DIFFERENTIAL INVOLVEMENT OF AMYGDALAR NMDA RECEPTORS IN VARIANTS OF ADOLESCENT CONTEXTUAL FEAR CONDITIONING

by

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ABSTRACT

The context preexposure facilitation effect (CPFE) is a variant of contextual fear conditioning that consists of three distinct phases in which learning the context (preexposure), the context-shock association (training), and retrieval of the context-shock association (testing) are separated by 24 hours. In contrast, in standard contextual fear conditioning (sCFC), learning of the context and formation of the context-shock association occur in the same training session. In adult rats, disrupting basolateral amygdala (BLA) activity or plasticity during training on sCFC impairs both post-shock and retention freezing (Maren et al., 1996). This manipulation on the training day of the CPFE disrupts retention freezing but effects on post-shock freezing are unknown (Matus-Amat et. al., 2007). Experiment 1 extends this literature from adult to adolescent rats and to the role of BLA in post-shock freezing during the CPFE. Experiment 2 directly compares the role of plasticity within the BLA on training day of sCFC and CPFE. Experiment 3 examines the role of BLA plasticity on retention of contextual fear when acquisition is not tested. In the first experiment, intra-BLA infusions of muscimol prior to the training day of the CPFE disrupted both post-shock and retention freezing in postnatal day (PD) 31-33 rats. In the second two experiments, intra-BLA infusions of APV prior to training day of sCFC disrupts retention but not post-shock freezing, while infusions of APV prior to training of the CPFE disrupt both post-shock and retention freezing. Our findings suggest that the BLA plays a different role in the CPFE vs. sCFC. Its role in the CPFE is similar in both adolescent and adult rats, while the role of the BLA in post-shock freezing during

sCFC may differ across age or across studies that employ different procedures or parameters.

Chapter 1

INTRODUCTION

Contextual fear conditioning is a useful paradigm for studying the neurobiology of learning and memory. There are two contextual fear conditioning paradigms: standard contextual fear conditioning (sCFC) and the context preexposure facilitation effect (CPFE). Standard contextual fear conditioning involves a 2-min exposure to a context followed by a brief foot-shock. Fear can then be measured immediately following the shock in a post-shock test or assessed 24 hours later in a retention freezing test. This paradigm requires learning of the context and forming a context-shock association within one trial, limiting researchers in their ability to distinguish between these individual components. The other fear conditioning paradigm, CPFE, overcomes this by separating the learning into three phases: preexposure, training, and testing. The preexposure day involves learning the context, the training day involves association of the retrieved context representation with immediate shock, and testing day involves the retrieval of this context-shock association.

Previous studies have shown the hippocampus to be involved in all three phases of the CPFE whereas the basolateral amygdala (BLA) plasticity on the training day of the CPFE is necessary for 24-hour retention (Matus-Amat et. al., 2004; Matus-

Amat et. al., 2007). One theory of contextual fear conditioning proposed by Maren and Fanselow (1996) holds that information about the context is sent from the hippocampus to the amygdala where it converges with information about the shock where the context-shock association is formed (Figure 1). This context-shock association is then sent to the periaqueductal gray (PAG) which generates the freezing response. This fear conditioning model has been widely accepted in the literature (Kim & Jung, 2006; Fanselow & LeDoux, 1999) and offers a framework for understanding both sCFC and the CPFE. Both phenomena involve hippocampal encoding of context, which subsequently serves as a conditioned stimulus for shock. However, in sCFC, the context is actively processed when shock is presented whereas, in the CPFE, the context representation is first learned incidentally (without reinforcement) and consolidated over 24 hr before it is retrieved and associated with immediate shock on the training day. These differences have led to the proposal that sCFC can be learned based on either "elemental" or "configural" context representations whereas the CPFE requires configural context learning (Rudy, 2009). As a result, the CPFE always depends on hippocampus whereas sCFC can be learned without hippocampus based on "elemental" context representations (Rudy, 2009). The question of whether BLA plays a similar role in these two variants of contextual fear conditioning has received little attention. Few, if any, studies directly

