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Modulation in NMDA receptor function alleviates safety learning deficit in females who experienced chronic stress during adolescence

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A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

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Abstract

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Anxiety disorders disproportionately impact women and are more prevalent in people with a history of chronic stress. Post-traumatic stress disorder (PTSD) is a type of stress and trauma-related disorder that is largely characterized by hyperarousal to fear-based cues. The inability to dissociate the fear-response from a non-threatening cue is known as impaired safety learning. Given that N-methyl-D-aspartate (NMDA) receptors are key mediators of learning behaviors, we examined the role of NMDAr function in deficient safety learning. Previous studies have shown that female rats with a history of stress have altered NMDAr gene expression and altered glutamatergic signaling; therefore, we hypothesized that alterations in NMDAr function in females with chronic adolescent stress accompanies the safety learning deficit they may exhibit as adults. To test this hypothesis, we exposed male and female rats to a mixed modality stress paradigm during adolescence. The mixed modality stress paradigm consists of repeated restraint, social defeat, and individual housing. Once in adulthood all animals were subjected to a fear conditioning procedure designed to test the ability of safety learning. For the fear conditioning procedure, we measured the rat's startle response to a light cue that had been previously paired with a shock stimulus. Following extinction of the learned behavior, we tested safety learning by presenting the previously shock-associated cue

with an acoustic startle probe. Females with a history of stress showed impaired safety learning by a failure to demonstrate reduced responses to the new association of the light to absence of shock. A separate cohort of rats were treated with the NMDAr agonist D-cycloserine (DCS) that has been shown to enhance fear extinction learning. Females treated with DCS that had a history of stress showed improved safety learning compared to the females who also had a history of chronic stress but did not receive DCS, suggesting that modulation of NMDA receptors can restore safety learning in females with a history of chronic adolescent stress. Males showed no difference between stress groups and non-stressed groups with the addition of DCS. The NMDAr subunit implicated in fear acquisition, NR2B, was shown to be present in equal quantities in both males and females of non-stressed (NS) and chronic adolescent stress (CAS) condition in the basolateral amygdala. However, in the central amygdala CAS males and females both had significantly less NR2B than their NS counterparts. These data suggest that chronic stress during adolescence modifies NMDA receptor function in a manner associated with impairments in learning through alterations in the NR2B subunit in the central amygdala. A better understanding of the mechanisms underlying stress-induced impairments in fear behaviors and safety learning will provide a foundation to examine alternative therapies for people living with fear-based disorders like PTSD.

Introduction

Stressors have the potential to serve as the catalyst for the development of many psychological disorders. Anxiety disorders are the most prevalent psychological disorders in both children and adults, and males and females (Beesdo et al., 2009). Over the past decade the number of people who have reported feeling anxiety has significantly increased among nearly every social demographic, with this trend predicted to continue to increase (Goodwin et al., 2020). Many of the various anxiety disorders have onsets primarily in adolescence or early adulthood (Paus et al., 2008).

PTSD is an anxiety-and-trauma related disorder, that unlike other mental health disorders, must be precipitated by a traumatic event. A person is diagnosed with PTSD if they have been exposed to a traumatic event and consequently have the following effects: intrusion of memories or reactions associated with reminders of the traumatic event; an avoidance of stimuli related to the event; changes in thoughts or feelings concerning the event, such as the inability to remember key aspects of the event or an enduring experience of anhedonia; and alterations in intensity of reactivity and arousal often represented by angry outbursts or hypervigilance (American Psychiatric Association, 2013). When these symptoms prove maladaptive to a person's life and endure longer than a month the criteria for a diagnosis of PTSD is fulfilled (American Psychiatric Association, 2013). In animal models of PTSD-like behavior, the main factors evaluated are anxiety-like behaviors, fear extinction, spontaneous recovery of fear memories and fear response. Researchers have often studied characteristics of PTSD through testing of an animal's acoustic startle response (ASR) after fear conditioning. Using this method researchers have been able to study an animal's expression of fear and extinction learning, after exposure to a threatening stimulus.

In addition to behavioral testing, PTSD-associated phenotypes are often studied by examination of mainly three areas of the brain, the prefrontal cortex, hippocampus, and amygdala. Elevated firing of cells of the medial prefrontal cortex (mPFC) has been associated with spontaneous recovery of fear memories (Milad & Quirk, 2002). Destruction of mPFC-amygdala pathways have resulted in impaired recall of fear

extinction (Yabuki & Fukunaga, 2019), which in conjunction with the decreased fear response seen in mice with inactivated basolateral amygdala (BLA) neurons (Sotres-Bayon et al., 2012), led researchers to believe that suppression of the amygdala works to suppress exaggerated fear responses. Patients diagnosed with PTSD also exhibited excessive amygdala activity in response to a fearful cue (Rauch et al., 2000; Stevens et al., 2013).

Women have a lifetime prevalence for PTSD of 9.7% with a majority of these cases caused by sexual violence, while men have a lifetime prevalence of 3-7% with these primarily arising from combat experience (Cloitre et al., 2019; Dobmeyer, 2017). Treatment for PTSD often involves some prescription of selective serotonin re-uptake inhibitors (SSRIs) and selective norepinephrine re-uptake inhibitors (SNRIs) in combination with therapy (Dobmeyer, 2017). Mice not expressing the serotonin 1A receptor displayed increased anxiety-like behaviors and increased fear retrieval implicating this receptor in PTSD symptoms (Klemenhagen et al., 2006; Parks et al., 1998). SSRIs work to increase the activation of serotonin receptors by blocking reabsorption of serotonin, consequently relieving some of the symptoms of PTSD. SNRIs work in a similar fashion for norepinephrine and its receptors. One prominent symptom of PTSD is an increased response to stimuli associated with a fearful event. This increased response to stimuli associated with the event underlies an issue in safety learning, that is the ability to form new memories disassociating an object/situation with danger, after previously having formed memories linking that object/situation with danger. Treatments like SSRIs work to reduce the anxiety and fear caused by this inability to form safe memories.

Examining factors that contribute to an impairment in safety learning is important for better understanding PTSD (Jovanovic et al., 2012). Stress during early life is associated with greater adult diagnosis of PTSD (Nemeroff et al., 2006). Adolescence is a specific period of development in which the effects of stress are magnified (Mousavi et al., 2019).

Adolescence

Adolescence is a time of rapid maturation in a person's early life (Steinberg, 2005). Puberty occurs during adolescence with adolescence describing a time period and puberty referring to the onset of physical maturation occurring at the behest of certain neural developments. Throughout adolescence, young people are often exposed a bevy of new experiences, such as an expanding circle of peers, more exposure to harmful substances, and an increase in academic pressures (Moffitt, 2017; Steinberg, 2005). These social changes, along with the physiological ones already naturally occurring, make adolescence important for neuronal maturation. The exact timeline for what qualifies as adolescence in humans is somewhat varied with researchers generally considering it within the age range of 10-19 years (Freeman et al., 2020). However, one thing that is generally agreed upon among those who study development is that adolescence is a time for numerous critical developments, especially in the brain (Steinberg, 2005). During adolescence there is an increase in the ratio of white matter to gray matter (Blumenthal et al., 1999; Sowell et al., 2002), which is indicative of more integration between different regions of the brain. The prefrontal cortex specifically has this increase in white to gray matter ratio and has increases in synaptic pruning as well (Sowell et al., 2002). These changes in matter ratio also result in an increase in conductivity, thus improving the efficiency of processing information. Increase in processing has been shown to be important for self-regulation, and planning (Steinberg, 2005).

All the remodeling that occurs as a natural progression of neural development also make this window of time particularly susceptible to the impacts of external stressors. Individuals who experienced severe stressors during adolescence were more likely to develop psychological disorders in adulthood than those who were not exposed to stressors of a similar severity (Beesdo et al., 2009). The amygdala is the region of the brain responsible for our learning of and response to uncertain or threatening stimuli (Cheng et al., 2007; Silvers et al., 2016). Compared to adults, the adolescent amygdala is more reactive, with a greater reactivity being correlated with greater risk of developing psychological disorders (Vasa et al., 2011). Amygdala activity is in part regulated by the

PFC, making the PFC's increase in white to gray matter ratio especially important in controlling emotional reactivity (Likhtik et al., 2014).

The most common psychopathology to develop in adolescents are those dealing with anxiety (Kessler et al., 2005). Anxiety is a neural response to anticipated stimuli believed to be dangerous and that the subject would want to avoid (Craske et al., 2009). The anxiety response is typically adaptive, as it primes the body to avoid a potentially dangerous event. The anxiety response becomes maladaptive when it begins to interfere with normal functioning. The anxiety response becoming maladaptive is exemplified in the case of generalized anxiety disorder (GAD) where non-dangerous stimuli are evoking a neural response reserved for potentially dangerous stimuli. This increased response to stimuli not directly associated with danger in patients with GAD shares a similarity with PTSD, with the main difference being that patients with PTSD have a specific event that spurred their bouts of hyperarousal (Dobmeyer, 2017).

In a study researching development of anxiety disorders in adolescents aged 14-17, researchers found that a majority of adolescents who developed an anxiety disorder would go through a sort of remission, with only twenty percent still meeting the criteria for their disorder in a two year follow up (Podossinov & Chekin, 1991). Despite this remission, in a study that included a ten year follow up on those who had been diagnosed with an anxiety disorder as adolescents, most of those who initially had a remission later on had some form of mental health disorder (Wittchen, 1988). These results mean that the development of anxiety disorders during adolescence is related to one's probability of developing a disorder as an adult. With adulthood being the period where people are more likely to face the situations that are often the leading causes of PTSD, such as combat or sexual violence, (Black, 2010; Miao et al., 2018; Street, 2016) examining adolescence, and its concurring developments are pivotal for understanding mental health in later life.

