

Long Term Effects of Chronic Variable Stress Administered during Different Developmental Stages in Mice

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LONG TERM EFFECTS OF CHRONIC VARIABLE STRESS ADMINISTERED
DURING DIFFERENT DEVELOPMENTAL STAGES IN MICE

by

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ABSTRACT
LONG TERM EFFECTS OF CHRONIC VARIABLE STRESS ADMINISTERED
DURING DIFFERENT DEVELOPMENTAL STAGES IN MICE

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Marquette University, 2011

A number of studies have suggested that the occurrence of past trauma can increase an individual's chance of developing PTSD from a new traumatic experience later in life. Trauma that occurs during childhood appears to have a particularly strong effect on this risk increase. Furthermore, conditioned fear responses have been shown to incubate over extended periods of time in animal models. To further investigate the role these phenomena play in the development of PTSD, this study exposed juvenile and adult mice to 7 days of chronic variable stress (CVS). One month later, a Pavlovian delay fear conditioning procedure was used to assess fear learning behavior, and anxiety levels were assessed with an Elevated Plus-Maze (EPM). It was hypothesized that mice who experienced CVS exposure as juveniles would show greater long-term levels of anxiety and long-term sensitization to later fear learning than mice who experienced CVS as adults. Furthermore, mice exposed to CVS, regardless of age, were hypothesized to show significantly enhanced anxiety and fear conditioning relative to control mice. Surprisingly, it was found that stress induced sensitization of fear conditioning deteriorated over the 30-day incubation period for both juvenile and adult mice, leading to no differences between groups, including controls, in fear learning behaviors. Adult stressed mice showed significantly greater anxiety levels than adult controls, while juvenile stressed and control mice showed no difference in anxiety. These results suggest possible neurological differences between juvenile and adult mice in regions involved in fear learning, such as the hippocampus, the central nucleus of the amygdale, and the bed nucleus of the stria terminalis. Alternative interpretations of the data are discussed. Despite failing to support the proposed hypotheses, this study suggests that a successful animal model of PTSD should consider the differential dynamics of associative and non-associative fear learning processes. Furthermore, the moderating effects of developmental stages on the effects of chronic stress should also be acknowledged and investigated further.

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Introduction

Post-traumatic Stress Disorder (PTSD) is characterized by exposure to a traumatic event, followed by distressing recollections of the event, avoidance of event-related stimuli, and heightened arousal (Vieweg et al., 2006). The development of PTSD and other types of anxiety disorders is thought to be the result of, or at least correlated with, maladaptive stress response mechanisms (Risbrough & Stein, 2006). Maladaptive stress processes may, in turn, result from past trauma or life stressor exposure. A number of studies have suggested that the occurrence of past trauma can increase an individual's chance of developing PTSD in response to a traumatic experience later in life (Breslau et al., 1999; Breslau, 2009). Breslau et al. (1999) found that multiple exposures to traumatic events can have an additive effect on the probability that an individual will develop PTSD from a later traumatic event. A history of multiple past traumas has also been shown to increase the severity of PTSD symptoms developed later in life (Gillespie et al., 2009).

Because of the large amount of development and change during childhood, as well as the particularly plastic nature of psychological processes during childhood, the long-term effects of childhood trauma on the development of later adult PTSD are of particular interest. For example, Vietnam Veterans who developed PTSD as a result of their combat experience were significantly more likely to have experienced childhood physical or sexual abuse than Veterans without PTSD (Bremner, Southwick, Johnson, Yehuda, & Charney, 1993). Similarly, childhood maltreatment is a significant predictor of adult PTSD (Grassi-Oliveira & Stein, 2008) and the presence of past childhood physical and/or

sexual abuse is associated with 2- to 3-fold increased symptom severity in PTSD patients (Gillespie et al., 2009).

Many animal studies have demonstrated behavioral effects of chronic stress. These effects usually involve modifications of later fear learning, which is the process of developing an aversion to a stimulus, such as a tone, when it is paired with an aversive consequence, such as pain. A common finding is that chronic stress paradigms such as chronic restraint stress and chronic unpredictable stress enhance subsequent contextual fear learning in rats (Conrad et al., 1999; Sandi, Merino, Cordero, Touyarot, & Venero, 2001). Chronic unpredictable stress has also been found to increase rats' burying behaviors and immobility in a defensive burying test, suggesting increased anxiety levels (Matuszeqich et al., 2007). Rats exposed to chronic mild stress have shown an impairment in their consolidation of fear extinction memories (Garcia, Spennato, Nilsson-Todd, Moreau, & Deschaux, 2008), and rats exposed to chronic restraint stress have shown an impairment in the recall of extinction memory (Miracle, Brace, Huyck, Singler, & Wellman, 2006). Short-term stress paradigms, such as acute shock stress and brief restraint stress, have also been shown to enhance contextual fear learning (Rau, DeCola, & Fanselow, 2005; Manzanares, Isoardi, Carrer, & Molina, 2005; Cordero, Venero, Kruyt, & Sandi, 2003).

In order for the results of studies using an animal model of PTSD to have any clinical utility, it must be shown that the behavioral and neurobiological processes of fear learning observed in animals are similarly present in humans. Not only have the effects of fear learning been shown to be similar across species, but recent studies utilizing neuroimaging have also demonstrated similar fear learning processes in humans

(Delgado, Olsson, & Phelps, 2006). These findings support the conclusion that the results of animal model studies can be reliably applied to our clinical understanding of human PTSD.

The neurobiological literature, though beyond the scope of the proposed study, demonstrates a possible connection between PTSD and stress. A primary physiological feature of PTSD is a reduction in hippocampal volume (Heim & Nemeroff, 2009; Gilbertson et al., 2002; Schuff et al., 1997). Various stressors have been shown to cause dendritic atrophy in the rat hippocampus (Conrad, Magarinos, LeDoux, & McEwen, 1999; Vyas, Mitra, Rao, & Chattarji, 2002; McKittrick et al., 2000). By altering hippocampal volume and structure, a history of stress may alter susceptibility to the development of PTSD.

When taken together, the above studies support the hypothesis that chronically stressful environments can alter an individual's behavior, especially in regard to future stress response patterns. Although several studies have examined the effects of stress administered during the juvenile developmental period (Avital & Richter-Levin, 2005; Toth et al., 2008; Sterlemann et al., 2008), no study to date has specifically compared the long-term fear-inflating effects of chronic stress experienced either in the juvenile or adult period on later fear learning.

