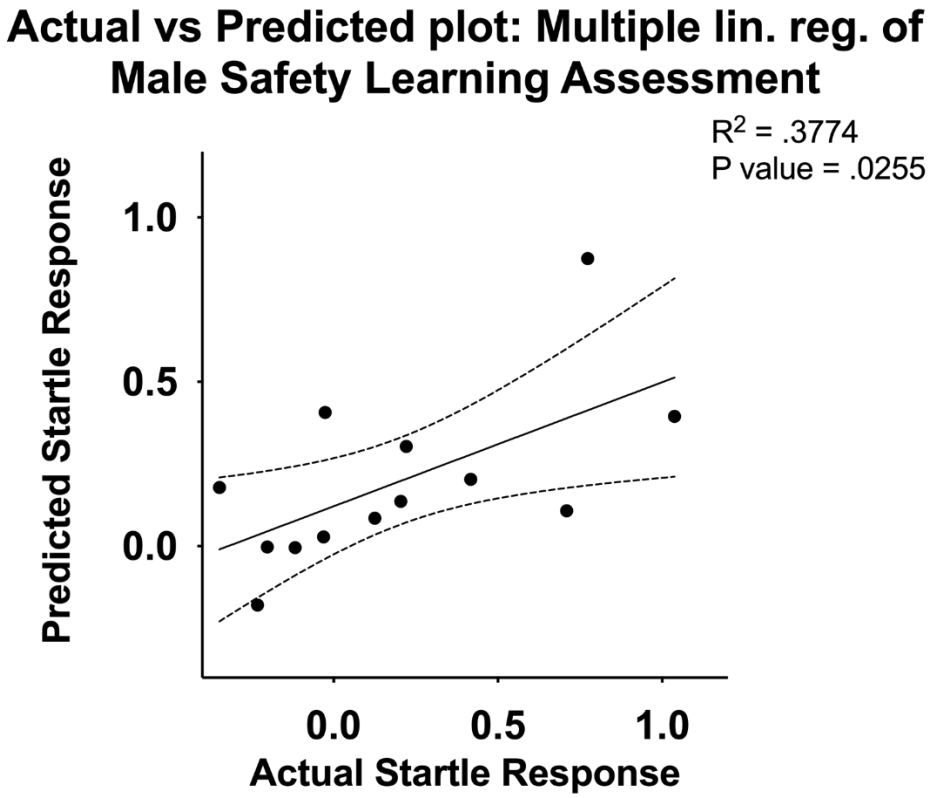

Figure 12: Above is the multiple linear regression for the actual vs predicted variables of the startle response for the safety learning assessment day within the males using average times of struggling during restraint, average times pinned during defeat, and average times of kicking during defeat as explanatory variables. There was a significant regression (P-value=0.0255) and a medium goodness of fit of (R^2=0.3774).

