SEXUAL CONSPECIFIC AGGRESSION RESPONSE (SCAR):

A NOVEL ANIMAL MODEL FOR SEXUAL ABUSE IN YOUNG WOMEN

By

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ABSTRACT OF THE THESIS

Sexual Conspecific Aggression Response (SCAR):

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Sexual abuse in adolescent girls and young women is unfortunately common and often leads to long-lasting deficits in thoughts and behaviors related to mental illness. In order to study the neuronal consequences of sexual abuse, we developed an animal model referred to as SCAR. The acronym stands for Sexual Conspecific Aggression Response, which indicates behavioral, cognitive and neuronal changes that occur after repeated exposure to sexually aggressive and experienced adult males. In the first set of experiments, pubescent females were exposed to an adult male aggressor for 30 minutes every three days over the course of adolescence (PND 35- PND 57). During adulthood, the female's ability to learn an associative response was examined. Overall, adult females that were exposed to the sexually aggressive males during puberty did not perform as well as females that were not exposed to the male and showed increased sensitized responsiveness to the conditioned stimulus. Thus, the aggressive encounters during puberty were sufficient to induce long-lasting effects on processes of learning and sensitization during adulthood. We also examined the effects of SCAR on the survival of new neurons in the hippocampus as a result of the training procedure. In previous studies, we find that learning keeps new neurons alive. However, adult females that were exposed to the aggressive adult males during puberty retained fewer new cells as a result of the training process. Since they did not learn as well, these results indicate long-lasting effects of this procedure not only on cognition but also on the structural integrity of the adult brain.

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I. Introduction

Sexual abuse in adolescent girls and young women is common and oftentimes produces long-lasting deficits in thoughts and behaviors related to mental illness. It is estimated that a staggering 44% of all female sexual abuse victims are under the age of 18 (US D.O.J). Studies report that women who were sexually abused as children are at a much higher risk for mental illnesses such as depression, post traumatic stress disorder and social phobias (Kessler 2003, Parker & Brotchie 2005) as well as a host of other mental and physical disorders such as drug abuse, addiction, alcoholism and eating disorders (Sherman & Silver, 2009, Parker & Brotchie 2010). Additionally, depressed patients are four times mores likely to commit suicide if they were victims of childhood sexual abuse (McCauley et al, 1997). Whereas sexual abuse during puberty is not perpetrated exclusively on girls, females are abused nearly three times as often as males (Sherman & Silver, 2009). Sexual abuse is one of the most stressful life experiences a young girl can endure. Although clinical examples are numerous, there is little basic research addressing the effects of exposure to a sexually aggressive male on processes of brain development and plasticity in females.

It is reported that stressful experiences during puberty can have long lasting and deleterious effects for both genders, especially on learning later in life (Beylin & Shors, 2003, Hodes & Shors, 2005). However, previous studies indicate that males and females learn very differently both during and after the critical period of puberty (Hodes & Shors 2005, Bangasser & Shors, 2008). For example, stress can have a detrimental effect on learning in females, one that does not occur in males (Wood & Shors, 1998). This deficit occurs only during adulthood; females that are exposed to a stressor and tested during puberty learn better than those that are unstressed. Therefore, there are reportable differences in the way that females respond to stress simply as a function of age.

As noted, there are lifelong effects of sexual aggression and abuse on mental health and brain function. Despite this problem, there are, to date, no animal models that examine these critical changes in brain connection and later behavior. To address this need, we developed a naturalistic stressor in order to study the neuronal and physiological consequences of sexual abuse. It is an ethologically relevant stressor that replicates some

aspects of real-world human experience and the reaction to such an experience. The SCAR experience consists of eight of the male-female rat pairings over the course of the female's puberty and adolescence. The males are sexually aggressive and experienced whereas the females are newly weaned and virgins. These females are then tested in adulthood to examine long-term effects of the experience. An animal model obviously cannot reproduce all the intricate higher order processes involved in human sexual abuse or trauma (such as shame and guilt, etc.). Therefore, SCAR is a putative model for some aspects of the physical response in the developing body to abuse. The acronym stands for Sexual Conspecific Aggression Response, which refers to the behavioral, cognitive, hormonal, and neuronal changes that occur after a female adolescent is repeatedly exposed to a rotation of sexually aggressive and experienced adult males.

The purpose of this study was to develop the SCAR model and to assess the long-term consequences of the SCAR experience during puberty on learned and unlearned behaviors in adulthood. Additionally, we examined long-term consequences of the model on an associative learning task and on the structural plasticity of the hippocampus. We hypothesized that the SCAR experience in puberty would have persistently negative effects on hippocampal neurogenesis cell survival and associative learning ability in adulthood. We hypothesized that these deleterious effects would last long after the SCAR exposure had ceased and would be expressed in adulthood as a result of exposure to SCAR during puberty.

II. Methods

Subjects. The female Sprague-Dawley rats were bred in-house and weaned from their mothers at the normal PND 28. Litters were split between conditions and were housed 3-4 per cage in a plastic box style cage (44.5 cm long × 21.59 cm wide × 23.32 cm high) and grouped by experimental condition until surgery, after which they were single housed to prevent damage to headstages. Animals had access to food and water *ad libitum*. The males were adults (>90 days) that were single housed in our male breeder colony and had histories of successful copulation. The animals were maintained on a 12 hour light- dark cycle with the lights turning on at 7am. At PND 35 the exposure procedures began for the experimental group and the control animals were handled. All experiments were

conducted in full compliance with the rules and regulation specified by the PHS Policy on Humane Care and Use of Laboratory Animals an the Guide for the Care and Use of Laboratory Animals.

Figure 1. Photos illustrating the physical dimorphism of the fully mature adult male (right) and the pubescent female (left) at the beginning of scar exposures (PND 35). Note the typical female weight of 99g and the typical male weight of 650g.

SCAR Exposures. Adult male breeders noted for their aggressive nature and history of successful breeding were chosen for the SCAR exposures. The female adolescent rats were placed in a plastic box style cage (44.5 cm long × 21.59 cm wide × 23.32 cm high) for 30 minutes every 3 days (see figure 2). Each box was unique to each male and was not washed over the course of the exposures. The pairings were done at an unpredictable time and the male breeders were alternated and counterbalanced. None of the adult males were related to any of adolescent females. There were a total of 7 exposures, lasting throughout puberty adolescence (PND 35-PND57). The encounters were filmed and monitored for any overly aggressive behavior that might have caused serious bodily harm. Later, the videos were evaluated for numbers of pins to quantify levels of aggression and ensure a consistently aggressive exposure experience. Control animals were handled and otherwise left in their home cages, group housed by condition.