The current study compared the fear-sensitizing effects of chronic stress first experienced as either a juvenile or an adult, after a delay of 30 days. Juvenile and adult mice were exposed to chronic variable stress (CVS) for 7 days. One month later, their fear learning behavior was assessed in a Pavlovian fear conditioning procedure, and anxiety levels were assessed in an Elevated Plus-Maze (EPM). During the CVS phase,

mice experienced a series of semi-random stressors designed to model the unpredictable nature of stressful life events. During the subsequent Pavlovian fear-conditioning phase, mice were exposed to a series of three tone-shock pairings and levels of context and tone fear were measured by freezing behavior. Total open-arm time over a 5-minute period in an EPM was used as the measure of anxiety (see Methods).

The current study utilized a long period of time between chronic stress and later trauma, which is particularly important due to the possibility of incubation effects. Incubation is commonly defined as a growth of fear over a period of time following aversive stimulation (McAllister & McAllister, 1967). During incubation, the period of time during which the fear increases is free of further exposures to the original aversive stimulus, and therefore the fear increase appears to be spontaneous (McAllister & McAllister, 1967). Utilizing galvanic skin response (GSR) as a measure of anxiety, Bindra and Cameron (1953) found that human participants experienced higher levels of anxiety in a signal/shock pairing exercise after a 10-minute rest period than during the initial phase of the exercise. These findings suggest that subjects' anxieties towards the signals incubated over the 10-minute rest period. Similar results have also been shown after a 30-minute delay (Golin, 1961).

More recently, studies utilizing animal models of anxiety have found additional evidence for incubation effects. Conditioned tone fear (Pickens, Golden, Adams-Deutsch, Nair, & Shaham, 2009) and context fear (Houston, Stevenson, McNaughton, & Barnes; 1999) in rats have been shown to incubate over prolonged periods of time, resulting in heightened fear responses to the aversive stimuli used. A similar incubation effect has been observed in mice for learned, non-associative types of fear (sensitization

and hyperarousal; Siegmund & Wotjak, 2007). This suggests that the effects of other phenomena known to lead to fear sensitization, such as chronic stress, may also incubate. No study has investigated whether or not stress-induced sensitization incubates in a fashion similar to conditioned fear, and the current study could detect the existence of this phenomenon in the mechanisms of chronic stress effects.

The present study also attempted to detect developmental differences in the hypothesized incubation of stress-induced fear sensitization. The human PTSD literature suggests that the sensitization effect of chronic stress is most severe when stress occurs in childhood (Grassi-Oliveira & Stein, 2008; Breslau et al., 1999). These findings indicate the possibility that incubation effects are more potent and active during childhood. It is hypothesized that the sensitizing effects of chronic stress will incubate in a fashion similar to conditioned fear, but that this incubation and its effects on later behavior will differ in severity depending on the developmental stage in which chronic stress is first experienced.

Avital and Richter-Levin's (2005) work may have the most resemblance to the current study. They found that the cumulative effect of stress experienced by rats during childhood and adulthood on anxiety behavior was greater than the cumulative effect of stress experienced twice in adulthood. However, they used an elevated platform and acute swim stressors, and measured anxiety behaviors rather than fear learning performance. Fear learning mechanisms may play a unique role in anxiety disorders such as PTSD (Lissek et al., 2005). Furthermore, the chronic variable stress paradigm, which was utilized in the current study is thought to be more representative of the chronic stress experienced by humans in adverse environments (Herman, Adams, & Prewitt, 1995). For

example, a child in an abusive household, or living in poverty, will likely experience a long-term pattern of traumatic experiences rather than just one or two instances of trauma. This chronic nature of the stress may have a markedly different impact on later fear learning than one or two traumatic instances.

The current study may generate experimental data complementing past human research that observed long-term effects of childhood stress on the development of PTSD later in adulthood. Incubation may be a driving force behind this phenomenon, maintaining and strengthening fear sensitization that was obtained as a child into adulthood. There are currently no studies in the PTSD literature that use an animal model to examine these long-term effects of chronic stress specifically on later fear learning. The majority of animal studies examining chronic stress effects on fear learning have a relatively short stress-to-conditioning interval (24-48 hours), whereas the current study had a stress-to-conditioning interval of 30 days. Therefore, this study was able to examine the durability and possible enhancement of chronic stress effects on fear learning over an extended period of time.

It was hypothesized in the current study that mice who experienced CVS exposure as juveniles would show greater long-term levels of anxiety, measured by an EPM test, and long-term sensitization to later fear learning, measured by freezing levels in a Pavlovian fear conditioning paradigm, than mice who experienced CVS as adults. Furthermore, mice exposed to CVS, regardless of age, were hypothesized to show significantly enhanced anxiety and fear conditioning relative to control mice that were not exposed to CVS.

Methods

This study compared behavioral measures in a fear-learning task 30 days after exposure to chronic variable stress, which involved exposing animals to multiple unpredictable stressors. It consisted of a 2 x 2 factorial design, with age and stress exposure as the independent variables and rate of freezing during a fear-learning paradigm as the dependent variable. Anxiety levels, measured with an Elevated Plus Maze (EPM) procedure, and animal weight were also recorded and analyzed.

Subjects

Subjects consisted of 32 male mice (C57Bl/6 strain) purchased from Charles-Rivers laboratories (Portage, MI), and were divided into 2 experimental groups and 2 control groups. Experimental Group 1 consisted of juvenile mice (aged 23 days, n=8), and Experimental Group 2 consisted of adult mice (aged 2-4 months, n=8). Both experimental groups were exposed to 7 days of a chronic variable stress procedure, from day 1 to day 7 of the study. Control Group 1 consisted of juvenile mice (aged 23 days, n=8), and Control Group 2 consisted of adult mice (aged 2-4 months, n=8). Neither Control Group was exposed to the chronic variable stress procedure. After an incubation period of 30 days, the anxiety levels (total seconds spent in open arms over a 5-minute period) of all groups were tested in an EPM procedure. All groups then underwent a Pavlovian delay fear conditioning procedure and subsequent fear tests, and the freezing rates of mice were measured.

All animals were housed in the Marquette University Vivarium. They were group housed within their assigned experimental or control groups (4 per cage), granted free access to food and water, and kept on a 12-hr light-dark cycle (lights on at 7:00 a.m.). Mice were randomly assigned to experimental and control groups.