The challenges faced by people that deal with these psychopathologies make studying factors that contribute to their development crucial, as a possible preventative measure for their occurrence. Many factors are positively associated with the development of anxiety disorders, such as low income (Wittchen et al., 1998), childhood separation

(Kessler et al., 1997), among others. A common thread between many factors contributing to these developments is the presence of stress.

Stress

Stress is defined as any threat to an organism's homeostasis (Seyle, 1956). Stress, like anxiety, is meant to be adaptive when experienced briefly. Acute stress even has memory enhancing effects (Roozendaal et al., 2009). People often find themselves feeling stressed more now than they did in past years and in different ways (Goodwin et al., 2020). Our body's stress response is primarily controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Toledo-Rodriguez & Sandi, 2011). The HPA axis is a feedback loop that consist of interactions between the hypothalamus, anterior pituitary gland, and the adrenal glands (Gjerstad et al., 2018). When we experience stress, afferent neurons stimulate the paraventricular cells of the hypothalamus causing them to release corticotropin releasing hormone onto the anterior pituitary gland (Antoni, 1986). Corticotropin releasing hormone then stimulates corticotropic cells of the anterior pituitary gland to then release adrenocorticotropin hormone onto the zona fasciuclata of the adrenal cortex (Gjerstad et al., 2018). The adrenal cortex then releases cortisol (corticosterone in rats) throughout the body having various effects, with the overall goal being to protect the host from detrimental effects from the stressor (Sternberg, 2012). Cortisol then acts on the hypothalamus to reduce the activity of this chain, so excess cortisol is not released. The impact a stressor has through this axis and all its corresponding parts, depends on a few factors, primarily the type of stressor, who experiences it, and the period in which one is exposed to it.

In examining the difference between the effect of different types of stress, a study that compared the effect of physical stressors versus those of psychological stressors found similar results in some areas such as a mutual increase in corticosterone in response to the stress compared to control animals (Mousavi et al., 2019). However they also found that the physical stress group had increased anxiety-like behavior compared to the remaining groups, while the psychological stress group had increased oxidative stress (Mousavi et al., 2019). Researchers studying effects of early life stress often find

different results based on whether the stressors were applied in a predictable manner or an unpredictable one (Koenig, 2007; Xu et al., 2014).The varied effects caused by different characteristics of the stressor signifies that a study looking to examine the impact of stress would need to incorporate different kinds of stressors to be comprehensive.

For the “who” of how stressors have different impacts, an important difference is the varied responses between males and females. Females experiencing stress during adolescence have a higher likelihood of developing psychiatric disorders than males who experience stress (Woodward & Fergusson, 2001). These higher rates are likely in part caused by a dysregulation of the HPA axis. (Toledo-Rodriguez & Sandi, 2011). This axis is influenced by hormones that females have in much higher concentration than males, like estradiol (Nillni et al. 2015). The fluctuation of estradiol that occurs during female’s estrous cycle can cause the HPA axis to become unstable (Maddox et al. 2018). This instability of the HPA axis can lead to an increase in the release of the molecules associated with the stress response (Maddox et al. 2018). These differences can make females more likely to experience the consequences of stress. This higher rate of stress in females makes it important to further examine what else may be contributing to this increase.

For examining the difference between the timing of when a stressor is experienced, we have already discussed how adolescence is a particularly vulnerable time due to the maturation occurring in the brain. Many of the afferent neurons that stimulate the hypothalamus have greater reactivity in adolescents than in adults (Vasa et al., 2011). Adults who experienced chronic stress during adolescence tend to display higher levels of anxiety than adults who did not and they also tend to develop more psychiatric disorders. (Paus et al., 2008; Woodward & Fergusson, 2001). This is likely because during adolescence, and even more so specifically in females, stress exacerbates the growth of the cells that are responsible for our learned responses to stressful and fearful events (Farrell et al., 2015; Suvrathan et al., 2014). These are the cells of the amygdala.

Amygdala

The amygdala is the region of the brain that is responsible for identifying and learning about important environmental conditions that may be relevant to spur action of some kind (Herry et al., 2007). These environmental conditions are emotionally charged, such as fears or stressors. The two primary regions of the amygdala that are associated with learning and responding to emotionally relevant stimuli are the basolateral amygdala (BLA) and the central amygdala (CeA) (Kim et al., 1993; Quirk et al., 1995; Walker & Carrive, 2003). The BLA is the main input region for sensory information coming from the thalamus and different regions of the cortex (Sotres-Bayon et al., 2012). The BLA sends this sensory information to the CeA which is the main output region of the amygdala. The CeA has different neurons connecting to areas such as the midbrain that work to control our physical response to the information gathered by the BLA (Walker & Carrive, 2003).

As the main input region the BLA is important in the largely implicit learning associated with individual's experiences of fears and stressors (Campeau & Davis, 1995). Lesions to the BLA blocked some of the memory enhancing effects caused by a response to stress (Roozendaal, 1997; Roozendaal & McGaugh, 1996). Often memory enhancing effects related to stressful stimuli are sensory, thus lack of emotional recognition is likely caused by the break in connection between the thalamus and the BLA. Rats who had bilateral lesions of the amygdala showed no recognition of emotional material, nor emotional responsiveness (Adolphs et al., 1997). These results, along with many others, demonstrate the amygdala's impact in emotional learning and response.

The amygdala undergoes substantial development as people age. Rapid development occurs immediately postnatally, and the amygdala is much more responsive during early childhood than during adulthood (Decety et al., 2012; Gee et al., 2013; Silvers et al., 2017; Vink et al., 2014). The connections the amygdala makes with the PFC significantly strengthens during late adolescence (Delgado et al., 2008; Motzkin et al., 2015). This strengthening is important because the PFC often acts as a regulator of our behavior that is evoked by strong emotional responses (Gabard-Durnam et al., 2014; Gee et al., 2013; Qin et al., 2014; Wu et al., 2016). Some of the riskier behavior that

increases during adolescence has been hypothesized to be caused by the uneven development in the PFC and amygdala (Tottenham & Gabard-Durnam, 2017). Specifically, researchers have hypothesized that since the amygdala develops more quickly than the PFC, children often do not have the same neural regulations on their emotional impulses that adults may have (Tottenham & Gabard-Durnam, 2017). This uneven development gives the amygdala relatively more leverage in our natural response to stimuli, and consequently giving it a bigger role in our following behavior. This unevenness between the amygdala and PFC and its results are even more exacerbated in adolescents who experience repeated stress (Suvrathan et al., 2014).

Stress increases the activity of the neurons of the amygdala, and the arborization of its excitatory neurons (Castillo-Gómez et al., 2017). Administration of corticosterone resulted in hypertrophy in the basolateral region of the amygdala (Mitra & Sapolsky, 2008). The amygdala's change in morphology in response to stress results in hyperactivity, partially due to the increase in synapses with cells of other regions (Thomas et al., 2001). This change in the amygdala is seen in those with anxiety disorders, which again occurs in greater frequency in females (Thomas et al., 2001). Given the amygdala's function, examining the proteins that play a role in our response to stimuli can give us greater insight into the effects of chronic stress on adolescent females. Since much of our response to threatening or uncertain stressors is determined by how our body has learned to react to them, to further examine the impact these stressors have on our behavior, it is important to look at a key component in the amygdala's process of learning, mainly N-methyl-D-Aspartic-Acid receptors (NMDArs).

NMDAr

The amygdala plays a crucial role in our learned responses to stressors. Learning is often done through a process called Long Term Potentiation (LTP). LTP is the strengthening of the connection between two cells and is the prototypical example of synaptic plasticity. The primary method of long-term potentiation occurs when glutamate is released from a presynaptic cell onto NMDArs.

N-Methyl-D-Aspartic-Acid receptors are protein ion channels that exist in various parts of the brain and allow responses to glutamate in conjunction with coagonist, such as glycine or D-serine, released by presynaptic cells (Paoletti & Neyton, 2007). NMDArs are a heterotetrametric ion channel composed of a combination of three different subunits, NR1, NR2, NR3 (Cull-Candy et al., 2001). NMDArs consistently contain two subunits of NR1, and for their remaining two subunits may contain a combination of NR2 or NR3, with there being four different subunits for NR2 and two for NR3 (Monyer et al., 1992). Version A of the NR2 subunit, also called NR2A, is the version of NR2 primarily found in mature synapses, and NR2B is the subunit often found in newly formed synapses (De Armentia & Sah, 2003). Upon birth, most of the synapses containing NMDArs have primarily the subunit combination of NR1/NR2B, with this often switching over to NR1/NR2A as the regions develop (Wang et al., 2014). An increase in NR2B in adults could be used as evidence of an increase in developing connections between cells. NR2B however is not only present in newly formed synapses. NR2B in the amygdala is prevalent in synapses concerning fear memories (Walker & Davis, 2008). NR2B antagonist administered to the lateral amygdala blocked fear acquisition in adult rats (Rodrigues et al., 2001), while NR2A antagonist reduced fear expression (Walker & Davis, 2008).