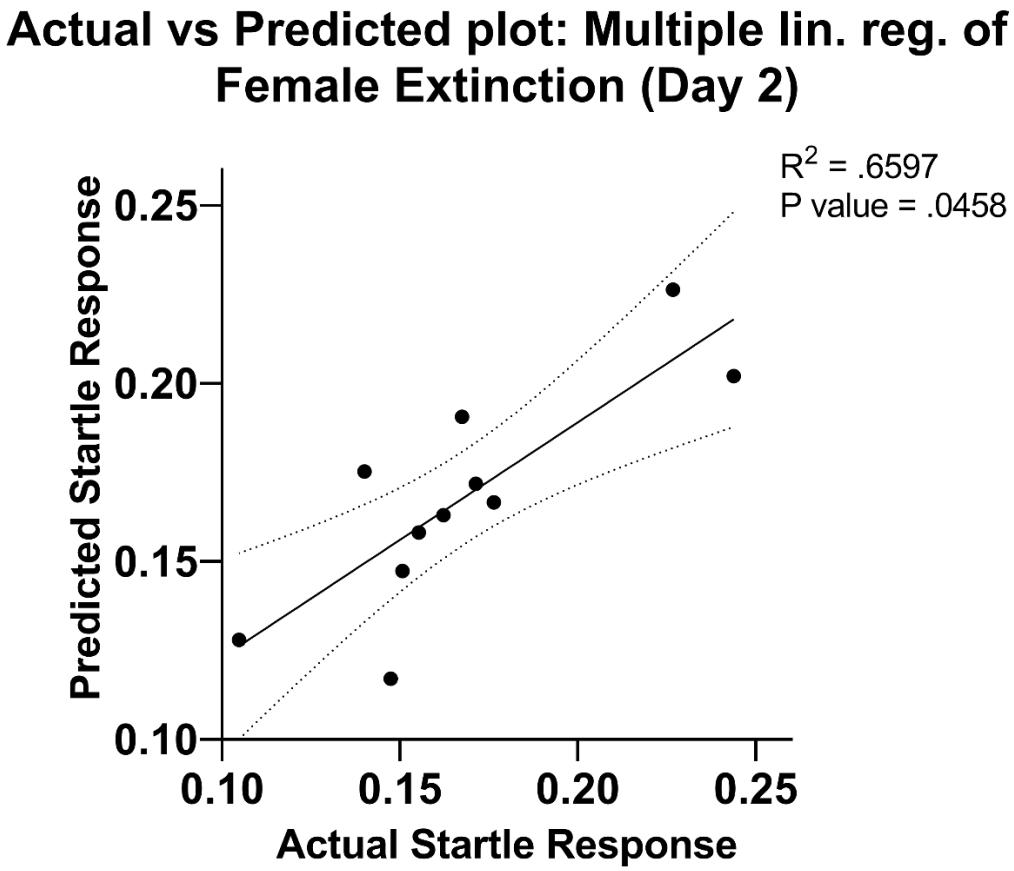

Figure 13: Above is the multiple linear regression for the actual vs predicted variables of the startle response for the second extinction day within the females using average times of struggling during restraint, average times pinned during defeat, and average times of kicking during defeat as explanatory variables. There was a significant regression (P-value=0.0458) and an almost strong goodness of fit of (R^2=0.6597).

Discussion

It is known that PTSD causes impairments in safety learning (Jovanovic et al., 2009). Within human models, the individuals with PTSD have an impaired ability to inhibit fear of a danger cue when paired with a safety cue showing the decrease in safety learning (Jovanovic et al., 2010). Similar animal models show that rats are able to safety learn and discriminate between danger and safety cues (Myers et al., 2004). Chronic adolescent stress causes impairments that can affect the HPA axis which can lead to increase in glucocorticoids that lead to the alteration of synaptic plasticity (Heck and Handa, 2018). Increased synaptic plasticity can lead to alterations in long-term potentiation, which can enhance fear memories due to heightened glucocorticoids (Izquierdo et al., 2016).

Unsupportive to the hypothesis that predicted that CAS would lead to impaired safety learning, chronic adolescent stress significantly increased the ability of the Wistar rats to successfully safety learn within the startle paradigm. The rats within the study proved to extinguish the fear of the conditioned stimulus across all groups, however during the safety learning assessment day within the startle paradigm, the CAS group had a lower startle response to the presence of the conditioned stimulus and therefore a heightened safety learning ability. Regarding the hypothesized decrease in female's ability to safety learn compared to males, sex did not have an effect of the outcome of safety learning.

There were no observable differences prior to chronic adolescent stress between the NS and CAS groups that would cause a difference in the outcomes of each group. The weights of the nonstress group were slightly more than the weight of the chronic

adolescent stress group, however, this would not be a likely reason for any of the differences in the startle responses between the two groups in either sex. CAS did not seem to cause an impairment within the safety learning mechanisms and did not show results consistent with previous work (Bourke and Neigh, 2011; Bekhbat et al. 2021, Hyer et al., 2021; Rowson et al., 2019).

Finally, the hypothesis predicting that the CAS data could be used in a predictive model for the startle response paradigm was not supported due to the lack of overall significance of the regression, as well as the weak goodness of fit. Although, the R^2 values for some of the regressions were significantly able to be predicted, due to the weak goodness of fit, the actual meaning of the regression analyses suggests possibility of other variables that affect startle response. This is important to understand that while each rat within the chronic adolescent stress group was exposed to the stressors, there was a range of actual stress occurring during these simulated events. The severity and incidence of traumatic stressors and the individual physiological and neurological responses to these stressors can explain how within a controlled paradigm, the ability to alter cognitive function and morphology can be limited.

Startle Response

The outcome of this study fails to support the initial hypotheses about chronic adolescent stress being a possible factor in the inability to successfully extinguish a fear and learn safety cues. In the final day of the startle paradigm, the safety learning assessment showed that the non-stress group had a significantly greater reaction to the acoustic cues paired with the conditioned stimuli than without it, which was opposite to

prior expectations. One possible explanation is that due to the startle paradigm being an acute stressor, the CAS group reacted in a way that resulted in them extinguishing the fear of the conditioned cue better than that of the non-stress group due to the increase in corticosterone that aided in the synaptic plasticity (Izquierdo et al., 2016). This increase in synaptic plasticity may have resulted in enhanced memory consolidation during the extinction days. In addition to the chronic adolescent stress not hindering the safety learning assessment, the females startle response did not differ between the males for the baseline, fear potentiated startle, or safety learning days, which is seen to be like a few other studies that show no sex differences in startle behavior (Bourke and Neigh, 2011). It is seen in some studies that the presence of a chronic stressor prior to the fear acquisition helped to facilitate extinction with the females (Baran et al., 2009). However, this lack of sex differences in chronic adolescent stress behavior is not consistent with many other behavioral findings (Hyer et al., 2021, Rowson et al., 2019; Bekhbat et al., 2021)