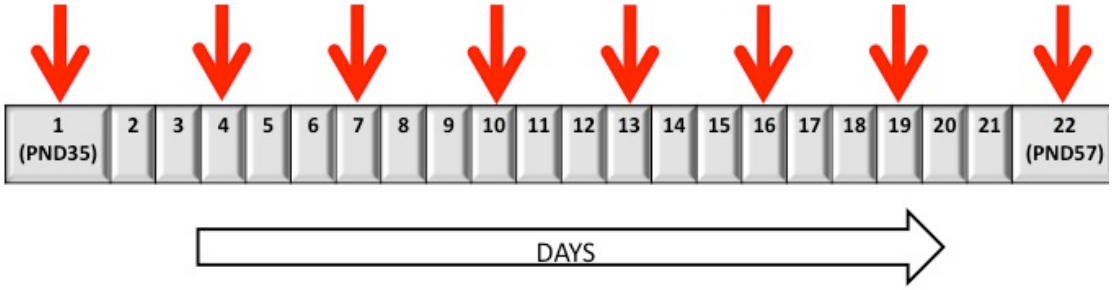


Figure 2. Timeline for SCAR exposures. Red arrows indicate days of exposures. The experience encompasses the entirety of puberty in the rat (roughly PND35- PND57).

Aggression Quantification. Videos were analyzed and total numbers of pins were recorded. A pin was classified as any time the aggressive male immobilized the female for more than 1 second, either against the floor or wall of the cage (Figure 3)

Vaginal Cytology. The estrus cycle begins once the female has sexually matured (approximately PND 35-40) and signals the increases in circulating sex hormones and the development of secondary sexual characteristics (Hodes & Shors, 2005; Wood & Shors, 1998). After vaginal canalization has completed and ovulation has begun, females exhibit a two-phase estrus cycle, alternating between estrus and proestrus (Ojeda & Urbanski, 1994). Later, as adults (PND >60) animals exhibit the full, four-phase female reproductive cycle

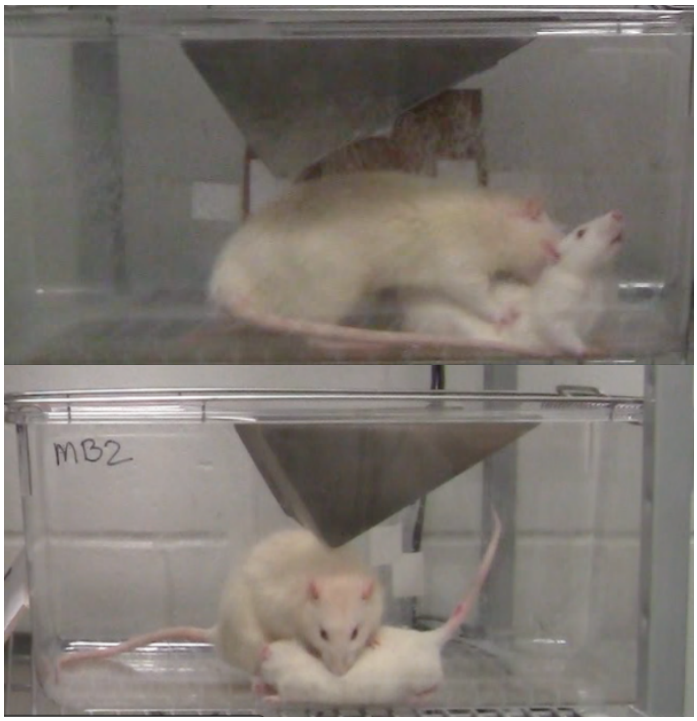


Figure 3. Representative photo stills from videos males and females taken from an average SCAR session. The males frequently dominated the females, pinning them and limiting the female's mobility.

including diestrus1, diestrus2, proestrus and estrus.

To examine the estrus cycle, females were lavaged with saline soaked cotton swabs and loose vaginal epithelial cells were collected and rolled onto slides to dry. Slides were dyed in Toulidine Blue to visualize the cells. We determined the stages of estrus via microscope by visual inspection of the cytology of the vaginal cells and charted the stages of estrus of each animal on each day of testing in adulthood. As estrogen levels rise, the number of large nucleated pink/purple stained cells increases. As these cells proliferate and form sheets of epithelial tissue the animal enters the Proestrous phase. Next, during Estrus estrogen levels fall and the vaginal epithelium sloughs off forming visible cell clumps that have a blue, unnucleated, cornified appearance when stained. After Estrous the animal undergoes Diestrus 1 and Diestrus 2. Diestrus 1 is marked by small, dark staining leukocytes and diestrus 2 is similar to Diestrus 1 only with fewer cells. Often in Diestrus 2 there are few cells present in the culture. As estrogen peaks in late Diestrus 2 it gives way to the Proestrus phase and the cycle begins again (Shors et al., 1998). The cycle repeats every 4-5 days in adulthood. Stages of estrus were not used as an independent variable but were charted to ensure that the animals were not pregnant and to evaluate whether or not they were cycling normally.

Cell Production and Labeling. BrdU is a thymidine analog that is incorporated into the DNA of a dividing cell during the S-phase of the cell cycle and marks cells that are actively proliferating at the time of the injection. For one week after BrdU injections, newly dividing cells are labeled in the brain. Successful learning of trace conditioning results in an increased number of surviving neurons in the dentate gyrus of the hippocampus (Gould, 1999; Shors, 2001), specifically in the granule cell layer (GCL). BrdU injections were given one week before the first day of trace conditioning in order to allow newly proliferating cells to be labeled.

To label newly produced cells, female rats were injected on PND65 intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, 200 mg/kg, Sigma, Atlanta, GA, USA) in physiological saline solution (pH 7.4). By sacrificing the animal three weeks after injection (PND 86) we were able to visualize the cells that were dividing during the

week following the injection and had survived and integrated into the existing hippocampal structure.

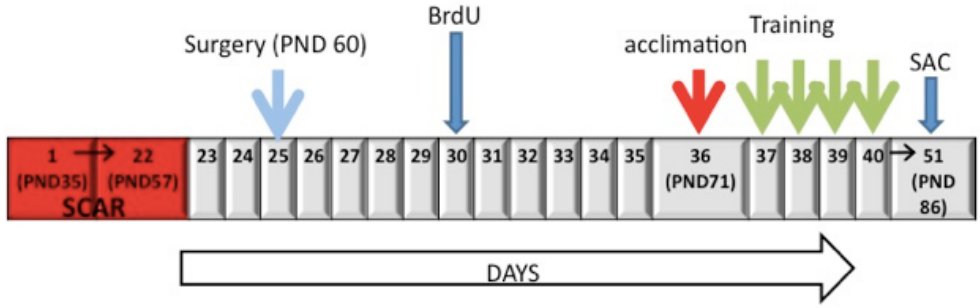


Figure 4. Neurogenesis and trace conditioning timeline. After the SCAR exposures, animals underwent surgery (after PND 60) and BrdU injections per this timeline. Green arrows indicate days of training. Note the additional time between training and sacrifice.