At rest, NMDArs are blocked by an Mg+. For NMDArs to be activated two things must happen simultaneously: glutamate must be released from the presynaptic cell onto the ligand binding domain of the NR2 subunit of the post synaptic cell, and the post synaptic cell must be depolarized (Luscher & Malenka, 2012). Once both events happen the Mg+ ion leaves the NMDAr receptor, and calcium, rushes in. Large amounts of calcium rushing into the cell causes a cascade of signaling proteins that ultimately result in the addition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPArs) (Luscher & Malenka, 2012). These actions by NMDAr and the addition of AMPArs to the synaptic membrane is the process referred to as Long Term Potentiation, which is the mechanism that strengthens connections between cells with these receptors in learning (Fiorenza et al., 2012). This process has been studied extensively in the hippocampus, and past research in this lab has shown that chronic stress during adolescence in females alters NMDAr function and enhances the activity of genes contributing to

activity at NMDAr synapses which was correlated with memory impairments. These results show that stress impacts learning through changes in NMDAr function and given the amygdala's role in learning in response to stressful events we could expect to find similar results in this region in regard to safety learning. To verify that any changes in memory are in response to changes in NMDAr function it would be necessary to determine the effects of pharmacological modulations of NMDAr. To do so we used D-cycloserine.

D-cycloserine (DCS) is a drug that has been shown to enhance the extinction of fear memories through working with NMDArs (Baker & Richardson, 2017; McCallum et al., 2010). DCS mimics the naturally occurring D-serine, which itself binds to NMDArs to facilitate the binding of glutamate (Davis et al., 2006). In clinical studies DCS has often been used in conjunction with exposure therapy or cognitive behavioral therapy, to varying degrees of success (De Kleine et al., 2012; Loerinc et al., 2015). In animal studies DCS has been used in fear conditioning procedures to enhance the effects of safety learning, promoting a decrease in anxiety-like behaviors, with many studies consistently verifying its effectiveness (McCallum et al., 2010; Tang & Graham, 2019; Weber et al., 2007).

Implication

I have discussed the vulnerability of adolescents to emotional stimuli. With the greater occurrence of some of the more insidious outcomes of stress, like anxiety disorders, that are more often found in females, it is important to study this process of learning. In looking at this process we will gain insight into why females experience anxiety disorders more, and how adolescence might be a sensitive period for this. I hypothesize that deficits in female's safety learning are caused by changes in NMDAr function. To verify that changes in NMDAr function are the causes it will be important to measure the changes in NR2B between females and males who have experienced stress and those who have not. It will also be important to see if DCS can assist in the extinction of fear memories in females who have issues in this regard. Since impairments in safety learning is an issue that creates some of the phenotypes associated with PTSD,

understanding what proteins, and protein subunits play a role in this impairment can narrow the focus of what possible treatments would address.

Methods

Subjects

Male and female Wistar rats were used for these procedures. Female pregnant Wistars were obtained from Charles River in Morrisville, NC. Animals were kept in cages in a room on a 14:10 light cycle, that stayed between the temperatures of 20 and 23 degrees Celsius. All animals involved had free access to both food and water. The animals used for the procedures were obtained following parturition by the mothers. Upon birth the litters were reduced to four of each sex. The animal's weights were recorded every week (shown in figure 2). For the first cohort with which we did the standard fear conditioning we had 36 rats in total. There were 20 males and 16 females. The two sexes were then evenly assigned to either the stress or non-stressed group. For the second cohort which underwent the fear conditioning procedure with the addition of DCS, we had 23 rats in total. There were 12 males and 11 females, with the males split evenly in the stressed and non-stressed group and the females having 5 in the stressed group and the remaining 6 in the non-stressed group. On postnatal day (PND) 23 all the pups were weaned and housed in pairs of the same sex. The non-stressed animals remained in this standard housing procedure. Upon PND 35 the rats in the stress group were moved to isolation housing. The chronic adolescent stress group started the stress procedure starting on PND 38 until PND 49, a period seen as analogous to the period of adolescence humans experience (Sengupta, 2013). To induce stress the animals underwent isolation housing, restraint, and a social defeat procedure. This stress paradigm, described in more detail below, has been well established in previous studies completed by researchers in this lab, resulting in increases in female's anxiety-like behavior as shown by their results on an elevated plus maze (Bourke & Neigh, 2011). For the restraint sessions the amount of observed

struggling-like behaviors were recorded. Behaviors considered to be indicative of struggling were any biting, pushing, or digging around the restraint barrier. These struggling behaviors and weights were recorded to ensure that the stress was equally potent between the groups.

Chronic adolescent stress

Restraint stress

For the restraint stress the animals were kept in narrow plastic restraint tubes (Braintree Scientific, Braintree, MA) for an hour. The animals underwent this hour of restraint once a day, on six different days dispersed between PNDs 38-49. Every two minutes of the restraint procedure the animals were observed to see if they were displaying a behavior indicative of struggling. Results from the restraint stress are shown in figure 3.

Social defeat

The following social defeat procedure was completed to place the Wistar rats in situations where larger Long Evans rats (Charles River, Morrisville, NC) would display stress-inducing dominance behaviors. Female Long Evans rats were ovariectomized. This exact protocol for this procedure has been taken from previous studies (Bourke & Neigh, 2011; Rowson et al., 2019).

For this procedure, a barrier was placed in the cage of the Long Evans and a Wistar rat was placed on the side opposing the Long Evans rat. After two minutes of this condition, the barrier was removed allowing the two rats to physically interact. The rats could interact for either five minutes or until the larger Long Evans rat successfully pinned the Wistar rat three times. A pin was defined as the Long Evans rat forcing the Wistar onto their back, temporarily immobilizing them. After the five minutes or three pins the barrier was placed back in the cage and allowed to sit for twenty-five minutes. The pairing between the Wistar and the Long Evans of the same sex were rotated, so each session the Wistar would have a different pairing from its previous one. The number of pins for

each day were recorded. This procedure was completed once on six different days throughout PND 38-49. Results from the social defeat stress are shown in figure 4.

Post-stress

Non-stressed animals were separated on PND 60. After the stress procedure was completed the stress animals received enrichment and all animals were undisturbed until startle fear conditioning, minus standard husbandry activities. On PND 83-92 the animals started the conditioning procedure.

Startle response & fear conditioning

To examine the animal's efficiency in extinction learning we measured their startle response through a fear conditioning paradigm. The animal's startle responses were measure with a Startle Response System (San Diego Instruments Inc., San Diego, CA) commonly referred to as a "startle box". The startle box consists of a narrow plastic tube in a larger box with a constant background white noise at 55dB. The plastic tube contains metal gratings that transmit the pressure placed on the gratings from movement to a separate system that records the data.

To start the animals were first acclimated to the room where the experiment would be carried out. For this the animals were brought from the vivarium to the experiment room where they remained in their cages for an hour, and then brought back down to their vivarium. This was done for two days. Then to habituate the animals to the startle boxes, on the following days the animals were brought up again to the experiment room and each spent five minutes in the startle box with the chamber lights turned on. This was also done for two days. Following the second habituation day the animals started the fear conditioning procedure, which was divided into several days: Day 1 for measuring the baseline; Day 2 for fear conditioning, Day 3 for fear potentiated startle, Day 4 and 5 for extinction training, and Day 6 for the safety learning test. The schedule and set up for the procedure are displayed in figure 1.

Baseline

For the baseline measurement the animals were brought up to the behavior room for thirty minutes to be allowed to habituate to the room before the experiments. The animals were then placed in the startle box which was then activated. The startle box would play an acoustic startle probe at either low, medium, or high volume (90dB, 95dB, 105dB respectively). An acoustic probe of randomized intensity between the three options was played every thirty seconds for thirty trials. Each volume received ten trials. This produced the baseline startle response for each animal at the different intensities.

Fear conditioning

For the fear conditioning day, the animals were placed in the startle boxes. When activated the metal grating at the bottom of the narrow tube containing the animals would deliver a shock of 0.6mA to the animals. The shock was always preceded by an LED light flashing on for three seconds before the shock. This was done to condition the animals to associating the LED light with the shock. The entire session for one animal lasted twenty minutes with ten trials of the shocks + light pairings dispersed randomly throughout this time, and the startle response recorded for each trial.

Fear potentiated startle

For this day, a similar procedure to the baseline day was performed however this time each acoustic probe was paired with the LED light on half of the trials. Again, the LED light, which is now functioning as a conditioned stimulus, would flash on for three seconds. This procedure is done to examine how the association of the LED light with the foot shock from the previous day influences the startle response. The light stays off for half of the trials as way of comparing the startle responses to see if the fear conditioning with the light alters startle responses.

Extinction training

For the extinction training days, the animals were placed in the startle boxes with the LED light flashing on every thirty seconds for three seconds without the shock. This was done to train the animals to no longer associate the light with the stressful shock.

Safety learning test

For this day, the same procedure from the fear potentiated startle day was repeated. An acoustic probe was paired with the LED light on half of the trials. The LED light would flash on for three seconds. This procedure was done to examine how the association of the LED light with the foot shock from the previous day influences the startle response. The light stayed off for half of the trials as a way of comparing the startle responses to see if the fear conditioning with the light alters startle responses. This was done to examine if the extinction training days reduced the startle responses from the initial augmented startle responses caused by the fear conditioning.