Chronic Variable Stress

The chronic variable stress procedure involved exposing the mice to 2 semi-randomly chosen stressful events each day, one in the a.m. and one in the p.m., for 7 consecutive days. At the end of the 7 days, each of 7 different stressors had been experienced a total of 2 times. The stressors included Swimming (placement in room temperature water for 5 minutes), Vibration (placement in a laboratory shaker for 10 minutes), Restraint (restraint in wire mesh for 30 minutes), Cold (placement in a cold room (4 degrees C) for 30 minutes), Ultrasound (placement in a bucket and exposed to a pest control ultrasound emitter for 10 minutes), Crowding (placement of 2 home-cages of mice within the same experimental or control group in a single cage overnight), and Isolation (placement of each animal in a separate cage overnight). The Vibration stressor was done with a Dubnoff metabolic shaking incubator (GCA Precision Scientific, Chicago, IL), and the Ultrasound stressor involved exposure to a Pest Chaser Ultrasonic Repeller (Lititz, PA). Each morning, all animals were tailmarked with colored sharpies to ensure proper identification. Stressors were administered in a different room than the conditioning and testing rooms, and all transportation to and from the various labs was done with wheeled laboratory carts. During these 7 days, Control Group animals were

not exposed to chronic variable stress, and remained in their home-cages. Furthermore, Control Group animals received the same transportation exposure as the Experimental Group animals, to ensure that any stress from the act of transportation was controlled.

The chronic variable stress paradigm used in this study is thought to be a more accurate model of real world stress than paradigms such as repeated restraint stress. Animals often habituate to repeated restraint stress, due to its repetitive, non-random nature (Fernandes et al., 2002, Bowmann, Beck, & Luine, 2003). The unexpected and variable stressors used in the chronic variable stress paradigm are much less likely to be habituated to, and more accurately represent the unpredictability of human stressors (Herman et al., 1995). Past research has demonstrated the chronic variable stress paradigm's effectiveness as an experimental chronic stress procedure (Kim et al., 2008; Molina, Suarez, & Rivarola, 2006; Zurita, Cuadra, & Molina, 1999). Furthermore, 7 days of exposure to a chronic variable stress paradigm is long enough to reliably cause behavioral changes in animals (Sanders, Stevens, & Boeh, 2010; Tauchi, Zhand, D'Slessio, Seeley, & Herman, 2008; Zurita, Martijena, Cuadra, Brandao, & Molina, 2000).

Incubation

Stress exposure comprised days 1-7 of the experimental procedure (see Figure 1 for a procedural timeline). The 30-day stress-conditioning interval occurred from experimental day 8 through 37. Past research has demonstrated significant incubation effects at or around 30 days following fear conditioning (Pickens et al., 2009; Siegmund

& Wotjak, 2007; Houston et al., 1999). This prolonged length of time is the most commonly used period for the detection of incubation effects. All groups experienced identical housing conditions in the Marquette University Vivarium as described above. All animals' tails were remarked with colored sharpies every 2 days during this interval, in order to ensure proper animal identification for later testing and behavioral analyses.

Figure 1. Procedural Timeline



Figure 1. CVS exposure occurred from experimental d1 through d7. The 30 day incubation period occurred from experimental d8 through d37. The EPM test occurred on d38, and fear acquisition training (ACQ) occurred on d39. A context fear test (CNTXT TST) was performed on d40, and a tone fear test (TONE TST) was performed on d41.

Elevated Plus Maze

On day 38, all groups were tested in an EPM procedure. Although anxiety levels were not the main focus of the proposed study, they are very relevant to the field of anxiety disorders. Measuring anxiety levels will provide further information about the effects of chronic stress incubation. The process of measuring anxiety through the use of the EPM test has been well validated (Pellow, Chopin, File, & Briley, 1985). Furthermore, the EPM test has been successfully used to detect anxiety levels experimentally elevated by stress (Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007). The EPM consisted of a plus-shaped maze with four black arms (50.8 cm

long by 10.2 cm wide; 114.3 cm from ground). Two arms had side walls, two were open, and all arms connected at an open center area (12.7 cm long, 12.7 cm wide). All testing was done under minimal lighting conditions, provided by two plug-in night lights. A low light environment was used because rodents, being nocturnal, show avoidant behaviors towards brightly lit environments (Jacob et al., 2009). Mice are able to escape bright light in an EPM by staying in the closed arms (Jacob et al., 2009). This might confound the exploration-related anxiety that is being looked for in the current study with light-escaping behaviors. Animals were placed individually on an open-arm of the maze facing the center, and allowed to explore the maze for 5 minutes. All behavior was recorded by an overhead camera for later scoring. Behavior was scored by assessing the total number of seconds, measured with a stop watch, each mouse spent in an open-arm during the 5-minute trial. The center of the maze was considered to be an open-arm location, given its lack of enclosure on any side. The position of a mouse was determined by the location of its back side, in order to standardize what counted as being in or out of an open-arm. Spending more total seconds in the closed arms of the EPM, as opposed to exploring the open-arms or center, is considered an anxious response, and therefore indicative of heightened anxiety levels.

Fear Acquisition

On day 39, all groups underwent a Pavlovian delay fear conditioning procedure. Pavlovian fear conditioning was chosen as a test of chronic stress effects because of its acceptance as a representative model of clinical anxiety (Risbrough & Stein, 2006).

Furthermore, Pavlovian fear conditioning has long been recognized as a method of eliciting anxiety and fear in animals, and is commonly used in studies examining the process of fear learning (Delgado et al., 2006). On the morning of testing, animals were tailmarked and transported from the Vivarium to our testing lab. They were trained 4 at a time, in identical training chambers (30cm X 24cm X 21cm; Med Associates Inc., St. Albans, VT). The ceiling and back wall of the chambers were white opaque plastic, the front door were made of clear plastic, and the side walls of the chambers were aluminum, with a speaker installed in one wall through which tonal stimuli were played. The floor consisted of 36 stainless steel rods, through which shocks were administered, with a metal waste pan beneath the grid of rods. All chambers were cleaned with a 1% solution of acetic acid, and a film of the solution was placed in each chamber's waste pan. The room in which the chambers resided was brightly lit with 8 100-Watt overhead incandescent bulbs, and background white noise (60 dB) was created by a standard HEPA air filter. After two minutes of exposure to the chambers in the absence of stimuli, three tone-shock pairings were administered. The tones were 28 s in duration, 2800 Hz in frequency, and 85 dB in intensity. Immediately following the tone, a 2 s foot shock was administered through the floor, with an intensity of 0.75 mA. There were 30 s inter-trial intervals, and mice were removed and returned to their home-cages 30 s after the last of the three tone-shock pairing. Freezing behavior (explained in the Data Collection and Analysis section) was measured during the baseline period, and during each tone exposure and inter-trial interval. This was measured in order to assess for differences in the speed or strength of fear learning between groups. The number of cage crossovers during the initial 2-minute baseline period were also recorded in order to determine the

baseline locomotor activity levels for all animals. This was measured by counting the number of times a mouse's back side crossed the center of the cage during the 2-minute baseline period. In order to ensure consistency and control of the background noise and test tone stimuli, these sounds were calibrated and monitored with a Radio Shack dB meter (A scale). Likewise, a storage oscilloscope (B&K Precision Corporation, Yorba Linda, CA) and a 10K Ω resistor was used to confirm shock intensity in each chamber before each round of training. All stimuli presentation and timing was controlled by a PC running MedAssociates software (Med Associates, Inc., St. Albans, VT), and all 4 chambers in each training session were recorded simultaneously by a video camera transmitting to a DVD recorder for storage.