Trace Conditioning of the Eyeblink Response. Trace conditioning is a form of classical conditioning wherein the conditioned stimulus (CS) and unconditioned stimulus (US) are separated in time. The animal's ability to associate the CS with the impending US is determined by how frequently the animal performs the conditioned response (CR) in anticipation of the US. In order to measure eyeblinks, animals were implanted with electrodes to record the electromyographic response. At PND 60 the female (now adult) rats were anesthetized with Nembutal (50 mg/kg; Henry Schein, Indianapolis, IN, USA) and supplemented with isoflurane inhalant (Baxter Healthcare, Deerfield, IL, USA) and oxygen. Heads were shaven, sterilized, and injected with Marcaine local anesthetic (.03mL each side of the scalp). Next, headstages (four-pin connectors with four wires) were surgically mounted to the skull using four skull screws and dental cement. Four wires were threaded perioribitally from the headstage into the orbicularis oris muscle. Two of the wires delivered eyelid stimulation during conditioning and the other two wires sent EMG (electromyography) data from the eyelid to the computer in order to assess the animal's eyeblink

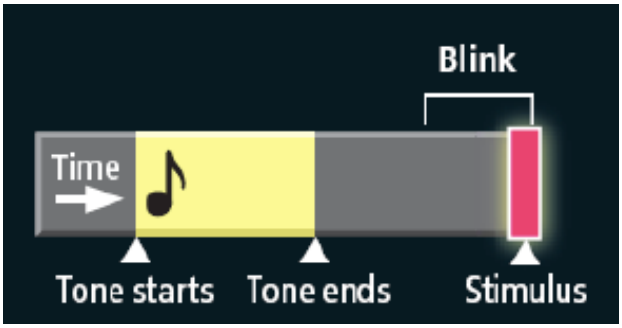


Figure 5. Trace conditioning paradigm. The CS begins and terminates before a stimulus free interval, followed by the US. (Figure credit: Shors, 2009)

response. After awaking from surgery the animals were given 1mL dose of acetaminophen and allowed a minimum of 5 days recovery before BrdU injections. After a minimum of 2 weeks post surgery and one week after BrdU injections (See figure 4), the females were tested on their ability to learn the association between a white noise tone (CS, 80dB) and a periorbital stimulation (US, .5mA). Animals from both groups were given one day of acclimation in the testing chambers wherein no stimuli was presented, followed by four days of trace conditioning during which EMG data was recorded.

Training consisted of 8 sessions comprising 800 trials (CS-US pairings), 200 trials (2 sessions) a day over four days. One trial consisted of a 240-ms white noise conditioned stimulus (CS, 80 dB) followed by a 500-ms stimulus-free trace interval, and immediately thereafter by the unconditioned stimulus (US), a 100-ms eyelid stimulation (0.5mA). The inter-trial interval was 25 ± 5s.

Sensitization Testing. Sensitization is a non-associative learning process during which repeated presentations of a stimulus result in a progressive amplification of a response. Previous studies have shown that repeated exposure to an uncontrollable stressor can result in the sensitization of norepinephrine activity in such a way that exposure to new stressors can trigger an exaggerated response in the previously stressed animal (Anisman, 2011). Measures of sensitization included blinking during the first 80ms of the CS (reflexive responses) as well as incorrectly timed blinks, which occurred during the duration of the white noise CS (240ms). The 80 ms window blinks are referred to as "sensitized" responses and the 240 ms window blinks are referred to as "alphas" for clarification. (Figure 6).

Perfusions. All animals were deeply anesthetized with a lethal dose of sodium pentobarbital (100mg/kg) and transcardially perfused with 4% paraformaldehyde. Brains were extracted and post fixed in 4% paraformaldehyde at 4 degrees Celsius for 24 hours before being transferred to phosphate buffer saline.

Tissue Sectioning and Immunochemistry for BrdU-labeled Cells. 40-µm coronal sections were taken throughout the entire rostro-caudal extent of the dentate gyrus of one hemisphere of each brain with a vibratome (Leica). Every 12th slice was mounted onto superfrost glass slides (Fisher, Suwanne, GA, USA) and allowed to air dry. Once dry, tissue was stained for the presence of BrdU positive cells using standard peroxidase methods. First, the tissue was treated with boiling 0.1 M citric acid solution (pH 6.0) for 15 minutes and placed in mailers holding 5 slides. Following several PBS rinses, the mailers were filled with a trypsin solution for 10 minutes, followed by 3 more PBS rinses and 2NHCl solution for 30 minutes. After more PBS rinses the slides were kept overnight on the shakerplate at 4 °C in primary anti-mouse antibody (1:200, Becton–Dickinson, Franklin Lakes, NJ, USA). The next day, slides were rinsed and treated with biotinylated anti-mouse antibody (1:200, Vector Labs, Burlingame, CA, USA) for 1 hour, followed by avidin–biotin–horseradish peroxidase (1:100, Vectastain ABC Kit, Vector Labs, Burlingame, CA, USA) for 1 h, and lastly in diaminobenzidine (DAB SigmaFast tablets, Sigma, Atlanta, GA, USA). After being rinsed with dH20, counterstained with cresyl violet and rinsed in increasing concentrations of ethyl alcohol, the slides were left in Xylene for 4 minutes. Slides were then coverslipped using permount glue and left to dry. Once dry, animal IDs were concealed and coded arbitrarily to eliminate any counter bias.

Light Microscopy and Cell Analysis. BrdU-positive cells in the dentate gyrus (DG= granule cell layer + hilus) of each representative slice were counted using a modified unbiased stereological protocol (West et al, 1991; Gould et al., 1999). Cell counts were obtained at 100x on a Nikon Eclipse 80i light microscope for both the granule cell layer (GCL) and hilus. Cells that were not in focus or outside of the anatomical boundaries of the GCL or hilus were not included. All cell counts were then multiplied by 24 (12 representative slices x 2 hemispheres) to estimate the total number of BrdU-labeled cells in the dentate gyri of each animal.

Statistical Analyses.

A. Eyeblink Data Analyses.

In order to assess the animals' performance on trace eyeblink conditioning, raw EMG data was analyzed for the presence of blinks. Eyeblinks were detected using MatLab (MathWorks, Natick, MA, USA) software running a program designed for our lab (West lab). For each trial, the mean and standard deviation of the EMG signal amplitude was calculated across the baseline period (500ms prior to CS). This value was then used along with the "multiplier" (number of standard deviations above mean baseline at which to set the blink threshold) to determine the threshold for blink detection. If the signal crossed the blink threshold and contained a minimum of 4 consecutive 1ms data points per 10ms window of time that crossed the threshold, it was counted as a blink.

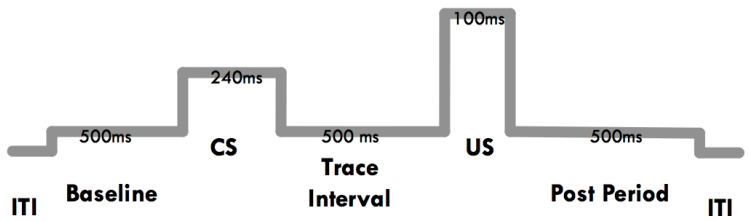


Figure 6. Diagram of the timing of each trial of trace conditioning.

A.1. Blink Detection Time Windows.