Drug

The second cohort to go through the startle conditioning received a subcutaneous injection of D-cycloserine (Sigma Aldrich, St. Louis, MO) dissolved in saline immediately following day one of extinction training (with a dosage of 15 mg/kg at a volume of 1ml/kg). This specific dosage and volume was used because it has shown effectiveness in previous fear conditioning studies (Graham & Scott, 2018; Ledgerwood et al., 2005; Lehner et al., 2010; Tang & Graham, 2019). The timing of the administration, which was immediately after the last extinction trial on day 1 of extinction training, was also chosen because past studies found significant results on memory with this timing (Graham & Scott, 2018; Ledgerwood et al., 2005; McCallum et al., 2010).

Histology

The day after the safety learning test the animals were euthanized by rapid decapitation. The brains were collected and bisected. The brains were kept in paraformaldehyde for at least twenty-four hours before they were then transferred over to phosphate buffered saline (PBS). After remaining in PBS for at least 24hrs the brains of the animals who did not receive DCS were eventually transferred over to a 20% sucrose solution, where they remained until they were saturated. When brains were fully saturated with the glucose solution the brains were then sectioned fully at 40µm on a Leica CM1950 cryostat. The region containing the amygdala was heated in a citrate buffer before being rinsed with PBS. The tissue was then placed in blocking buffer at room temperature for an hour. Afterwards the tissue was taken and incubated overnight with a monoclonal anti-NMDAR2B antibody (BioLegend Cat. No. 818701). On the following day, the slices were rinsed with phosphate-buffered saline (PBS). Then the slices were incubated overnight with Alexa Fluor 488 goat anti-mouse secondary antibody (ThermoFisher AB_2534088) at room temperature. On the following day, the slices were rinsed again in PBS. The slices were then coverslipped with Vectashield HardSet Antifade Mounting Medium with DAPI (Vector, cat # H-1500).

Imaging

The stained slides were imaged on a Zeiss LSM 700 by taking 10µm image stacks with 1µm intervals at 63x oil immersion. The images taken were of the basolateral and central regions of the amygdala. Regions were identified using the Paxinos and Watson Rat Brain Atlas (6th edition). Amygdala sections were selected based on Figure 57. The basolateral amygdala was located by following the external capsule to its most ventral point. The central amygdala was located by moving medially and imaging directly ventral to the commissural stria terminalis. Two image stacks per section and two sections per region were taken totaling four image stacks per region. The lab member taking the images was blinded to the experimental conditions. These image stacks were then uploaded into the program Volocity (Quorum Technology Inc., Ontario, Canada) which was used to determine the density of NR2B as indicated by the volume of

fluorescence within the image stack detected by the program. Parameters within Volocity were set to exclude objects larger than 490μm³ and to count intensity threshold between 30,345-65,535 for the BLA and 44,525-65,535 for the CeA. These settings were implemented based on negative control images to exclude blood vessel labeling and background labeling, respectively. The volume of fluorescent labeling for each image stack was recorded on an excel sheet. Volumes across the four images per region were averaged and each averaged value per animal was used for analysis.

Statistics

All data was analyzed using GraphPad Prism Software (San Diego, CA). To determine the difference in densities we used a two-way Analysis of variance (ANOVA). We also used a two-way ANOVA to analyze the difference in startle responses for the fear conditioning. We used a three-way ANOVA to analyze the difference in startle responses on the baseline, and fear potentiated startle day. We used a four-way ANOVA to analyze the difference between the startle responses on the safety learning test day, and startle responses for the extinction training days. Significance was analyzed using an alpha level of 0.05. Tukey's test for post-hoc analyses were performed for the days in which cue level was a factor, namely the baseline, fear potentiated startle, and safety learning test day.

Results

Weight & Stress

Males had significantly greater weight than females ($F_{(1,32)} = 30.232$; $p < 0.001$), as shown in figure 2. There was no difference between non-stressed and chronic adolescent stress ($F_{(1,32)} = 0.001$; $p = 0.970$), and there was no interaction between sex and stress ($F_{(1,32)} = 0.001$; $p = 0.970$). As displayed by figure 3 there were no significant differences between males and females for average amount of struggle behaviors per session of restraint stress ($F_{(1,10)} = 0.149$; $p = 0.707$). For the social defeat procedure males had significantly more pins per session than females did. ($F_{(1,10)} = 6.974$; $p = 0.025$), as displayed in figure 4.

Fear conditioning and startle

Startle responses were taken from the average max mV from a subject's session on a given day. The weight corrected startle was calculated by taking the average mV from a subject's session and dividing it by their terminal weight in grams. For the fear potentiated startle day and safety learning test the proportional startle was calculated by taking the average startle response on a conditioned stimulus run and subtracting that from the average startle response without the conditioned stimulus. This number was then divided by the average startle response without the conditioned stimulus to obtain the final proportional startle result.

Baseline

Figure 5 displays the results from the baseline day. The results from the ANOVA showed females had a significantly higher startle response than males ($F_{(1, 165)} = 6.878$; $p = 0.0095$). There was also a main effect of decibel intensity indicated by the low, medium, and high cue level ($F_{(2, 165)} = 65.72$; $p < 0.0001$). There was no effect of stress

($F_{(1, 165)} = 0.04552$; $p = 0.8313$). There was no significant interactions between sex and stress ($F_{(1, 165)} = 3.296$; $p = 0.0713$), sex and cue level ($F_{(2, 165)} = .4289$; $p = 0.6520$), and sex, cue level, and stress ($F_{(2, 165)} = 0.2929$; $p = 0.7465$). A Tukey HSD Post-Hoc test showed that high acoustic weight corrected startle response was significantly higher than the low acoustic response with a mean difference of 2.703 and a p value less than 0.001. The high acoustic weight corrected startle response was also significantly higher than the medium acoustic response with a mean difference of 1.889 and a p value less than 0.001. The medium acoustic weight corrected startle response was significantly higher than the low acoustic response with a mean difference of 0.8148 and a p value of 0.003.

Fear conditioning

Figure 6 displays the results from the fear conditioning day. The results from the ANOVA showed that females had a significantly higher startle response than males ($F_{(1, 55)} = 139.134$; $p < 0.001$) with no effect of stress ($F_{(1, 55)} = 0.506$; $p = 0.48$). There were no significant interactions between sex and stress ($F_{(1, 55)} = 0.300$; $p = 0.586$).

Fear potentiated startle

Figure 7 displays the proportional startle results from the fear potentiated startle day. The results from the ANOVA showed a main effect of cue level when taking into account the trials with and without the conditioned stimulus ($F_{(2, 165)} = 13.87$; $p < 0.001$). There was no main effect of sex ($F_{(1, 165)} = 0.874$; $p = 0.351$), or stress ($F_{(1, 165)} = 0.346$; $p = 0.557$). There were also no significant interactions between sex and stress ($F_{(1, 165)} = 2.721$; $p = 0.101$), stress and cue level ($F_{(2, 165)} = 1.207$; $p = 0.302.$), sex and cue level ($F_{(2, 165)} = 0.725$; $p = 0.486$). There was also no interaction between sex, stress, and cue level ($F_{(2, 165)} = 0.534$; $p = 0.587$). A Tukey HSD Post-Hoc test showed that the proportional startle response to the high acoustic cue was significantly lower than the proportional startle response to the low acoustic cue, with a mean difference of -1.049 and a p value less than 0.001. The high acoustic proportional startle response was also

significantly lower than the medium acoustic response with a mean difference of -0.641 and a p value of 0.004. The low acoustic proportional startle response was not significantly higher than the medium response with a mean difference of 0.408 and a p value of 0.101.

Extinction training

Figure 8A and figure 8B display the results from day 1 and day 2 of extinction training. For the extinction training days there was a significant effect of sex with females having higher a higher startle response than males ($F_{(1, 106)} = 43.551$; $p < 0.001$). There was also a main effect of day with the day 1 results being lower than the day 2 results ($F_{(1, 106)} = 4.41$; $p = 0.038$). There was no effect of stress ($F_{(1, 106)} = 0.048$; $p = 0.828$). There was no interaction between sex and stress ($F_{(3, 106)} = 0.034$; $p = 0.854$), sex and day ($F_{(1, 106)} = 0.832$; $p = 0.364$), stress and day ($F_{(1, 106)} = 0.000$; $p = 0.982$). or sex, stress, and day ($F_{(1, 106)} = 0.034$; $p = 0.854$).