Contextual Fear Test

On day 40, all groups were tested for learned contextual fear in response to the context in which fear conditioning occurred. This involved placing the mice back into the training chambers for 5 minutes, while presenting no stimuli. All conditions of the chambers and the training room were identical to those found in Day 39's fear conditioning procedure. After 5 minutes, animals were returned to their home-cages. Freezing behavior (explained in Data Collection and Analysis section) was measured over the 5 minutes of context exposure. All sessions were recorded as in the fear conditioning procedure.

Tone Fear Test

On day 41, all groups were tested for learned tone fear in response to the test tone stimulus within a novel context. Testing chambers differed markedly from those used for fear conditioning; they contained no stainless steel floor grid or waste pan, and had a white curved vinyl sheet inserted in order to make the interior hemi-cylindrical. One wall of each chamber had a speaker installed, through which the test tone was administered. All chambers were cleaned with a 5% Simple Green solution before each group of mice was tested. The room housing the chambers was lit by 8 40-Watt overhead incandescent bulbs, and background white noise (50 dB) was created by a standard HEPA air filter. After two minutes of exposure to the new context, a tone identical to the training tone was administered for 3 minutes, after which animals were returned to their home-cages. Freezing behavior (explained in Data Collection and Analysis section) was measured during the 2-minute baseline period and the 3-minute tone exposure period. All sessions were recorded as in the fear conditioning procedure.

Data Collection and Analysis

The main behavioral measure in this study was the amount of freezing the mice exhibited during fear conditioning, context testing, and tone testing. All session recordings were digitized to QuickTime files at 1 Hz on a Macintosh G5, and a human observer blind to experimental conditions counted the number of frames in which each animal moved during the session. An animal's amount of freezing was quantified as the

percentage of total frames in which no movement was observed for any given session. During the training sessions, freezing levels were averaged for each 28 s tone exposure and for 30 s after each shock in order to examine the strength and speed with which fear was being acquired. Freezing levels were averaged for the 5-minute context tests. For the tone tests, average freezing levels were separately calculated for the 2-minute baseline period and the 3-minute tone exposure. Anxiety levels were quantified as the total number of seconds spent in the open arms or center during the 5-minute EPM test. This was scored using the recorded EPM video by a human observer blind to experimental conditions.

Freezing behavior in the training session was analyzed using a mixed-design ANOVA, with mouse age and presence of chronic variable stress exposure as between-groups factors and temporal period over the three tone-shock pairing trials as a repeated measure. Freezing behavior in the context testing session was analyzed using a 2 x 2 ANOVA, with mouse age and presence of chronic variable stress exposure as the independent variables. Freezing behavior in the tone testing session was analyzed using a mixed-design ANOVA, with mouse age and presence of chronic variable stress exposure as between-groups factors and temporal period between baseline and tone exposure as a repeated measure. Open-arm time in the EPM was analyzed using a 2 x 2 ANOVA, with mouse age and presence of chronic variable stress exposure as the independent variables.

Weight was measured over the 7-day CVS procedure, as well as after the 30-day incubation period, in order to confirm the effectiveness of CVS. Weight-loss measures can be used as a physiological sign that an animal is under significant stress, with stressed animals typically weighing less than control animals (Choi et al., 2008; McLaughlin,

Baran, Wright, & Conrad, 2005). Although animals were randomly assigned to groups, unexpected differences occurred at baseline (CVS D1) between the groups. As such, weight analyses utilized change scores, where weight at subsequent timepoints was calculated as the difference in weight from baseline, rather than using actual weight. This conversion was intended to prevent preexisting group differences from confounding our weight-change analyses.

Results

Conditioning

A Mixed-design ANOVA was performed on freezing behaviors in the training session, with mouse age and presence of chronic variable stress exposure as between-groups factors and temporal period over the baseline and three tone presentations as the repeated measure (see Table 1 for means and standard deviations). There were no significant effects of either age ($F(1,28) = .899, p=.351, \eta^2=.031$, observed power = .150) or stress ($F(1,28) = .268, p=.609, \eta^2=.009$, observed power = .079) on freezing behaviors. There was a significant effect of the temporal period across the training trials ($F(3,84) = 35.206, p<.001, \eta^2=.557$, observed power = 1.00), suggesting that learning of the tone-shock pairing took place.

Table 1

Means (and Standard Deviations) of Percent Freezing Behavior During Conditioning

Age	Condition	Point in Training			
		Baseline	Tone 1	Tone 2	Tone 3
Adult	CVS	0.42 (0.63)	0.42 (1.18)	10.83 (10.95)	17.50 (13.42)
	Control	0.31 (0.88)	0.00 (0.00)	6.67 (5.63)	15.83 (17.62)
Juvenile	CVS	0.00 (0.00)	0.42 (1.18)	6.67 (4.71)	12.91 (13.15)
	Control	0.00 (0.00)	0.42 (1.18)	0.83 (2.36)	18.33 (10.39)

Context and Tone Tests

A 2 x 2 ANOVA was performed on freezing behavior within the context testing session, with mouse age and presence of chronic variable stress exposure as the independent variables (see Table 2 for means and standard deviations). There were no significant effects of either age ($F(1,28) = .217, p=.645, \eta^2=.008$, observed power = .074) or stress ($F(1,28) = .015, p=.904, \eta^2=.001$, observed power = .052) on freezing behaviors in response to the training context.

A mixed-design ANOVA was performed on freezing behavior within the tone testing session, with mouse age and presence of chronic variable stress exposure as between-groups factors and temporal period between baseline and tone exposure as the repeated measure (see Table 3 for means and standard deviations). There were no significant effects of age ($F(1,28) = .033, p=.858, \eta^2=.001$, observed power = .053) or stress ($F(1,28) = .015, p=.902, \eta^2=.001$, observed power = .052) on freezing behaviors in response to a baseline trial and training tone exposure. There was a significant effect of temporal period on freezing behaviors, with freezing being significantly greater during tone exposure than during the baseline period ($F(1,28) = 75.78, p<.001, \eta^2=.730$, observed power = 1.00). This suggests that some memory of the tone-shock pairing was present.