For the baseline and fear potentiated startle days, the outcome was as expected within prior studies (Myers and Davis, 2004). Both males and females on the baseline day that were in the CAS group had a higher startle response compared to the NS group. A similar difference in baseline testing has been seen among humans who have PTSD and a control group that does not (Bremner et al. 2005). As seen in Figure 7, during the fear potentiated startle day, the NS group was lower than the CAS group, however the only time the CAS group was significantly higher was when the medium cue was played. After running an A priori test, the same size needed to see significance was 14, so the results we see were not underpowered. Although not significantly

different, the males within the CAS group had a higher startle response to the conditioned cue as opposed to the NS group during the fear potentiated startle. The significance being present in the medium cue is possibly the cause of it being the ideal dB for startle responses, as a similar level of dB has been used in other studies (Myers and Davis, 2004). A low cue may be too low to elicit a proper startle response and a high cue may result in a ceiling effect of being too loud to see a difference in startle responses.

On both extinction days, the females had a higher startle response when compared to males. This heightened extinction within females is supported by other studies (Baran et al., 2009). In Figures 6 and 8, the females' startle response for the fear conditioning day and extinction days was almost doubled that of the males. This difference has been seen in other studies that found that there was an inhibited ability to extinguish a fear which is possibly caused by the hormonal response to stress that can potentially delay the return of hormones back to a baseline level (Glover et al., 2016; Heck & Handa, 2019).

However, in this experiment, the key factor that would model PTSD-like behavior is the inability to successfully transfer the inhibition the fear from a safety cue as seen on the last day during safety learning, in which the females and males were similar in startle response. Therefore, this experiment failed to model PTSD-like behavior within the rats by not exemplifying an impairment of safety learning due to sex or chronic adolescent stress.

One limiting factor that could explain the results of this experiment being inconsistent with previous work is the lack of aggressive behaviors the chronic adolescent stress rats experienced while undergoing social defeat. While being within the cage of a territorial matured Long-Evans rat causes adverse social stress due to the agonistic behavior towards the Wistar rat (Bolhuis et al., 1984), the wistar rats within this study did not show many signs of distress while in the cages or interacting with the Long-Evans. In addition to a small proportion of Wistar rats successfully being pinned throughout the social defeat days, additional signs of distress would include avoidance or tail rattling, both of which did not occur often during their time within the foreign cages. This lack of a severe adverse stressor within this cohort may explain that the chronic adolescent stress group were not stressed enough to cause increases in their risk to develop psychological disorders such as anxiety or PTSD. In some studies, involving rodents, there is a longer time exposed to the stressors which results in a more adverse stressor (Ayash et al., 2020).

While this study has contradicting results, it is worth noting there may be things that can be expanded upon as a result of this study. Originally, this study was a part of a larger study that included the use of the drug, D-cycloserine (DCS). Including this drug and expanding in a different way may result in a different perspective of how safety learning may be affected. Although the injection was controlled and uniform throughout the cohorts, the injection method used in this experiment particularly could have had some impact on the outcome of the startle responses in the safety learning day. The injections were done by a subcutaneous injection of DCS or saline.

Regression

Within this study, it was interesting to look at the regression analyses of using the CAS data as a predictive factor of the startle response outputs for each day. This stress paradigm has been used before within the lab as a standard way to model chronic adolescent stress, but the actual data has not been analyzed in a regression in this way before (Bourke and neigh, 2011; Bekhbat et al., 2021).

By using simple linear regression, the startle response each of the days were not reliably predicted by using the measured data from the CAS paradigm. Having a strong goodness of fit, or R-squared value, is important for determining if the model is an accurate model for the data set. As seen in Figure 10, even though the P-value of the explanatory variable and startle response is significant on the baseline day, there is a weak goodness of fit, so there is not a reliability using the average times struggling during restraint as an explanatory variable in a prediction model. Again, in Figure 11, within the males on the fear conditioning day, there is significance in the predictability model using the average number of pins during defeat as an explanatory variable, however the R-squared value is very weak and therefore not reliable. The average number of kicks during defeat is not a significant or reliable predictor for startle response using simple linear regression.

Multiple linear regression was also run to see if the three explanatory variables would be a better model that shows the affect the CAS paradigm would have on the startle response paradigm. As seen in Figure 12, there was significance in the actual versus predicted plot of the males' startle response on safety learning assessment,

however the goodness of fit was a medium reliability, and therefore not a strong model predicting the startle response during the safety learning assessment. In Figure 13, there was significance in the actual versus predicted plot of the females' startle response on the second extinction day, as well as the goodness of fit being a better model of reliability, however, does not reach the rule of thumb that establishes that the model is a strong fit.

Conclusion

The long-term psychological effects of chronic stress within childhood increases the risks of developing cognitive disorders, and should therefore be a priority to those researching neuroscience. Although this experiment failed to model the impaired safety learning seen within those who experience chronic adolescent stress, creating models that can simulate PTSD to further understand the complex mechanisms happening is an extremely crucial step into figuring out how to help the ever-growing amount of people with mental illness. More work should be done in the evaluation of how chronic adolescent stress may increase or impact the risk of developing PTSD and anxiety disorders later in life.

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