Safety learning test

Figure 9 displays the results for the males from the safety learning test day, and figure 10 displays the results for the females. There was a significant interaction between all four variables, sex, stress, DCS, and cue level ($F_{(2, 152)} = 3.259$; $p = 0.041$). There was also a significant interaction between sex, stress, and DCS ($F_{(1, 152)} = 4.488$; $p = 0.036$). CAS females who did not receive DCS had a significantly higher startle response than NS females who also did not receive DCS ($F_{(1, 41)} = 15.696$; $p < 0.001$). CAS females who received DCS had no significant difference from NS females who received DCS ($F_{(1, 27)} = 0.025$; $p = 0.877$). There was no significant interaction between sex, stress, and cue level ($F_{(2, 152)} = 0.842$; $p = 0.433$); sex, DCS, and cue level ($F_{(2, 152)} = 0.179$; $p = 0.837$); and stress, DCS, and cue level ($F_{(2, 152)} = 0.506$; $p = 0.604$). There was a significant interaction between sex and cue level ($F_{(2, 152)} = 4.866$; $p = 0.009$). There was no interaction between sex and stress ($F_{(1, 152)} = .678$; $p = 0.411$), sex and DCS ($F_{(1, 152)} = 0.001$; $p = 0.972$), stress and DCS ($F_{(1, 152)} = 1.690$; $p = 0.196$), stress and cue

level ($F_{(2, 152)} = 0.054$; $p = 0.948$), DCS and cue level ($F_{(2, 152)} = 0.200$; $p = 0.819$). The ANOVA showed no main effect of sex ($F_{(1, 152)} = 1.098$; $p = 0.296$), DCS ($F_{(1, 152)} = 0.429$; $p = 0.514$). There was a main effect of stress ($F_{(1, 152)} = 5.456$; $p = 0.021$) with CAS having a higher response, and there was a significant effect of cue level ($F_{(2, 152)} = 9.817$; $p < 0.001$) with low acoustic startle group having the highest proportional response. A Tukey HSD Post-Hoc test showed that males low proportional startle response was significantly greater than their high proportional startle response with a mean difference of 0.968 and a p value less than 0.001, and their medium proportional startle response with a mean difference of 0.637 and a p value of 0.005. Non-stressed males who did not receive DCS had a significantly higher proportional startle response at a low acoustic tone than at a high acoustic tone with a mean difference of 1.127 and a p value of 0.012.

Immunohistochemistry

Figure 11A and 11B display the results for the volume of NR2B in the BLA and CeA. An ANOVA performed on volumes obtained from the immunohistochemistry staining procedure found that for the basolateral amygdala there was no main effect of either sex ($F_{(1, 19)} = 0.055$; $p = 0.818$), or stress ($F_{(1, 19)} = 2.302$; p 0.146). There was also no interaction ($F_{(1, 19)} = 0.436$; $p=0.517$). For the central amygdala there was no main effect of sex ($F_{(1, 18)} = 0.453$; $p=0.509$). There was a main effect of stress ($F_{(1, 18)} = 5.423$; $p=0.032$), with the CAS males and females having significantly less NR2B than their NS counterparts. There was no interaction ($F_{(1, 18)} = 0.2417$; $p=0.6289$).

Figures

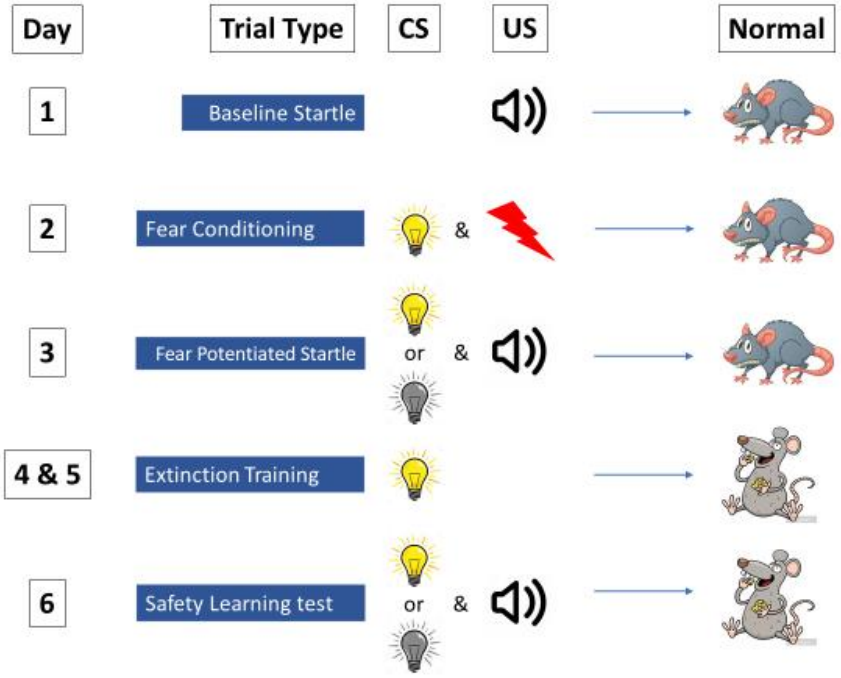

Figure 1: Displays the set up for the procedure for the fear conditioning days, including the pairings of the conditioned stimulus (CS), unconditioned stimulus (US), and a general expectation for how the animal should react.

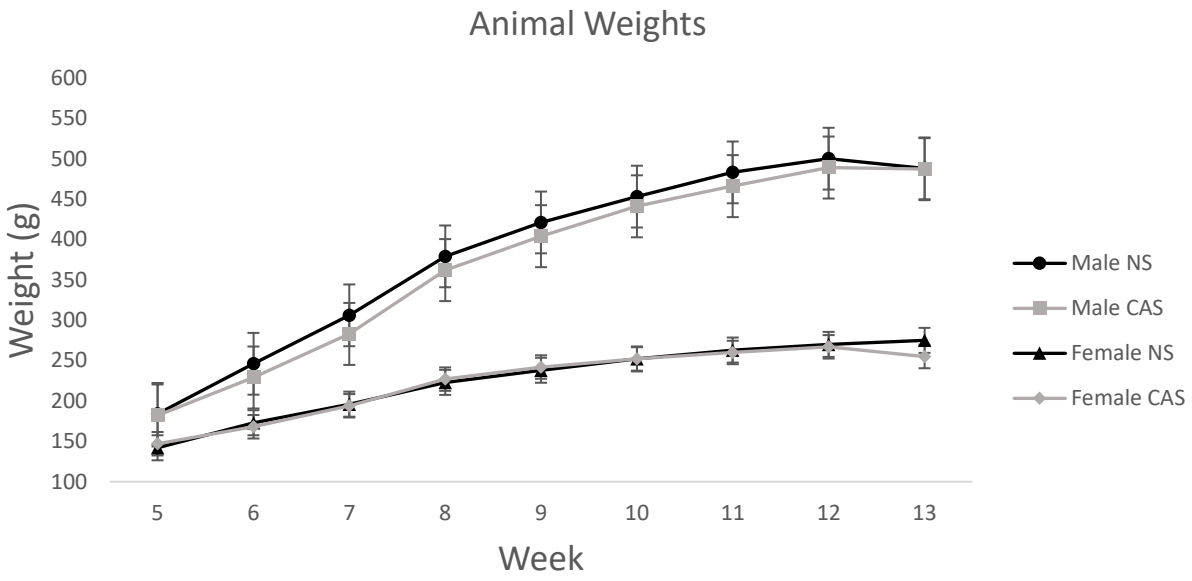

Figure 2: Displays the average weights for both males and females, chronic adolescent stress (CAS) and non-stressed (NS) groups. There were no significant differences between stress groups ($p > 0.05$). Males had significantly higher weight than females. Data are presented as mean ± SEM.

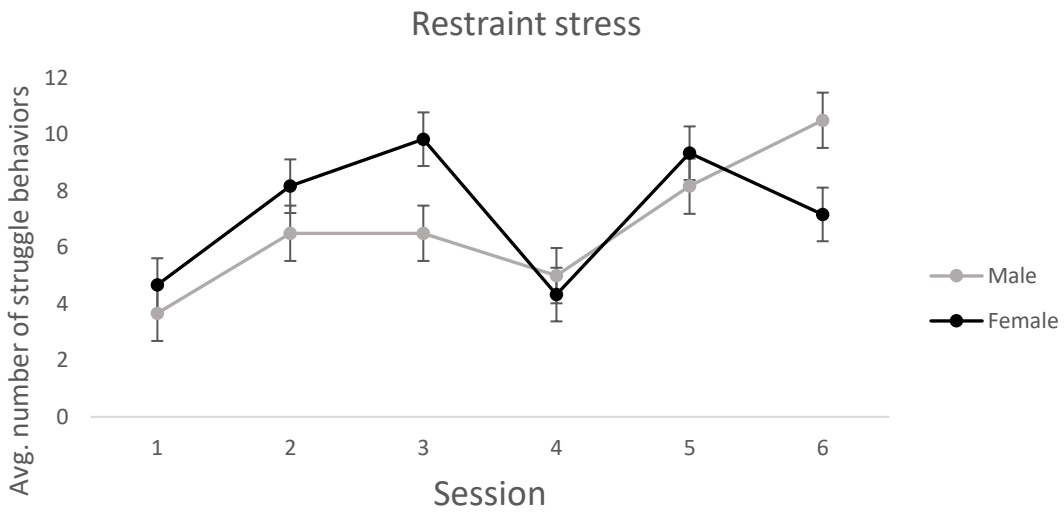

Figure 3: Displays the number of struggling behaviors exhibited per session averaged between all animals of their respective group, for both males and females of the chronic adolescent stress groups. There were no significant differences between males and females ($p > 0.05$). Data are presented as mean ± SEM.