Total numbers of blinks were recorded for both the 250ms and 500ms before the US for presence of a CR. Sensitized responses and alpha responses were quantified during the 80ms and 240ms after the onset of the CS, respectively. It may seem redundant to perform analyses that examine overlapping

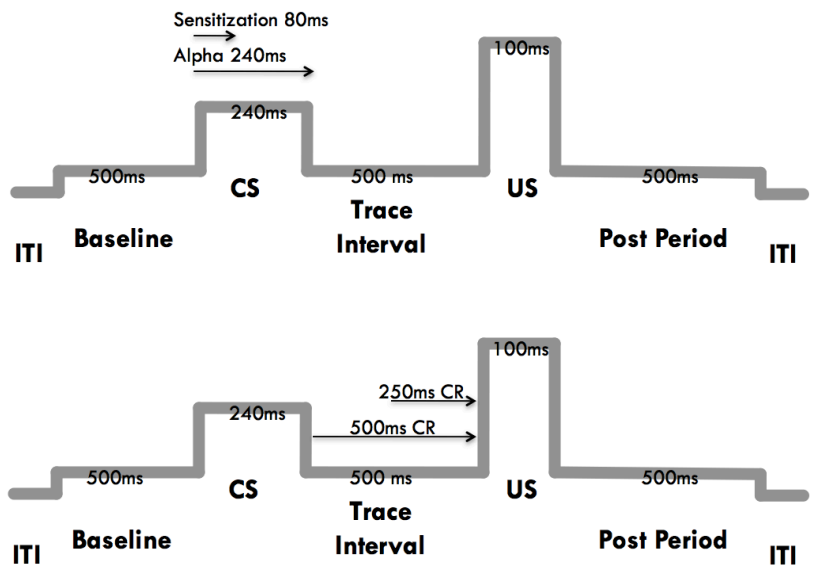


Figure 7. Timing diagrams of the detection windows for each of the 4 analyses. Blinks during CS (above) were either classified as sensitized or alpha responses. Blinks during the trace interval (below) were calculated during both 250ms and 500ms before the onset of the US.

time periods (i.e. 80ms and 240ms after the CS onset for sensitized responses and 250ms and 500ms before the US for CRs). However, this technique allowed for further exploration and information about what exactly was happening at what time for each trial for each group. Analyses were performed as to the numbers of blinks per 100 trials between groups and within groups across all 8 sessions over 4 days. Statistics were generated using repeated measures ANOVAs with PASW (SPSS, Chicago, IL, USA) software. Separate repeated measures ANOVAs were performed between groups for each analysis window (sensitization, alphas, 250ms and 500ms). Where interactions were detected, independent repeated measures ANOVAs were conducted for both the SCAR and no SCAR conditions.

B. Neurogenesis Data Analyses

Cell counts were compared between groups (SCAR and no SCAR) with independent sample T-tests: one of the hilus, one of granule cell layers, one of the entire dentate gyri using PASW (SPSS) software. Tests were performed with the designated .05% significance level. Animals that did not have any BrdU positive cells (n=3) were excluded from the analyses. The reason for the failure of BrdU to label cells in these animals is not precisely known but several theories are proposed. Because BrdU is incorporated during the S phase in all newly dividing cells, absence of any BrdU positive cells indicates a procedural failure of some kind and would not be plausible if the BrdU was successfully incorporated and tissues properly stained. However, some resources suggest a common reason for this failure is due to incorrect injection wherein the syringe is not placed introperitoneally but rather subcutaneously or in an organ. Additionally, it is possible that the heat when mixing the BrdU was too high and caused precipitation of the drug. (Pitulescu et al, 2010).

III. RESULTS

1. The SCAR Experience

During the SCAR experience, females were placed into a cage with a male aggressor. Interactions were videotaped to quantify levels of aggression. After the first exposure females were visibly resisitant to enter the cage and often vocalized audibly during the encounter. Earlier exposures were marked by freezing behavior and the males were able to pin the females for longer periods of time. Puberty in the female rat is defined by the progressive canalization of the vaginal canal and transition from a 2 stage estrous to an adult 4 stage estrous (Ojeda & Urbanski, 1994). As this physical maturation occurred, we observed more forceful pins as the females occasionally attempted to aggress upon the male. We also observed more mounting behavior, both because the male was able to successfully restrain the female and attempt mounting (due to her larger size) and presumably due to the sexual maturity of the animal. This experience differs from normal sexual activity in the uncontrollability of the stressor, the number and frequency of aggressors, and the age of the female. Behavioral receptivity in the female is marked by lordosis and pacing behaviors. These behaviors do not occur until well after the earliest markers of puberty: raised levels of lutenizing hormones opening the vaginal canal, followed by first ovulation. (Urbanksi & Ojeda, 1987).

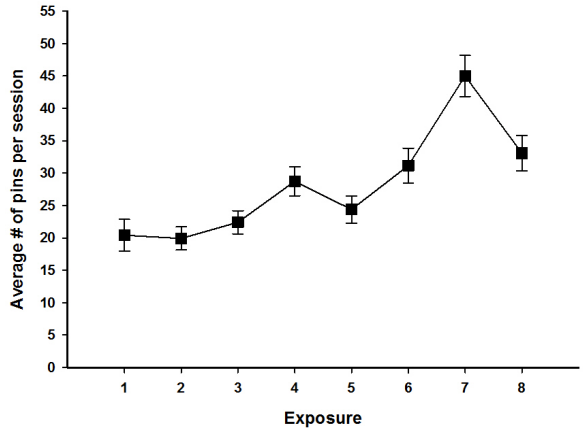


Figure 8. Aggression analyses. Line graph depicting the average number of times the female subject was pinned by the male aggressor per SCAR session across all animals. The average was 28.14 or roughly 1 per minute of the 30-minute exposure.

In Sprague-Dawley rats, vaginal opening occurs at roughly 39 days, followed by first ovulation 2-3 days later (Urbanksi & Ojeda, 1987). After that, females are only in "heat" or sexually receptive every 4 to 5 days (Ojeda & Urbanski, 1994). The females may be physically sexually mature for later part of the SCAR experience but are not during the first several presentations. At the beginning of the SCAR experience, females

are only one week weaned from their mothers. The aggressors are the first (unrelated) animal interaction they have had.

2. SCAR in puberty increases sensitized responses to the CS when tested in adulthood.

Females that had been subjected to the SCAR exposures during puberty emitted significantly more sensitized responses during the conditioned stimulus period of trace conditioning than did the no SCAR females. This effect was unexpected. The sensitized responses were first noticed as the SCAR animals emitted many blinks that occurred during the conditioned stimulus, a phenomenon which we did not see in the no SCAR animals. The sensitized blinks occurred early (within 80 ms after CS onset) and therefore would be considered unconditioned or perhaps even startle responses to the CS.

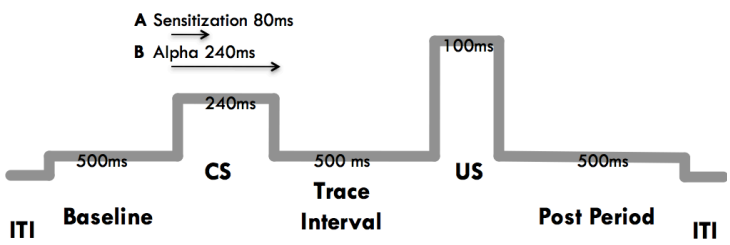


Figure 9. Diagram of the timeline of a trace conditioning trial. Results below reflect the number of blinks in the 80ms window (A) or 240ms window (B) after the onset of the CS.

2A. SCAR animals emitted more sensitized responses to the CS than the no SCAR group across all sessions.