Table 2

Means (and Standard Deviations) of Percent Freezing Behavior During Context Testing

Age	Condition	
	CVS	Control
Adult	43.54 (15.89)	44.83 (17.62)
Juvenile	41.42 (14.72)	41.54 (17.33)

Table 3

Means (and Standard Deviations) of Percent Freezing Behavior During Tone Testing

Age	Condition	Point in Testing	
		Baseline	Tone
Adult	CVS	14.27 (10.90)	26.88 (13.07)
	Control	13.13 (10.36)	24.31 (14.42)
Juvenile	CVS	8.02 (7.36)	27.15 (14.29)
	Control	12.81 (5.06)	27.92 (12.97)

Anxiety Levels

A 2 x 2 ANOVA was performed on time spent in the open-arm of the EPM, with mouse age and presence of chronic variable stress exposure as the independent variables (see Table 4 for means and standard deviations). There were no significant main effects of either age ($F(1,28) = .273, p=.606, \eta^2=.010$, observed power = .08) or stress ($F(1,28) = 2.526, p=.123, \eta^2=.083$, observed power = .336), but there was a significant interaction between the effects of age and stress on open arm time ($F(1,28) = 5.123, p=.032, \eta^2=.155$, observed power = .589). To clarify the interaction, the data were split by age, and a one-way ANOVA was performed on open arm time, with stress as the independent

variable. For adult mice, there was a significant effect of stress on open arm time ($F(1,14) = 7.524, p=.016, \eta^2=.350$, observed power = .723), with control adults spending significantly more time in the open arm than stressed adults. For juvenile mice, there was no significant effect of stress on open arm time ($F(1,14) = .224, p=.643, \eta^2=.016$, observed power = .073) (See Figure 2).

Table 4

Means (and Standard Deviations) of Total Seconds Spent in EPM Open-Arm

Age	Condition	
	CVS	Control
Adult	47.88 (14.84)	90.75 (41.65)
Juvenile	67.25 (31.19)	59.75 (32.17)

Figure 2. Time Spent in the Open Arm of an Elevated Plus Maze

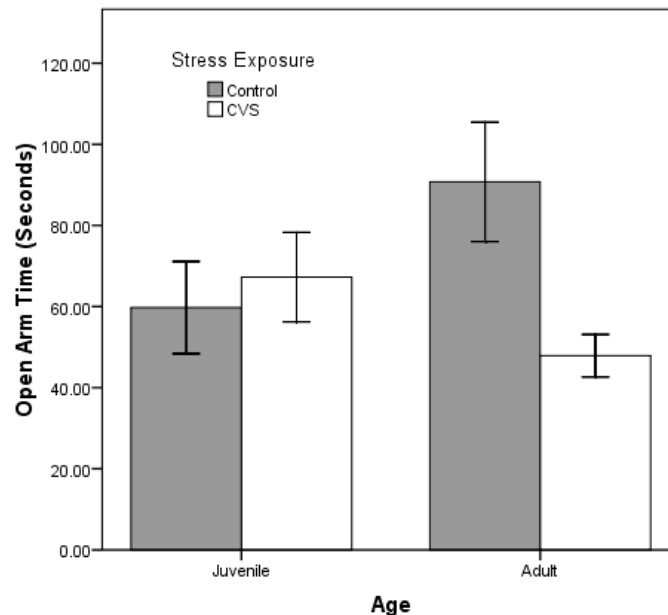


Figure 2. Amount of time spent by mice in the open arm of the elevated plus maze. Greater amounts of time spent in the open arm indicates lower levels of anxiety, and vice versa. Adult control mice displayed significantly greater amounts of open arm time than did adult stressed mice, which control and stressed juvenile mice did not differ in open arm time. Error Bars: +/- 1 SE.

Crossovers

A 2 x 2 ANOVA was performed on the number of cage crossovers during the 2-minute pre-training baseline period, with mouse age and presence of chronic variable stress exposure as the independent variables (see Table 5 for means and standard deviations). There was a significant main effect of stress on crossovers ($F(1,28) = 4.533$, $p=.042$, $\eta^2=.139$, observed power = .538), with control mice making significantly more cage crossovers than stressed mice (see Figure 3). No significant interaction between age and stress was present ($F(1,28) = 1.74$, $p=.20$, $\eta^2=.058$, observed power = .247).

Table 5

Means (and Standard Deviations) of Total Cage Crossovers Made in Training Baseline

Age	Condition	
	CVS	Control
Adult	8.63 (3.93)	12.88 (4.39)
Juvenile	11.13 (3.04)	12.13 (2.17)

Figure 3. Number of Cage Crossovers Made During the Two Minute Pre-Training Baseline Period

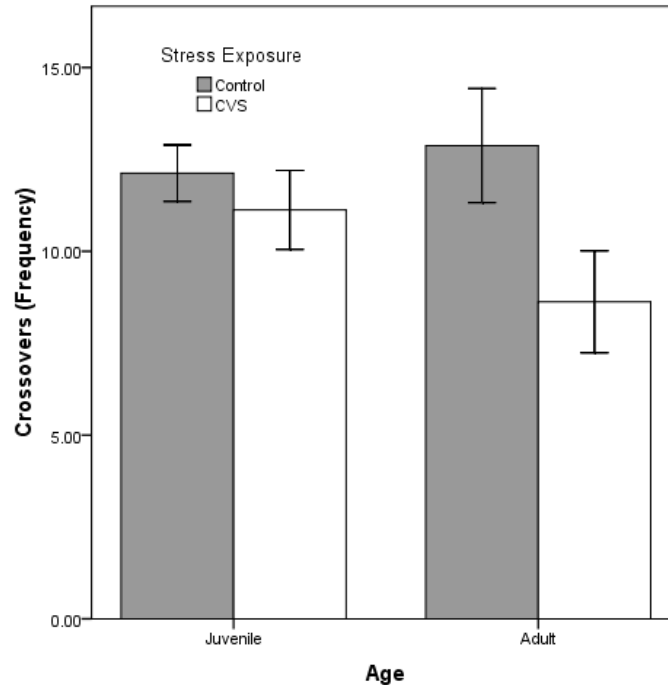


Figure 3. Number of cage crossovers made by mice during the two minute baseline period just before training begins. More crossovers should be an indication of greater exploratory behavior, and therefore lower levels of anxiety. Control adults show a near significantly greater number of crossovers than stressed adults, while control and stressed juveniles do not differ in crossover behavior. Error Bars: +/- 1 SE.