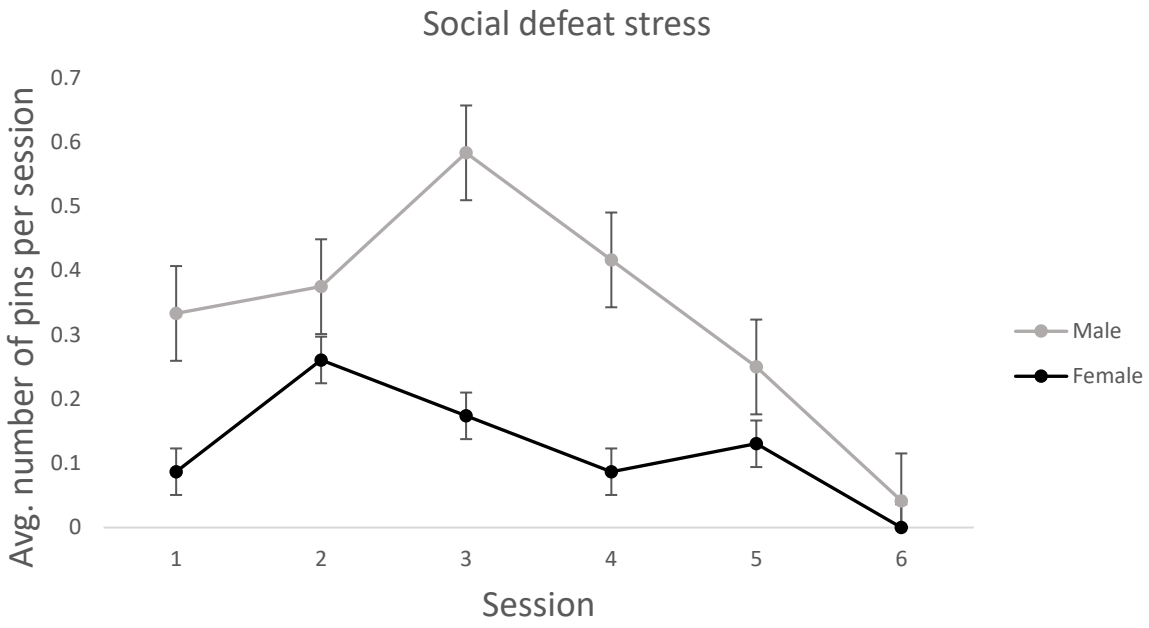

Figure 4: Displays the average number of pins per session for the male and female chronic adolescent stress animals for the social defeat stress. Males had significantly more pins than females consistently across days of exposure ($p < 0.05$). Data are presented as mean ± SEM.

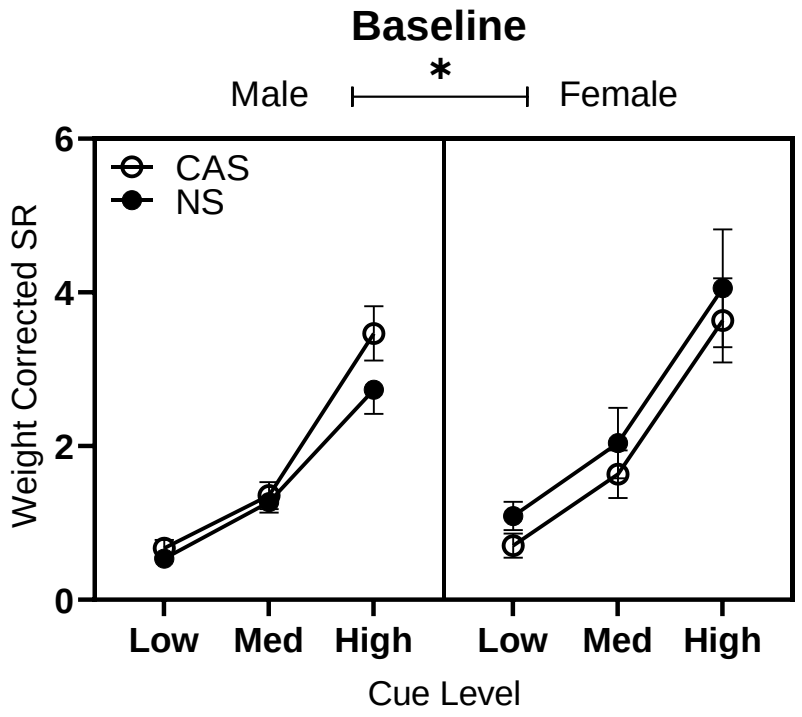

Figure 5: Females had a significantly higher weight corrected startle response during the baseline day. There was no difference between chronic adolescent stress (CAS) groups and non-stressed (NS) groups. This figure displays the mean and standard error of mean for the startle results. * represents a significant effect at an alpha level of 0.05.

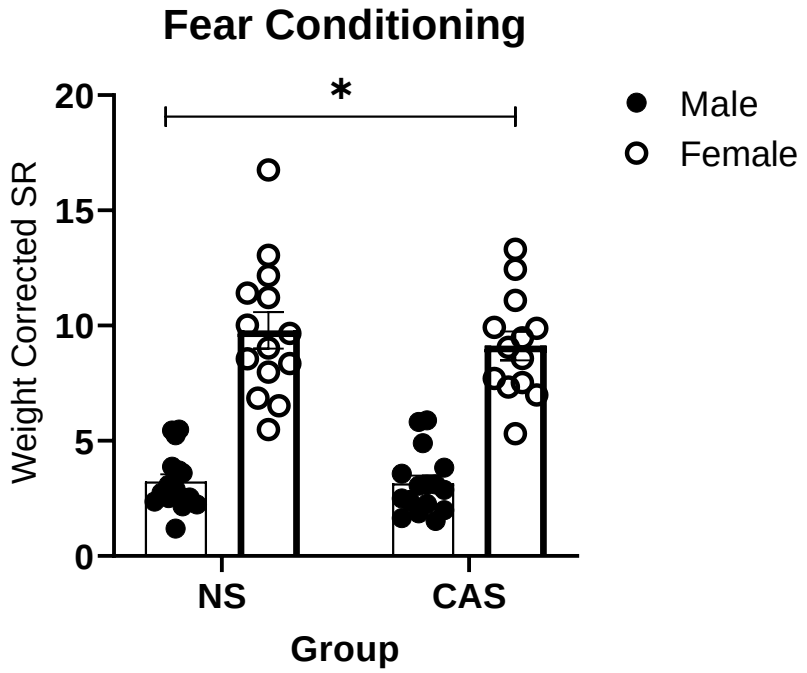

Figure 6: Females had a significantly higher weight corrected startle response during the fear conditioning day. There was no difference between chronic adolescent stress (CAS) groups and non-stressed (NS) groups. This figure displays the mean and standard error of mean for the startle results. * represents a significant effect at an alpha level of 0.05.

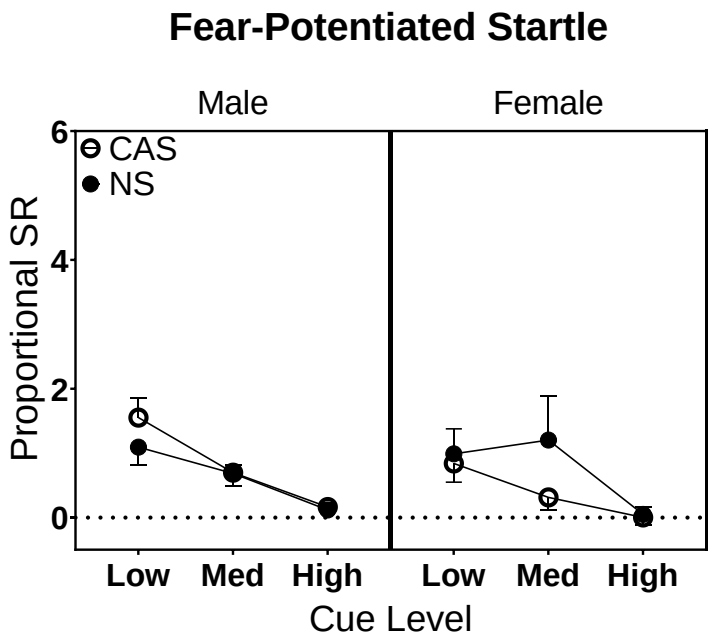

Figure 7: There were no significant differences in proportional startle responses between males and females during the fear-potentiated startle day ($p > 0.05$). There was no difference between chronic adolescent stress (CAS) groups and non-stressed (NS) groups. This figure displays the mean and standard error of mean for the startle results.

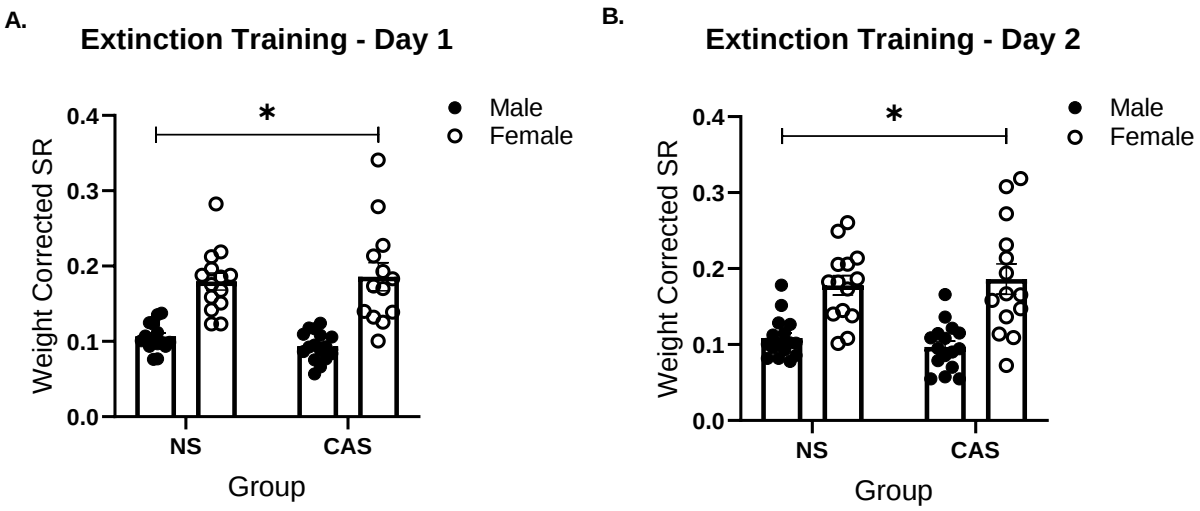

Figure 8A and Figure 8B: Females had a significantly higher weight corrected startle response during the extinction training days. There was no difference between chronic adolescent stress (CAS) groups and non-stressed (NS) groups. Figure 7A displays the results from the first day of extinction training while figure 7B displays the results from the second day. Both figures display the mean and standard error of mean for the startle results. * represents a significant effect at an alpha level of 0.05.