After 8 sessions over 4 days of trace conditioning animals' numbers of blinks during the CS were quantified. A repeated measures ANOVA was used to assess the differences between groups (SCAR or no SCAR) and over time (across sessions). Results indicated that the between subjects factor of group was significant $F(1,13)=6.95$, $p=.021$ at the $p<.05$ level. Furthermore, the analysis revealed a within subjects effect of session $F(7,91)=4.30$, $p<.001$, but no significant session by group interaction $F(7,91)= .49$, $p=.841$. Follow up repeated measures ANOVAs for each group indicated a significant effect of session in the no SCAR condition $F(7,42) = 3.13$, $p<.001$ but not in the SCAR condition $F(7,49) = .24$, $p=.097$. This suggests that the SCAR group continued to have a roughly equivalent number of sensitized responses over the course of training, which was not the case for the no SCAR group. Each session, over each day, SCAR animals emitted

significantly more reflexive blinks during the start of the CS than did the no SCAR group. Additionally, the graph reflects a kind of saw tooth pattern wherein the group means for the second session of each day was lower than the group mean for the first session of each day (because there were 2 sessions a day over 4 days, trials 200, 400, 600 and 800 were all preceded by trials 100, 300, 500 and 700, respectively.)

2B. Alpha responses

Next, we compared groups in terms of number of blinks during entirety of the CS window (240ms), including the sensitized blinks occurring in the first 80ms. Results were similar to those found above (2A, figure 10) but the differences between groups were even more pronounced. We chose to examine the entire CS because use of the stringent parameters by which we counted blinks may have missed blinks that started but did not terminate during the first 80 ms. Additionally, examining if the animals had later timed responses which still occurred during the CS was of interest. A repeated measures ANOVA between groups across sessions indicated that the between subjects

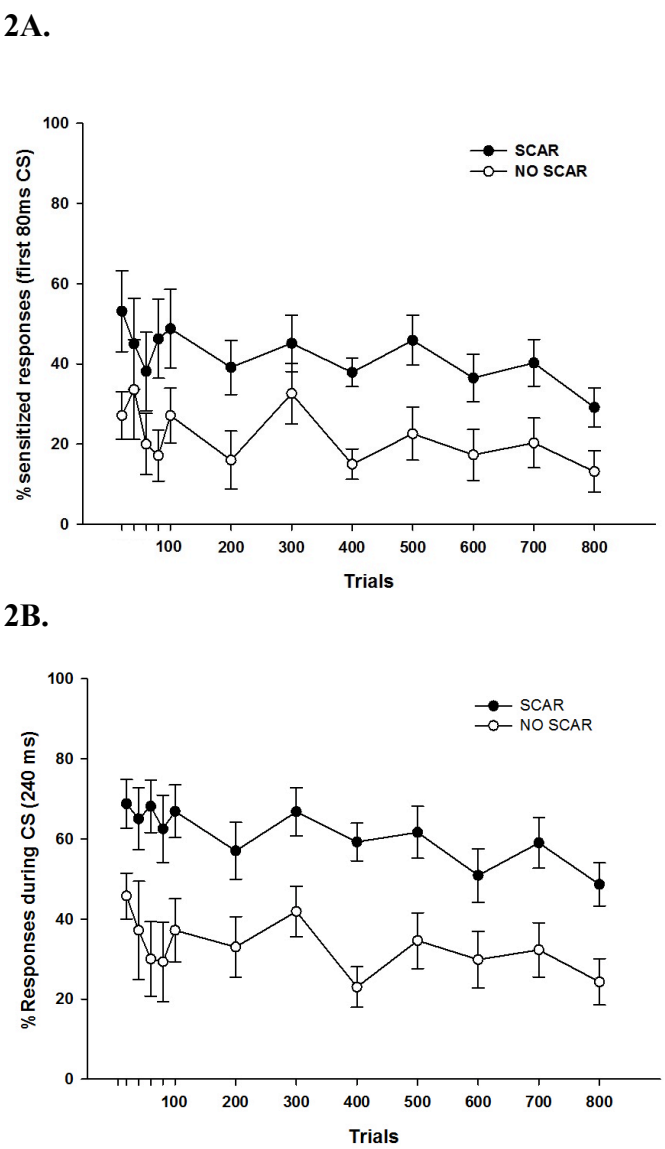


Figure 10. Effects of SCAR procedure on sensitized responses during trace eyeblink conditioning in adulthood. Animals were trained on 200 sessions a day for 4 days and blinks during specific time windows of the CS (white noise tone) were quantified. Significantly more ($p<.05$) sensitized and/or poorly timed responses were observed in the SCAR animals than in the no SCAR animals. The effect persisted over all sessions for both the 80ms (A) and 240ms (B) analysis windows.

factor of group was significant F(1,13)=6.95, p=.021 at the p<.05 level. Furthermore, tests indicated a within subjects effect of session F(7,91)=4.30, p<.001, but no session by group interaction F(7,91)= .49, p=.841. Follow up repeated measures ANOVAs for each group found no effect of session in the SCAR group F(7,49) = .24, p=.097 and did find a significant effect of session in the no SCAR condition F(7,42) = 3.13, p<.001.

3. No SCAR animals showed a normal learning acquisition curve over sessions whereas the SCAR animals did not.

After 4 days of 2 sessions a day (100 trials a session) of the associative learning task of trace eyeblink conditioning we measured the animals' ability to properly emit a precisely timed CR. We conducted repeated measures ANOVAs to examine the SCAR and no SCAR animals' performance on this task in order to determine whether or not the animals had successfully learned the association between the CS and US as demonstrated by a well timed CR (blink) before the impending US. Additionally, the animal was considered to have learned if it progressively increased the number CRs over time. As demonstrated in Figure 11, after quantifying the number of blinks during both the 250ms (A) and 500ms (B) before the US, the data suggests that the SCAR animals did not acquire the conditioned response over time. Therefore did not show normal learning acquisition curves. In contrast, animals in the no SCAR condition displayed normal learning curves across sessions for both analyses.

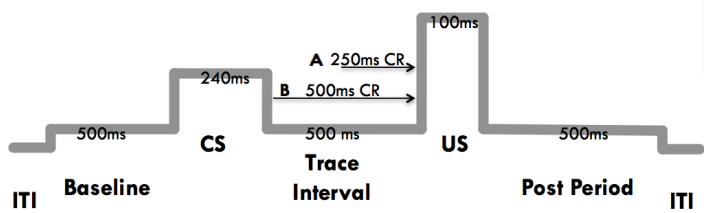
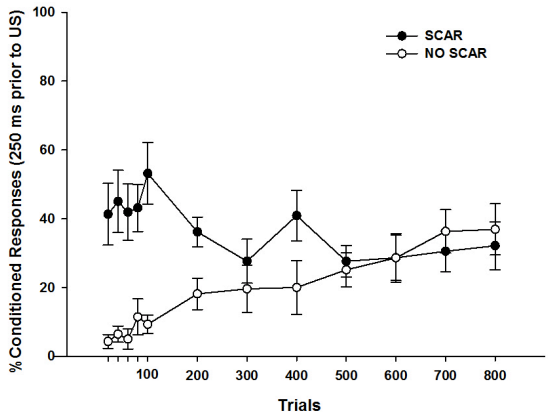


Figure 11. Diagram of the timeline of a trace conditioning trial. Results below reflect the number of blinks in the 250ms window (A) or 500ms window (B) before the onset of the US.