Weight

A mixed-design ANOVA was performed on difference-from-baseline weights, with age and stress as between-groups factors, and temporal period during the CVS procedure (CVS D3, D5, and D7) as a within-groups factor (see Table 6 for means and standard deviations). There were significant main effects of age ($F(1,28) = 774.73$, $p < .001$, $\eta^2 = .97$, observed power = 1.000), with juveniles showing greater weight change than adults; stress ($F(1,28) = 80.38$, $p < .001$, $\eta^2 = .74$, observed power = 1.000), with stressed animals generally showing lower weight relative to controls; and time

($F(1.44,40.43) = 278.60, p < .001, \eta^2 = .91$, observed power = 1.000), with difference-from-baseline weights increasing over the duration of the CVS procedure. Using a Greenhouse-Geisser correction, there was also a significant Time x Age x Stress interaction (see Figure 4; $F(1.44,40.43) = 3.69, p = .047, \eta^2 = .116$, observed power = .56). Follow-up analyses revealed that there were significant differences between the body weight change of control and stressed juvenile mice at CVS Day 3 ($F(1,28) = 30.13, p < .001, \eta^2 = .52$, observed power = 1.000), Day 5 ($F(1,28) = 67.18, p < .001, \eta^2 = .71$, observed power = 1.000), and Day 7 ($F(1,28) = 95.44, p < .001, \eta^2 = .77$, observed power = 1.000). In each comparison, stressed juveniles exhibited reduced weight gain compared with control juveniles. At this developmental stage, juvenile mice should be gaining weight. As such, these results suggest that stress inhibited normal weight gain. The body weight difference-from-baseline values of control and stressed adults also differed significantly at DVS Day 5 ($F(1,28) = 11.11, p = .002, \eta^2 = .28$, observed power = .90) and Day 7 ($F(1,28) = 13.92, p = .001, \eta^2 = .332$, observed power = .95), but not at Day 3 ($F(1,28) = .91, p = .35, \eta^2 = .031$, observed power = .151). In adults, stressed animals lost weight over time relative to controls. Finally, the difference between the juvenile groups at D7 was more than 2.5 times greater than the differences observed between adult groups.

A 2 x 2 ANOVA was also performed on difference-from-baseline weights after the 30-day incubation period, with age and stress as the independent variables (see Table 6 for means and standard deviations). There was a significant effect of age on weight change ($F(1,28) = 518.49, p < .001, \eta^2 = .95$, observed power = 1.000), with juveniles showing greater weight change than adults, as expected. However, the effect of stress on

weight change was no longer present following the 30-day incubation period ($F(1,28) = .55, p=.46, \eta^2=.019$, observed power = .11), nor was there a significant interaction between stress and age ($F(1,28) = .057, p=.81, \eta^2=.002$, observed power = .056).

Table 6
Means (and Standard Deviations) of Weight Change

Age	Condition	Day			Post- Incubation
		CVS Day 3	CVS Day 5	CVS Day 7	
Adult	CVS	0.13 (0.48)	-0.49 (0.29)	-0.49 (0.32)	2.42 (0.55)
	Control	0.29 (0.21)	0.28 (0.37)	0.46 (0.36)	2.65 (0.83)
Juvenile	CVS	1.48 (0.32)	2.86 (0.46)	4.24 (0.65)	12.46 (2.03)
	Control	2.41 (0.29)	4.74 (0.63)	6.73 (0.62)	12.90 (1.10)

Figure 4. Body Weight Change Values Over Seven Days of CVS

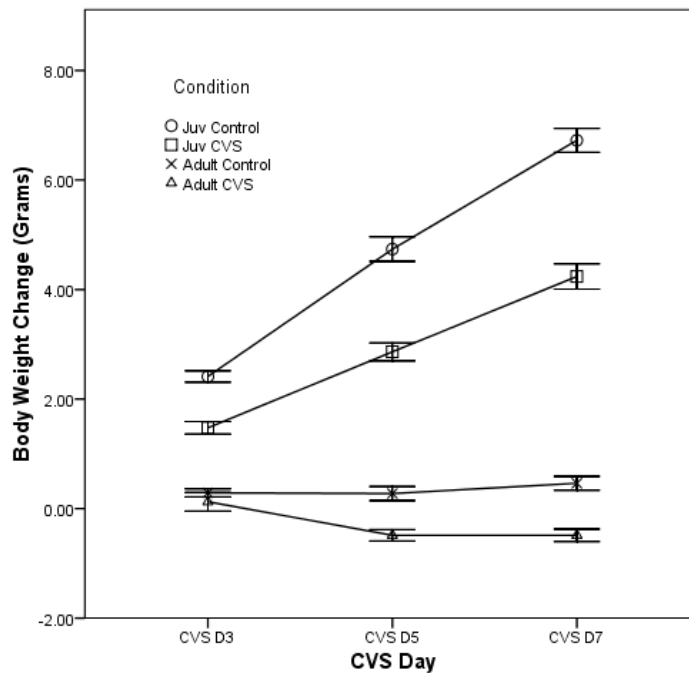


Figure 4. Body weight change is shown for mice on days 3, 5, and 7 of CVS exposure. Weights recorded on CVS D1 were used as weight baselines for all animals. There was a significant difference in the body weight change of control and stressed juvenile mice (top two lines) at days 3, 5, and 7 of CVS, with stressed juveniles showing less weight change than control juveniles. The body weight change of control and stressed adults (bottom two lines) differed significantly at days 5 and 7 of CVS, but not at day 3, with stressed adults showing lower weights than control adults. Error Bars: +/- 1 SE.

Discussion

The present study revealed that, contrary to what was expected, chronic stress-induced sensitization of fear learning, as measured by acquisition, context exposure, and tone exposure freezing behaviors, did not incubate differentially depending on the age in which stress was experienced. In terms of fear learning, stress-induced sensitization seemed to have deteriorated over the 30-day incubation period for both adult and juvenile mice, resulting in control and stress group subjects to display identical fear learning profiles. Similarities between control and stress group fear learning profiles also suggested that no detectable incubation occurred over the 30-day period, irrespective of age. In terms of non-associative measures of anxiety, adult stressed mice exhibited significantly greater anxiety than adult control mice, suggesting that a non-associative sensitization effect persisted over the course of 30 days. Interestingly, this sensitization effect was absent in the juveniles, where no difference in EPM behaviors existed between control and stressed mice. Finally, stressed mice had significantly different weight change over the course of the CVS procedure than control mice, suggesting that CVS was sufficiently stressful to the animals. In adults, stress led to weight reduction from baseline over time. In juveniles, stress attenuated the weight gain that occurred over time in controls. The effect of stress was also amplified in juvenile mice versus adult mice. However, after a 30-day incubation period, the effects of stress on weight had dissipated in both age groups. These results suggest that the stress-induced weight effects were state-dependent, rather than durable effects that persist in the absence of active stressors.

Our hypothesis that mice who experienced CVS exposure as juveniles would show greater long-term levels of anxiety and long-term sensitization to later fear learning than mice who experienced CVS as adults was not supported. Furthermore, the data did not support our second hypothesis that mice exposed to CVS, regardless of age, would show significantly enhanced anxiety and fear conditioning relative to control mice.