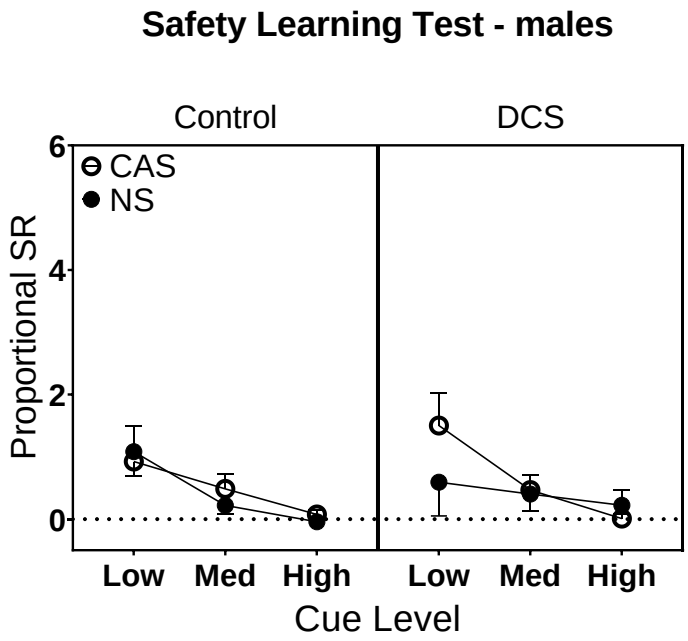

Figure 9: There was no significant difference in the proportional startle result between the males who had been administered D-Cycloserine (DCS) and the control males ($p > 0.05$). There was no difference between chronic adolescent stress (CAS) groups and non-stressed (NS) groups. This figure displays the mean and standard error of mean for the startle results. * represents a significant effect at an alpha level of 0.05.

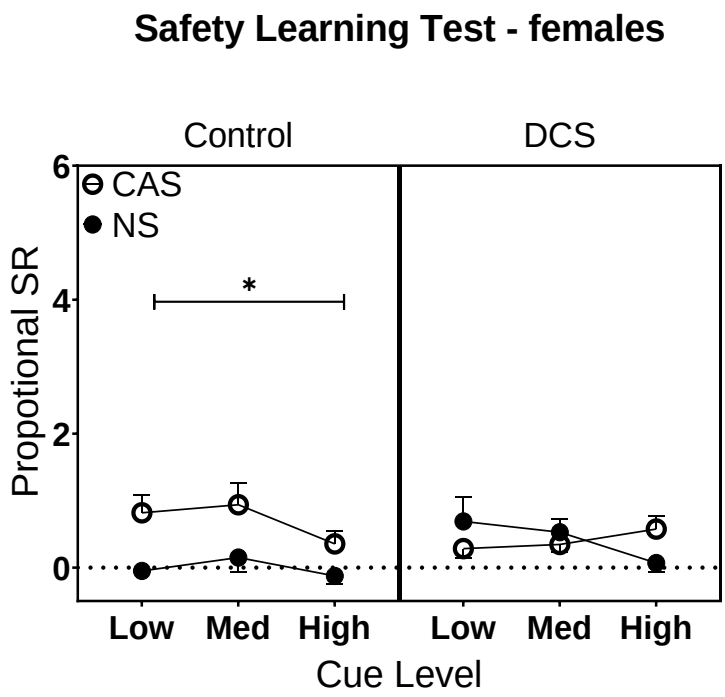

Figure 10: Chronic adolescent stress (CAS) females who did not receive D-cycloserine (DCS) had a significantly higher proportional startle response than non-stressed (NS) females who also did not receive DCS. There was no significant difference between the DCS CAS females and DCS NS females ($p > 0.05$). This figure displays the mean and standard error of mean for the startle results. * represents a significant effect at an alpha level of 0.05.

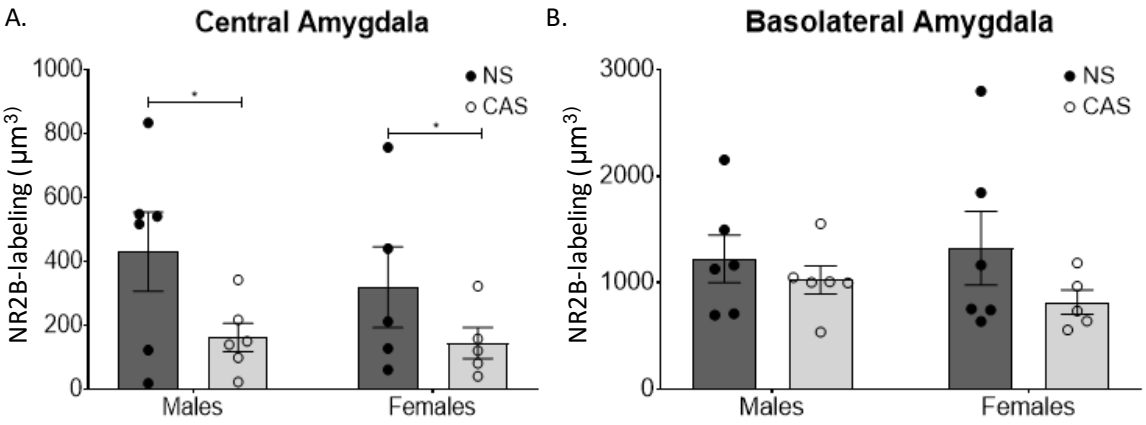

Figure 11A and 11B: For the central amygdala (A.) the animals that underwent chronic adolescent stress (CAS) had significantly less NR2B as indicated by staining compared to the non-stressed (NS) animals. For the basolateral amygdala (B.) there were no significant differences in NR2B staining between males and females as well as CAS and NS ($p > 0.05$). This figure displays the mean and standard error of mean for the NR2B-labeling results. * represents a significant effect at an alpha level of 0.05.

Discussion

Females who experienced chronic adolescent stress displayed behaviors indicative of impaired safety learning compared to females who also went through chronic adolescent stress but were injected with the NMDAr agonist DCS. Since one of the characteristics of PTSD is an exaggerated response to stimuli associated with that initial fear-inducing event, the results from this study support the notion that this impairment in safety learning may be linked to NMDAr activity. Given NR2B's role in fear acquisition in the amygdala and how fear memories need to be inhibited in some capacity to allow for safety learning behaviors to predominate, examining trends in NR2B is critical. The results displaying no differences in NR2B in both the central and basolateral amygdala suggest that the impairment in safety learning is separate from any alterations in fear acquisition.

We can be confident that both groups of animals found the CAS procedures stressful as both males and females displayed no discernible pattern in weight gain, with the stressed animals weighing slightly less than the non-stressed. Testing safety learning in conjunction with startle responses and NR2B allows us to examine the issue on a behavioral and neurobiological level allowing us to get a more comprehensive idea of how the two components may interact to impact safety learning.

Fear conditioning and startle

Females at baseline had a significantly higher startle response compared to males regardless of stress condition. Females tend to have a greater HPA axis activation in response to acute stressors (Heck & Handa, 2019), so their higher responses to an acoustic tone, which at certain levels can be a mild stressor, is unsurprising. Female's baseline results point to their propensity to develop deficits in safety learning as past research has shown that higher baseline startle response after a mild stressor is associated with PTSD phenotypes (Russo & Parsons, 2017).

For the fear conditioning procedure females again had higher responsivity than males with there also not being a significant difference between stress conditions. The

mechanism behind the response to fearful stimuli differ based on whether the stimulus is conditioned or unconditioned (Tovote et al., 2015). In the fear conditioned day, the foot shock serves as an unconditioned stimulus. In both in vivo and vitro studies unconditioned stimuli have been found to inhibit both inhibitory interneuron subgroups of the amygdala, the parvalbumin and somatostatin neurons (Bienvenu et al., 2012; Muller et al., 2006, 2008). Those inhibitory neurons typically moderate amygdala activation, however the resulting disinhibition caused by an unconditioned stimulus is partially the reason behind the strong activation of the amygdala in response to fearful events. This greater activation results in fearful stimuli creating not only strong memories, but also physiological responses from other stimuli merely associated with the fear-inducing unconditioned stimulus. While individuals who experienced chronic stress during adolescence have all the requisite morphology (such as a hypertrophied amygdala) (Mitra & Sapolsky, 2008) to produce a level of excitation exceeding their non-stressed counterparts, it is likely that the magnitude of the overexcitation, caused by the disinhibition of these amygdala interneurons creates somewhat of a ceiling effect. Meaning that the magnitude of the stimulus is likely too large, thus masking any differences that may have occurred. It is therefore likely that we would see a difference in responses if the voltage of the shock was decreased, however given that the fear conditioning day's purpose is to prepare the rats for following conditioning days, reducing the voltage would be unnecessary for this experiment.