3A. Conditioned Responses During the 250ms Before the Unconditioned Stimulus. In analyzing the number of blinks during the 250 ms window before the US in both the SCAR and no SCAR conditions across sessions, we detected several significant differences between groups in terms of performance on the trace conditioning task. A

repeated measures ANOVA revealed both a significant effect of session F(7,91)=2.96, p<.01 and an interaction between session and group F(7,91)=3.13, p=.005. Somewhat surprisingly, because we expected dramatic differences in terms of learning, there was not a significant between-subjects effect of group F(1,13)=1.49, p=.24. To further investigate the interaction between group and session we ran independent repeated measures ANOVAs on the SCAR and no SCAR groups. As expected, the no SCAR group had a significant effect of session F(7,42)=4.65, p=.001, suggesting they successfully learned the conditioned response. However, the SCAR group did not show a significant effect of session and thus did not show a normal learning curve over time (7,49)=1.20, P=.32. Therefore, the SCAR animal's performance, while variable, was not consistently increasing over subsequent sessions, which would indicate a successful learning of the association. Because learning of the association is measured in terms of incremental gains in CR production over time, and the SCAR animals showed no effect of session, we saw no evidence of learning.

3A.



3B.

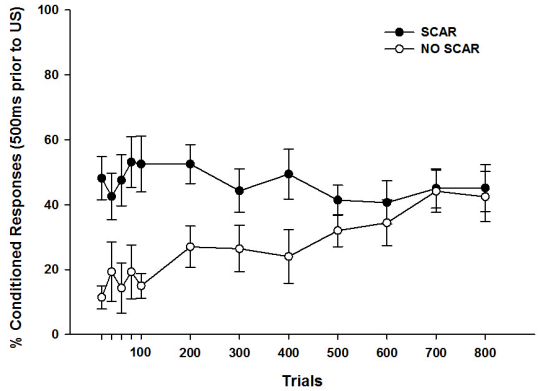


Figure 11. Animals from both the SCAR and no SCAR conditions were tested on their ability to learn a classically conditioned association between a white noise tone (CS) and a periorbital stimulation (US) in a trace paradigm. While the no SCAR animals were able to properly learn the association over time and emit precisely timed CRs, the SCAR animals were not. These findings were illustrated in both the 250ms (A) and 500ms (B) analyses.

3B. Conditioned Responses During the 500ms Before the Unconditioned Stimulus. Our analysis of the 500ms before the US revealed findings that

were similar to our analysis of the 250ms before the US (which was expected as the two time periods overlap) but with some notable differences. In contrast to the previous analysis, when looking at this timeframe we found no within subject effect of session across groups $F(7,91)=1.38$, $p=.22$. However, we did find a significant session by group interaction $F(7,91)=2.84$, $p=.01$ as well as a between subjects effect of group $F(1,13)=5.32$, $p=.038$. In order to further explore the interaction and the differences between groups we conducted independent repeated measures ANOVAs for both the SCAR and no SCAR conditions. As we previously determined in the 250 ms analysis (3A), the no SCAR condition successfully learned and showed a significant effect of session $F(7,42)=3.43$, $p=.005$. Once again, the SCAR animals who had been exposed to the aggressive males in puberty did not show an effect of session suggesting they did not properly learn the association of the CS and US in terms of producing a well timed CR over sessions $F(7,49)=.67$, $P=.69$. This means in the strictest terms that the SCAR animals did not learn, even when looking at this larger, more generous timeframe of what was counted as a conditioned response.

4. SCAR animals had fewer surviving neurons in the hippocampus after trace conditioning than did the no SCAR animals.

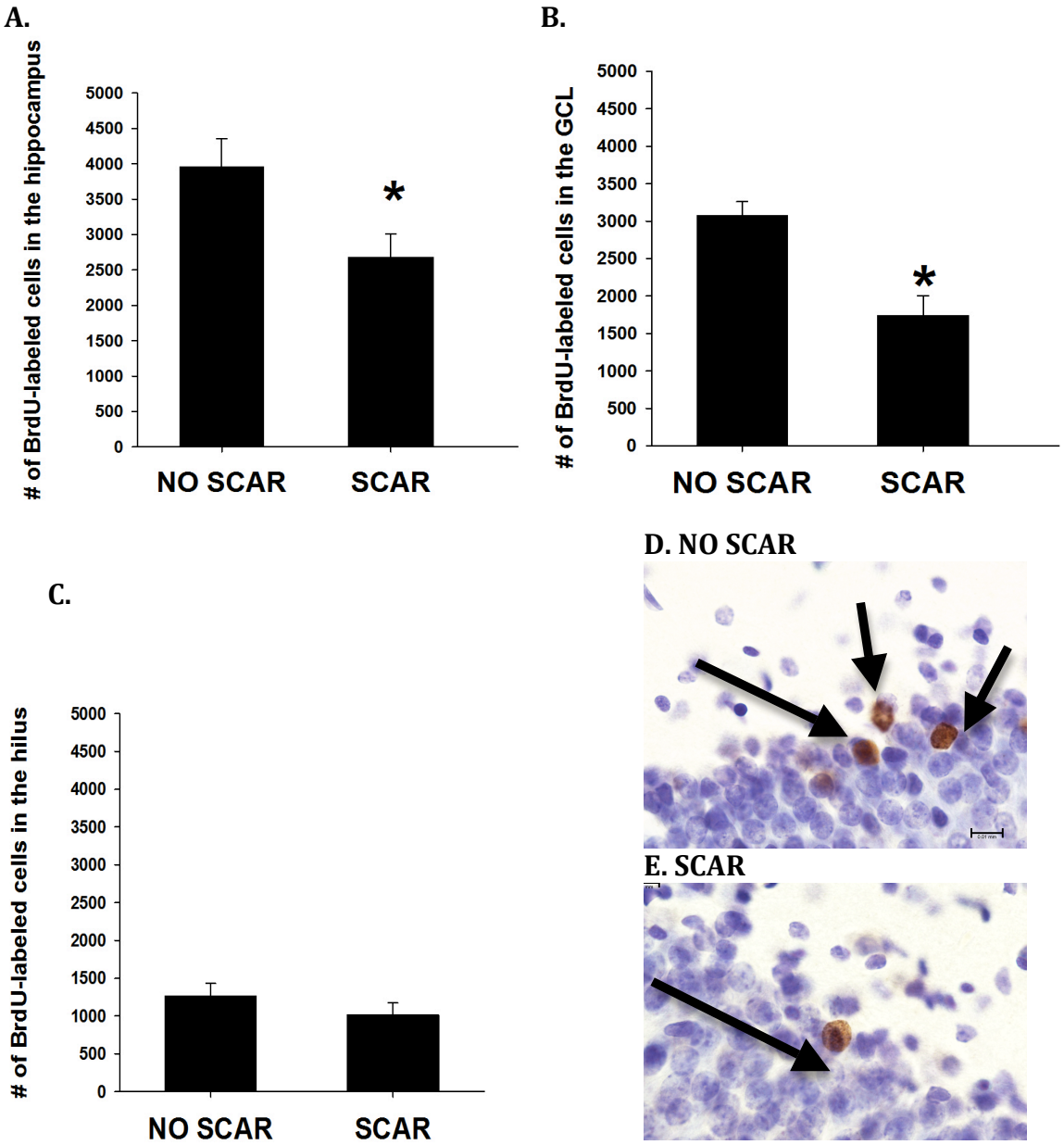


Figure 12. Bar graphs comparing the mean +/- SEM of BrdU labeled cells in the (A) entire hippocampus, (B) the GCL and (C) the hilus. Significantly fewer neurons were estimated in animals that had endured the SCAR experience when analyzing both the entire hippocampus (p= .025) and the GCL (p= .008) but not when analyzing the hilus (p= .287). Representative photomicrographs of dentate gyrus of the hippocampus taken from a no SCAR (D) and a SCAR (E) animal. Black arrows point to BrdU positive cells.