The current study failed to replicate the findings of Avital and Richter-Levin (2005) showing that juveniles were more susceptible than adults to the long-term behavioral effects of stress. This may suggest that methodological factors can best explain the findings in the current study. One of the greatest shortcomings of the study is the relatively small sample size. Past incubation research has typically used 12 or more animals per experimental cell (Pickens et al., 2009; Siegmund & Wotjak, 2007; Houston et al., 1999), whereas the current study only used 8 per cell. It is possible that this study simply did not have the power to detect existing differences between groups. However, Avital and Richter-Levin (2005) also used 8 animals per experimental cell. Another possible explanation for our failure to replicate their findings is that different forms of stressors could lead to different long-term sensitization effects. Avital and Richter-Levin (2005) used acute exposure (three 30-minute exposures in a single day) to a platform elevated above water, which may impact animals differently than seven days of variable chronic stressors. The variable nature of stressors in the current study may lead to weaker long-term behavioral effects than repeated exposure to the same stressor, or acute exposure to a single intense stressor. Differences have been found in the structural impacts of various stressor paradigms, which may support this possibility. For example, chronic restraint stress leads to dendritic atrophy of CA3 pyramidal neurons in the

hippocampus, and dendritic arborization in the basolateral complex of the amygdala (BLA), while chronic unpredictable stress leads to BLA bipolar neuron atrophy, and has relatively little effect on CA3 pyramidal neurons (Vyas et al., 2002).

The use of mice in the current study may also explain the lack of replication of Avital and Richter-Levin (2005), who used rats as subjects. Difficulties have been reported with mice when studying incubation, including the incubation effect fading after 14 days (Pickens et al., 2009). It was expected that chronic variable stress in juveniles would incubate in a robust enough manner to avoid fading over 30 days in the current study, but that expectation may have been in error. A replication of this study with a rat model and an increased sample size may lead to different results.

In contrast to the previously discussed limitations of this study, which may have precluded support for the experimental hypotheses, it is instead possible that the results are valid. Thus, it is valuable to also consider the importance of the findings in that vein.

For example, a discussion of the distinction between anxiety and fear learning is helpful in interpreting the findings of the present study. Davis and Shi (1999) proposed that the fear/anxiety response, a great deal of which occurs in the amygdala, is comprised of two separate pathways. The central nucleus of the amygdala is thought to be responsible for conditioned, associative fear learning, while the bed nucleus of the stria terminalis (BNST) is responsible for the elicitation of non-associative anxiety (Davis & Shi, 1999). According to this model, the tone-shock fear learning component in the current study directly involved the central nucleus, while the non-associative tasks, such as the EPM and initial baseline training cage exposure, directly involved the BNST.

Pego et al. (2008) found that 28 days of chronic unpredictable stress exposure led to a volumetric increase in the BNST of rats, but not the amygdala. Furthermore, this chronic stress led to increased anxiety reactions, while not altering fear learning behaviors (Pego et al., 2008). Perhaps the unique impact of chronic stress on the BNST, but not the amygdala, could explain the long-term durability of non-associative anxiety sensitization observed in the stressed adult mice in the current study. A volumetric increase in the BNST is likely to have long-lasting behavioral effects, while mechanisms responsible for the stress sensitization of fear learning seen in past research (Sanders et al., 2010; Conrad et al., 1999; Sandi et al., 2001) may be shorter lived, explaining the presence of anxiety sensitization and the absence of fear learning sensitization 30 days post-stress.

The finding that CVS exposure was able to significantly sensitize the anxiety responses of adult mice after a period of 30 days, but was unable to have the same effect for juvenile mice, suggests that a developmental factor exists that moderates the BNST's long-term consolidation of stress-induced, non-associative sensitization. Differences in anxiety responses between mice that were either stressed as juveniles or adults may be explained by examining the developmental time-line of certain regions essential to fear and anxiety responses. The BNST develops from approximately embryonic day 13 to 20 (Bayer, 1987). Given its prenatal maturation, the BNST of juvenile and adult mice in the current study would have been structurally and functionally equivalent, and therefore cannot likely account for observed behavioral differences.

The hippocampus is important for many aspects of learning and memory (Jarrard, 1993). More specifically, it plays a role in managing fear memories that are first formed

during the occurrence of a traumatic event (Vieweg et al., 2006). Developmentally, the mouse hippocampus is still showing marked growth and change on postnatal day 30 (Mody et al., 2001). Given that juvenile mice in the current study were just finishing their exposure to seven days of CVS at that time, it may be possible that an immature hippocampus in the juveniles interfered with whatever mechanisms led to long-term anxiety sensitization in adult mice.

Another region relevant to the topic is the central nucleus of the amygdala, which, as discussed earlier, is important for the process of associative fear learning (Davis & Shi, 1999). The central nucleus does not fully mature until postnatal day 35 (Canada's Michael Smith Genome Sciences Centre, n.d.). Again, CVS exposure in the juvenile mice was completed prior to the time this structure fully develops.

Given the preceding information, underdevelopment in the 23 to 30 day old mouse hippocampus, central nucleus of the amygdala, and perhaps other structures, may have led to interference in the mechanisms through which chronic stress is able to modify the BNST, and subsequently sensitize later anxiety behaviors in response to the EPM.

Several possibilities exist as to why an incubation effect was not elicited in the current study. The first may be that non-associative, chronic stress-induced sensitization does not incubate in the same manner as associative fear memories. Very little research has been conducted on the long-term nature of stress-induced sensitization. Siegmund and Wotjak's (2007) findings demonstrate the incubation of non-associative sensitization resulting from a single, discrete trauma. They showed that the hyperarousal characteristic of PTSD can be elicited from a single foot-shock in mice, and that this hyperarousal increases to a peak over the course of 28 days, as tested by freezing in response to a novel

context and novel tone (Siegmund & Wotjak, 2007). The possibility exists that this hyperarousal from a discrete trauma may be markedly different from the anxiety and fear sensitization caused by non-discrete, chronic, repeated exposures to unpredictable stressors. Siegmund and Wotjak's animal model of PTSD begins at the point in time where the trauma thought to give rise to the disorder occurs, whereas the model examined in the current study begins with stressful conditions thought to increase the response an organism may have to a discrete traumatic event in the future. In other words, the neural mechanisms responsible for trauma-induced non-associative sensitization may be altogether different from those responsible for CVS induced sensitization, and therefore may be differentially susceptible to the phenomenon of incubation.

Although purely speculative, perhaps the phenomenon of incubation requires a discrete stressful event, something explicit that can form a concrete memory. If this is the case, then the sensitization resulting from chronic stress may be too general and system wide, and may lack enough of a discrete memory aspect with which possible incubation mechanisms can interact. Further research is needed on the neurobiological mechanisms of incubation for the above hypothesis to have any validity.