For the fear potentiated startle session there was no significant effect between stress groups or sexes for their proportional startle results. Again, this is likely because the acquisition of fear memories happens through such a robust activation, that the changes in the amygdala because of CAS are somewhat superfluous in their role in expressing this fear so shortly after those memories were formed. The training days following the potentiated startle day is important for creating memories that serve to somewhat mollify the stronger fear induced ones.

For the safety learning test day there was a significant interaction between sex, CAS, and DCS, with females who experienced CAS having higher responses in the safety learning test day than NS females. CAS was shown to impair safety learning in females.

This is likely because adolescence is a vulnerable time in which the amygdala is developing rapidly (Tottenham & Gabard-Durnam, 2017). Previous studies have seen that repeated stress causes an increase in amygdala dendritic growth and connections amygdala neurons make with other regions of the brain such as the thalamus, hippocampus, or prefrontal cortex (Tovote et al., 2015). While males have more dendritic material, females experience a greater shift in response to stressful events (Farrell et al., 2015). Plasticity caused by conditioned responses develops quickly in the amygdala (Gabard-Durnam et al., 2014; Silvers et al., 2016) and expected unconditioned stimuli (such as the repeated foot shocks in this procedure) specifically signals afferents to the basolateral amygdala (Johansen et al., 2010). The periaqueductal gray (PAG) is a midbrain structure that is also the target of efferent fibers from the central amygdala (Johansen et al., 2010) and contributes to our reflexive response to threatening stimuli. If the amygdala is hypertrophied because of stress, and even more so in females (De Armentia & Sah, 2003; Farrell et al., 2015) and thus forming more connections, I believe that the greater physical response we see in females is directly related to outputs to the PAG.

The more physiological responses to the stimuli are also likely increased in CAS females in response to a stressor in adulthood. Previous research in this lab showed that skin conductance responses were higher in CAS females and predictive of their startle responses. This higher startle response is associated with greater rates in PTSD and other anxiety disorders that are more prevalent in females (Butler et al., 1990; Russo & Parsons, 2017) . Since NMDArs are mainly responsible for the process of learning from aversive situations, an impairment in a process involving learning from emotionally salient stimuli would likely need to include NMDArs of the amygdala. Therefore, an inability to sufficiently form new memories involving the dissociation of a conditioned stimulus from the unconditional fear-inducing stimulus would be caused by fear memories being more resilient or dysfunction in the process of extinction.

DCS works as an NMDAr agonist, and in this fear conditioning procedure it was administered immediately after fear extinction training. Past DCS experiments have enhanced extinction training results by administering DCS (Graham & Scott, 2018; Tang

& Graham, 2019). In an experiment where animals were given the NMDAr antagonist, MK801, animals fear memory retention was impaired. In a follow up experiment where animals were given MK801, and then DCS, the researchers saw that that there was no longer an impairment (Baker & Richardson, 2017). From those results we were confident that changes in safety learning following extinction training were caused by activity in the NMDAr. We saw that CAS resulted in females having poor safety learning efficiency, however when administered DCS that deficiency was corrected. DCS improving the safety learning efficiency scores in CAS females in this study supports the notion that female's impairment in safety learning caused by chronic stress during adolescence was based on NMDAr's activity.

NR2B Immunohistochemistry

CAS reduced NR2B in the central amygdala but had no impact on NR2B in the basolateral amygdala. There was no effect of sex, nor an interaction between sex and stress in either region.

I believed that CAS females would have a higher amount of NR2B. Females who experience stress tend to have multiple changes in the amygdala that equip that region to respond in greater magnitude to stressful stimuli (De Armentia & Sah, 2003; Puralewski et al., 2016). These effects have been replicated by various studies, and NR2B specifically has been shown to play a role in the processes in the BLA and CeA that are increased in response to stress, such as long-term potentiation (Zhuo, 2009). Also, the amygdala contains specific cells responsible for extinction of fear memories that connect to various regions of the brain, and that also inhibits the cells in the amygdala responsible for fear memory (Tovote et al., 2015). I proposed that we would see an increase in NR2B because with the increase in volume, and connections amygdala neurons are making as reported in these previous studies, the consistently high startle response seen in CAS females of the first cohort could be viewed as indicative of more persistent fear memories being formed. Also upregulation of NR2B was also seen to enhance synaptic plasticity and fear learning (Wang et al., 2014) with NR2B mRNA also being increased in the dorsal hippocampus as a result of chronic

stress (Pacheco et al., 2017). Since NR2B has a function in fear learning and has seen increased expression because of stress, I believed we would find increased NR2B in the amygdala as a result of our chronic adolescent stress procedure.

Downregulation of NR2B after the confrontation of a stressor, as a protective measure against overexcitation, has been seen in previous studies (Zinebi et al., 2003), and may account for the decrease in NR2B in the central amygdala present in this study. NR2B has a prominent role in learning in response to fearful stimuli, and the BLA is the main region of the amygdala responsible for this learning, therefore it is possible that NR2B expression in the BLA was increased in response to the learning that occurs following exposure to a stressor during adolescence. If this increase in NR2B expression occurred, it would likely have been decreased in adulthood as a protective measure after exposure to the fear conditioning procedure. The more probable cause behind the lack of difference in the NR2B results in the BLA is that since the startle results showed no difference between stress groups in the fear conditioning and fear-potentiated startle days, the chronic adolescent stress did not result in fear memories being encoded more strongly. As previously mentioned, NR2B activity is associated with fear conditioning, and the cells in the amygdala responsible for extinction memory are separate from those involved in fear conditioning. I believe it is likely that the safety learning impairment seen in CAS females is strictly due to the stressor's impact on the extinction memory subpopulation of cells. In other words, there was no enhancement of the fear memory cells function by CAS in females, only a degradation of the function of extinction memory cells. And likely a degradation of those specific extinction cells that make connections to areas like the PAG for controlling motor responses to threatening stimuli. If CAS caused dysfunction in extinction cells indiscriminately, we would likely see a disinhibition of the fear memory cells as a result. Thus, there would have been an observable difference between the CAS and NS females on the fear potentiated startle day since the CAS would have resulted in greater fear memory cell activation.

The CeA is the main output region of the amygdala. While NR2B is primarily studied in its role in fear learning, there is evidence for it also playing a role in fear extinction (Sotres-Bayon et al., 2007), and a display of fear extinction behaviors would be

governed by the CeA. The decreased amount of NR2B in CAS animals compared to NS animals is likely indicative of the process of fear extinction occurring less efficiently, resulting in those with this decrease in NR2B having greater startle responses on the safety learning day. Females have less somatostatin and GABA producing enzymes in their amygdala than males (Seney et al., 2013) which also may account for female's increased responses to fearful and stressful stimuli since the disinhibition by these cells can allow the PAG to activate. Somatostatin and GABA are produced by interneurons in both the BLA and CeA (McDonald, 1985; Sah et al., 2003), however, the CeA is composed of mainly inhibitory interneurons while in the BLA inhibitory interneurons are vastly outnumbered by glutamatergic excitatory neurons (Sah et al., 2003). The greater presence of these inhibitory interneurons in the CeA of females when compared to the CeA of males may explain why despite both CAS males and females having lowered CeA NR2B, only CAS females had impaired safety learning, indicated by greater startle responses, on the safety learning test day than the NS controls.

Future directions and implications

While the results from the safety learning day did support my hypothesis, to further solidify the validity of the results I would like to expand the experiment, mainly increasing the number of females present in both the CAS and NS groups. I also would like to add a saline group as a control as opposed to having our control be a group of animals who did not get an injection. For the NR2B study I believe it would be beneficial to expand the observation of NMDAr in general to different regions mainly the prefrontal cortex, since it is so instrumental in regulating amygdala activity. For this it would likely be important to look at the NR1 subunit as opposed to the NR2B, since NR1 is included in all NMDArs. Also, past research in this lab showed that females who experienced chronic adolescent stress had increased NMDAr transmission along with overall enhancement of glutamatergic synapses compared to non-stressed females and males. While the study just mentioned was examining the hippocampus, I believe we would see similar results if we were to also look for these same changes in the basolateral and central amygdala. While looking at the amount of subunit is important, I feel taking

measures a step further and examining the actual activity that occurs in these NMDAr synapses would be important to get an even more clear idea of how exactly stress changes NMDAr function.

The continued exaggerated startle reaction to a stimulus associated with a fearful event is a symptom of PTSD (American Psychiatric Association, 2013). Since stress during adolescence increases likelihood of developing anxiety disorders such as PTSD, and since fear and safety learning are primarily conducted by NMDArs in the amygdala, examining how the activity of these receptors is changed by repeated stress is important for learning how to best treat people who are at risk for developing these impairments in safety learning. The results support the idea that NMDAr function is related to impairments in safety learning. Furthermore, the results also support the notion that DCS is an effective treatment for PTSD symptoms in women. This finding is significant because most clinical trials examining the effects of DCS on PTSD have somewhat ambiguous results (Mataix-Cols et al., 2017), which may be due to a difference in how DCS effects women, as opposed to men. More work looking into the exact way chronic stress during adolescence changes NMDAr function could produce a solid foundation for developing treatments and practices to help people with PTSD alleviate the negative drawbacks associated with safety learning impairments.

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