Three independent T tests were conducted to compare the numbers of BrdU labeled cells between animals in the SCAR (N=9) and no SCAR (N=8) conditions. Successful learning of trace conditioning results in an increase in the number of surviving cells in the hippocampus, specifically in the granule cell layer (GCL). We found significant differences in the total numbers of BrdU positive cells estimated in the hippocampus between the SCAR (M=2674.67, SD=991.01) and no SCAR (M=3954, SD=1121.95) conditions; T(15) = -2.50, p=.025. There were also significantly fewer BrdU labeled cells estimated in the GCL of animals in the SCAR (M=1664, SD=542.93) condition versus the no SCAR (M=2688, SD=834.94) condition; T(15) =-3.03, p=.008. We did not detect statistically significant difference between SCAR (M=1010.67, SD=486.177) and no SCAR (M=1266, SD=463.917) conditions in terms of the estimated number of BrdU labeled cells in the hilus; T(15) =-1.10, p=.287.

This evidence suggests that the no SCAR condition animals were rescuing significantly more cells in the weeks following BrdU injections. This hypothesis is bolstered by the fact that we detected the greatest difference between groups in the region most effected by learning enhanced cell survival (the GCL) and did not see a difference in the area typically unaffected by such enhancement (the hilus). This effect was robust, as the total number of surviving cells estimated in the GCL of the no SCAR animals was almost twice that of the SCAR animals.

V. DISCUSSION

5A. Establishing the SCAR model.

Sexual abuse is a common stressful event that occurs more often in women than in men. Some CDC reports estimate that worldwide, 1 in 4 girls are sexually abused in some way before the age of 18 (Andrews et al., 2006). The true number of incidents, however, is thought to be even higher because many of the cases of sexual abuse are not reported for fear of shame, embarassment or other repercussions. The statistics on unreported abuse are even worse for adolescents, who often don't report abuse especially if thought of as culturally and socially acceptable or will bring shame to the family. For example, a 2007 national survey in Swaziland carried out by UNICEF, the CDC and a Swaziland action group found that 1/3 of females under the age of 18 responded to a

survey that they had been the victims of sexual violence during their youth (UN Secretary General's study, 2006).

While much attention has been paid to the general issue of childhood sexual abuse (CSA), which encompasses all abuse under the age of consent (18), significantly less research has focused on abuse that occurs during the specific window of puberty. We know that it is during this time that females transition from responding to stress with an enhancement in learning (similar to males) to a deficit (similar to adult females) (Hodes & Shors, 2001) so we suggest it is a key window that merits much further investigation. It is for these reasons that we developed the SCAR model and used it exclusively in pubescent animals to investigate how this new model of stress in puberty would affect associative learning and adult hippocampal neurogenesis later in life.

In this study, we hypothesized that the SCAR experience in puberty would have significant negative effects on ability to learn an associative learning task and subsequent adult hippocampal neurogensis. These hypotheses were confirmed by both the SCAR animals' inability to properly acquire the association during trace conditioning and by the dramatic difference in number of surviving cells in the hippocampus between the SCAR and no SCAR groups. Our hypothesis that the animals that had endured the SCAR experience would be more prone to sensitized responses was also confirmed, looking at both the 80 ms reflexive response time window and the longer time window including the entire CS. We expected to see some sensitized responses in the first few sessions for the SCAR group but never predicted how long-lasting and salient the differences between groups would be on this measure.

5B. Aggression and Social Stress.

Animal models of social stress and physical subjugation have been used in research for some time. Other studies have examined the effect of a male resident intruder and one very recent study has even looked at a process similar to the SCAR model in both females and males and called the process Juvenile Social Subjugation or JSS (Weathington et al, 2012). These researchers reported that when exposing males and females to aggressive males, females were much more likely to express depression-like and anxiety-like behaviors. They also reported that the females had larger stress induced

cortisol responses and heavier adrenal glands (Weathington et al, 2012) when compared with both the socially subjugated males and the female controls. Obviously, any act of physical domination can be stressful, however the sexual nature of the male's attempts to mount and force intromission in the female (as well as sustained periods of sniffing and licking of genitals) as we saw in the SCAR model reflects a sexualization not seen in the prototypical male-male resident intruder models.

One of the most valuable aspects of this model is that SCAR reflects a relatively normal experience that could occur with animals in their natural habitat. While the resident intruder model has been used for many years as a model of social stress, it has never, to our knowledge, been used in this way to examine this population in terms of age range or gender. SCAR model also differs from a typical resident intruder model in that the female rats are placed into the home cage or territory of the aggressor wherein the adult male aggresses upon a series of females. The timeline of exposures over the course of puberty was vital because puberty is a time that is particularly important in terms of changes in the brain, physiology and behavior (Veenit et al., 2013). This transition to adulthood is critical in the formation of the mPFC, hippocampus, amygdada and other structures. It is also a time during which the regulation of the hypothalamic-pituitary-adrenocortical (HPA axis) undergoes significant remodeling and functional changes (Spear, 2000; Suzuki et al. 2005). Previous animal studies have demonstrated that administration of the stress hormone cortisol during this sensitive period can have profound effects on novelty seeking, aggression, and social-exploration in adulthood. (Veenit et al, 2013).

Our data suggests that the animals did, in fact, find the SCAR experience stressful as it interfered with their later ability to learn and to then save newly developed neurons. The effects of the experience in puberty also manifested themselves in adulthood in the form of hyperarousal as demonstrated by sensitized responses. Future studies using SCAR will certainly examine corticosterone responses as well as adrenal gland weight and anxiety-like and depression-like behaviors to further demonstrate the validity of the stressor.

5C. Sensitization.