Another confusing aspect of the current study is that in adult mice, chronic stress sensitization was able to persist over the 30-day incubation period for anxiety levels, as measured by behavior in the EPM, but not for freezing behaviors in response to a fear conditioning paradigm. A possible explanation involves corticosterone, which plays a key role in conditioned fear, as well as the potentiation of anxiety reactions, freezing behaviors, and fear learning (Korte, 2001). In addition to detecting the differential effects of stress on the central nucleus and BNST discussed above, Pego et al. (2008)

demonstrated that chronic administration of corticosterone was able to completely replicate the effects due to chronic stress. Sandi et al. (2008) also found that chronic unpredictable stress leads to neurological alterations in amygdalar corticosterone systems, which directly correlate with enhanced contextual fear conditioning, and that these changes could be prevented through the administration of a corticosterone antagonist. Other researchers have found similar results (Blank et al., 2002; Corodimas et al., 1994).

Given a previous demonstration of sensitized associative fear learning with CVS using procedures identical to those in the current study (Sanders et al., 2010), perhaps short-term, post-stress potentiation of fear learning relies on corticosterone mechanisms, while long-term sensitization relies on structural changes caused by those mechanisms. Pego et al.'s (2008) finding that stress and corticosterone administration structurally modified the BNST, but not the central nucleus, may explain why the current study observed long-term sensitization of non-associative, BNST-related anxiety behaviors, but not associative, central nucleus-related fear learning behaviors. An assumption of this hypothesis is that the corticosterone systems of mice return to baseline, pre-stress functioning before the end of the incubation period we employed. This would be necessary to account for the sensitization of anxiety behaviors in the current study, but not the fear learning behaviors. If corticosterone systems were still functioning after incubation as they were just after chronic stress, we would expect to see sensitization of both associative and non-associative fear, because the effects of corticosterone systems are numerous and system wide (Korte, 2001). A long-term structural manipulation of the BNST, in the absence of abnormal corticosterone functioning, could explain the specific and isolated sensitization of non-associative anxiety in adult stressed mice.

A significant attenuation of weight gain in juvenile mice exposed to CVS, and significant weight reductions in adult mice exposed to CVS confirmed that the CVS procedure was subjectively stressful. Past studies have used weight loss or stunted weight gain as a physiological indicator of CVS effectiveness (Choi et al., 2008; McLaughlin et al., 2005). The weight effects appeared most pronounced in juvenile mice. This increased impact of stress on juvenile body weight is most likely because the juvenile mice were still developing, and as such, major physiological changes were ongoing. Juvenile body weight may be more sensitive to external stressors during this developmental stage, or perhaps the normal changes taking place during this period simply made the effects of CVS on body weight more easily detectable.

Unexpectedly, the present findings do not compare with the human PTSD literature. Human PTSD research has repeatedly shown that past traumatic experiences and chronic stress have sensitizing effects on the possibility of developing PTSD in the future (Grassi-Oliveira & Stein, 2008; Breslau et al., 1999; Bremner et al., 1993). Furthermore, it has been shown that this sensitization is most severe when the antecedent stressful conditions occur during childhood (Grassi-Oliveira & Stein, 2008; Breslau et al., 1999). The current study failed to demonstrate long-term, stress-induced sensitization of the fear learning system that is believed to be heavily involved in the etiology of PTSD (Lissek et al., 2005). Furthermore, we also failed to demonstrate a heightened risk for sensitization in juvenile mice. In addition to the possibilities discussed above, one reason for this failure may involve differences in the perceived intensity of stressors thought to lead to sensitization in human and animal research. Qualitatively, our CVS paradigm is quite mild compared to the stress of chronic physical and sexual abuse thought to

sensitize later PTSD development in the human literature. Perhaps a distress threshold exists that must be exceeded in order for chronic stress to elicit enduring and incubatable sensitization to later fear learning events. If this is the case, the current CVS paradigm may be stressful enough to elicit short-term fear learning sensitization (Sanders et al., 2010), but not long-term sensitization. Although the present study successfully sensitized non-associative fear in adult mice, it is well established that associative fear learning mechanisms are integral to the development of PTSD (Lissek et al., 2005). Therefore, non-associative fear sensitization may be longer lasting in nature, but is not sufficient in and of itself to elicit PTSD-like fear responses.

Much future research is needed in order to discern the specific mechanisms behind chronic stress-induced sensitization, its incubation, and its durability. A study involving regular corticosterone level readings over the course of a 30-day incubation period following chronic variable stress exposure would give valuable insight into the temporal durability of stress-induced corticosterone system manipulations. With this information, we could begin to differentiate between hormonally and structurally mediated stress effects. Neurobiological measures of corticosterone receptor densities at 24 hours post stress and 30 days post stress may also give us similar information. To support the hypothesis that stressed juveniles did not exhibit anxiety sensitization because of underdeveloped brain regions responsible for learning and fear responses, a future study could begin CVS exposure at 30, 35, 40, and 45 days of age. Doing this may allow us to detect a gradual increase in the ability of mice to consolidate anxiety sensitization, which would theoretically correlate with the maturation of regions such as the hippocampus and amygdala.

Although the current study did not observe developmental differences in the incubation of stress-induced sensitization, it arrived at several findings that may aid a future animal model of PTSD. These findings must be interpreted with caution however, due to the study's small sample size. In adult mice, non-associative anxiety sensitization due to chronic stress appears to be maintained for a much longer duration than associative fear learning sensitization. For reasons yet unknown, juvenile mice did not exhibit similar sensitization effects. These findings bring into focus the importance of acknowledging the differential mechanisms of associative and non-associative fear when developing a model of PTSD. Additionally, age of exposure must be considered because the stage of brain development may moderate, in various ways, how each of these sets of mechanisms function. The current body of PTSD literature suggests that many factors, including associative fear, non-associative fear, chronic stress, acute stress, incubation, and developmental period, may all play a role in the mechanisms of the disorder. It is therefore crucial for an animal model of PTSD to account for these factors and explain how they interact with one another. Ultimately, an accurate and detailed animal model will allow for the manipulation of the neurobiology of PTSD, as well as enlighten researchers and clinicians to factors that could improve the treatment of PTSD in humans.

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Note: The Mouse Atlas of Gene Expression is a project developed by Canada's Michael Smith Genome Sciences Centre and supported by Genome Canada, the British Columbia Cancer Agency, British Columbia Cancer Foundation, and the National Cancer Institute (USA). The Mouse Atlas of Gene Expression strives to establish a comprehensive atlas of gene expression over development in the mouse.

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