One of the hallmarks of juvenile sexual abuse in adulthood (and other forms of PTSD) is a high score on the sensitization measure of Acoustic Startle Response (ASR) (Isserlin, 2008, Morgan et al, 1997). The ASR is "characterized by an integrative, reflex contraction of the skeletal musculature in response to a sudden intense stimulus" (Jovanovic et al., 2009). It is mediated by a relatively simple and primitive limbic response that follows a pathway through in the ponto-medullary brainstem. It is therefore a sympathetic nervous system response and is thus below the level of conscious awareness or volition (Koch, 1999.) The response is mediated by the cochlear root nucleus' transmission to the caudal pontine reticular nucleus then directly to the motor neurons that carry out the response (Koch, 1999). Startle is thought to have evolutionary advantage, priming the animal to respond when faced with a sudden intense stimulus. The frequency and intensity of the startle response is very responsive to external factors, meaning it can be increased or decreased due to a host of variables, one of which is early life stress (Jovanovic et al., 2009) For these reasons, we quantified both the blinks that were entirely during the first 80ms and the blinks during the entirety of the white noise tone CS or what we call "alpha" responses after remarking that we observed a disproportionately large number of blinks during this time in animals that had been subjected to SCAR in puberty.

Alpha conditioning (training to blink to the CS) has historically been described in literature (Gormezano & Moore, 1969; Kandel & Spencer, 1968) as distinctly separate from "Beta" conditioning (classical conditioning), operating on fundamentally different brain mechanisms (Skelton et al., 1988). This distinction is because of the difference in both the necessary and sufficient brain mechanisms to form the associations as well as the fact that during alpha conditioning, the response is an intensification of the preexisting response (essentially a startle response) whereas classical or "beta" conditioning requires a newly learned and precisely timed CR in response to the CS and is more difficult to acquire. In short, this phenomenon of parsing apart alpha and beta responses is eloquently described in 1988 by Dr. Ronald Skelton when he writes that one of his concerns using alpha conditioning as a measure of learning is that "After all, alpha conditioning could be accomplished simply by enhancing the prepotent alpha response pathway, whereas

classical conditioning presumably requires a new pathway for the CR" (Skelton et al, 1988). The data suggests that our SCAR experience during puberty enhanced the animal's prepotent startle or sensitized responses to the CS.

In the present study, we specifically analyzed the 80ms window of blink responses during the CS, which would have been too quick for a deliberate or voluntary muscle response to begin. The SCAR-exposed animals displayed a significantly increased number of sensitized blinks across the 4 days of sessions. The phenomenon did not extinguish over time as one might expect from a normal, healthy population. This is corroborated in human literature which suggests that the auditory startle response in victims of PTSD seems to be one of the longest lasting and most difficult to extinguish physiological manifestations of the disease (Jovanovic, 2009.) Additionally, Many studies suggest that both traumatized animals and humans display enhanced fear recall and emotional arousal when recovering from chronic stressors (McGuire, 2010; Sax & Strakowski 2001, Strakowski et al, 1996).

Previous human research suggests that early childhood abuse can have profound impacts on the development of the limbic system (Teicher et al, 1993; Elzinga et al, 2010) and thus the development of the HPA axis regulated "fight or flight" response. As previously noted, the Acoustic Startle Response (ASR) is both an indicator of (Isserlin, 2008; Morgan et al, 1997) and perhaps even a predictor for (Shalev, 2001; Griffen, 2008; Pole et al, 2009) PTSD. Our findings are in line with human research that suggests that women who had experienced sexual or physical abuse in childhood had larger pituitary-adrenal and autonomic responses to stress than women who had not been abused as children (Heim et al, 2000). Exaggerated startle responses are a cardinal symptom of PTSD and are included in the DSM IV as a primary symptom of the disorder as well as an indicator of vulnerability to the disease in prospective studies from adults who have experienced trauma (DSM IV). Recent work with Veterans has also pointed to sensitization as an important characteristic of PTSD (Anisman, 2011; Burriss et al, 2007). Our SCAR model also corroborates human studies in which combat veterans suffering from PTSD show increased responsivity to the conditioned and the unconditioned stimuli (Buriss et al, 2007; Meyers et al, 2012) during trace eyeblink conditioning.

5D. Associative Learning and Neurogenesis.

Because puberty is a particularly sensitive time for stressors in terms of sex specific effects on learning (Wood & Shors, 1998; Hodes & Shors, 2005) we examined the SCAR and no SCAR animal's ability to properly emit a conditioned response following a white noise tone in anticipation of a periorbital stimlulation. As previously described, we know that females and males respond differently to stress in terms of learning (Beck & Servatius, 2003, Hodes & Shors, 2005). Additionally, several studies suggest that sexual abuse in childhood can have profound effects on learning and reward salience in adulthood (Pechtel et al, 2010) in humans. For these reasons, as well as the hippocampus' vulnerability to stress effects (Kim et al, 1996; Shors 2006) we chose to focus on the hippocampally dependent task of trace eyeblink conditioning. Trace conditioning is thought to be somewhat analogous to declarative learning in humans (Clark & Squire 2001). While other studies have examined the effects of juvenile social subjugation and social stress on mood disorder like symptoms, we focused here on the cognitive deficits from such an experience.

Females that were exposed to the aggressive adult male during puberty emitted more CRs during adulthood. They also emitted significantly more sensitized and alpha responses. Therefore the high numbers of CRs in the SCAR group was likely due to the increased responses to the CS alone. Learning of the eyeblink task is defined as the progressive increase in correct responses over time, which the SCAR animals do not display. That is to say, that the animal is in fact blinking, but it is not doing so in a well timed manner in anticipation of the impending US, which is how we define a conditioned response. Along theses lines, it is fair to say that the animals did emit a response but not a conditioned response.

As previously described, successful learning of the trace conditioning paradigm rescues new neurons from death in the hippocampus (Cameron & McKay, 2001, Gould et al, 1999). Others have reported that this pertains only to neurons born during a particular period of time (Sisti et al, 2007, Anderson et al. 2011) provided the learning is both effortful and successful (Curlik & Shors, 2007). Unfortunately we do not yet know with certainty if the animals exposed to SCAR had fewer surviving new hippocampal neurons as a result of the animal's inability to learn the trace task or if they were perhaps

producing fewer neurons. Our data strongly suggest that the difference between groups in terms of number of BrdU-labeled cells was a result of enhanced cell survival in the no SCAR condition after successful learning. This is theory is supported by both the fact that the SCAR animals did not learn (and thus would not have rescued cells) as well as the fact that the greatest disparity between groups was found in the GCL, the area most affected by learning derived enhancement of cell survival. However, further investigation needs to be done to confirm these findings. An additional study wherein the SCAR and no SCAR groups are sacrificed at the same time point as these animals but do not undergo trace conditioning is necessary to obtain a baseline measure of neurogenesis.

5E. CONCLUSIONS

The effect of SCAR exposures during puberty on associative learning and neurogenesis is robust, pronounced and long lasting. In fact, we saw less variability within groups than we had previously anticipated due to expected resiliency in the SCAR group and uncontrolled stress in the no SCAR group. We predict that this model will allow for further understanding into an insidious and widespread phenomenon that affects many young girls and women around the world by isolating the brain regions and behaviors most impacted by trauma similar to SCAR. With more insight into the behavioral and neurodevelopmental processes underlying SCAR, as well as to how these processes manifest themselves in both the developing and adult female, we can someday help to understand how to tailor treatments that are gender and trauma specific in humans. In doing so, we wish to advance understanding of this unfortunately widespread phenomenon and shed some light on the hormonal, neurological and behavioral changes in adult female survivors of juvenile sexual abuse.

VI. GRANTS

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