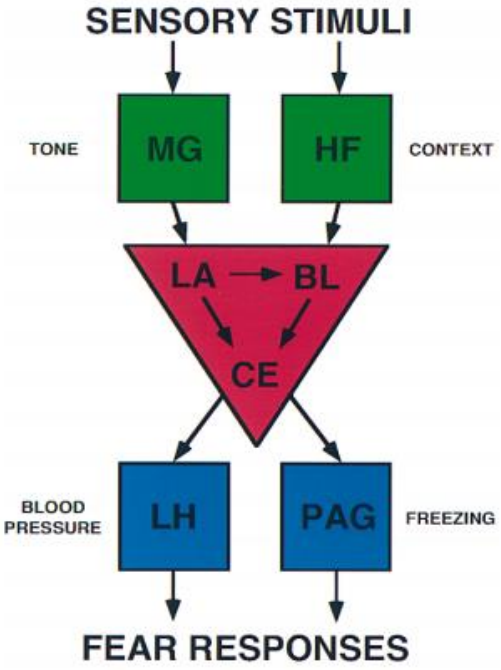

Figure 1 Schematic of the fear circuit adapted from Maren and Fanselow, 1996. Information about the context is sent from the hippocampus to the amygdala where it is associated with the shock. This context-shock association is then sent the PAG which produces the freezing response.

compare the role of BLA in these two variants in the same experiment (or under similar conditions across experiments). In addition, only a few studies of sCFC and virtually no studies of the CPFE have used post-shock freezing to assess the role of BLA in acquisition of contextual fear. It is more common to use 24-hr retention as a measure of "acquisition." The present study is the first to contrast post-shock freezing with 24-hr retention to assess how BLA manipulations affect acquisition of context fear during the CPFE vs. sCFC. Finally, current views of the neurobiology of

contextual fear conditioning are based on studies of adult rats. The present study extends this literature to adolescent rats (aged PD31-33).

The current set of experiments attempt to further explore the role of the BLA in adolescent contextual fear conditioning. Experiment 1 examines whether the BLA is involved in the training day of the CPFE by infusing the $GABA_A$ agonist muscimol into the BLA prior to training of the CPFE. Experiment 2 sought to examine the role of plasticity within the BLA on the training day of contextual fear conditioning by infusing NMDA receptor antagonist APV into the BLA prior to training day of sCFC and the CPFE. Experiment 3 sought to confirm the findings from experiment 2 by removing the post-shock freezing measure and examining retention freezing without potential prior influences from the post-shock freezing test. Together, Experiments 2 and 3 offered a within-subjects and between-subjects comparison of post-shock vs. retention test freezing.

Chapter 2

EXPERIMENT 1: INVOLVEMENT OF THE BLA IN ADOLESCENT CONTEXTUAL FEAR CONDITIONING

Introduction

Experiment 1 sought to examine the overall role the BLA plays in adolescent contextual fear conditioning by administering the $GABA_A$ agonist, Muscimol, prior to the training day of the CPFE.

Materials and Methods

Subjects

Animal husbandry was as described in our previous reports (Heroux, Robinson-Drummer, Rosen, & Stanton, 2016; Robinson-Drummer, Dokovna, Heroux, & Stanton, 2016). There were a total of 40 adolescent Long Evans rats (21 females and 19 males) derived from 9 separate litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. To achieve time-mating, females were housed with breeder males overnight and, if an ejaculatory plug was found the following morning that day was designated as gestational day (GD) 0. Dams were housed in clear polypropylene cages measuring 45 cm × 24 cm × 21 cm with standard bedding and access to *ad libitum* water and rat chow. Animals were maintained on a 12:12 h light/dark cycle with lights on at 7:00 am. Date of birth was designated as PD 0. Litters were culled on PD3 to eight pups (usually 4 males and 4 females) and were paw-marked with subcutaneous injections of non-toxic black ink for later identification. Pups were weaned from their mother on PD21 and housed with same-

sex litter mates (4 animals per cage) in 45 cm × 24 cm × 17 cm cages. On PD29 animals were individually housed after stereotaxic surgery in small white polypropylene cages (24 cm × 18 cm × 13 cm) with ad libitum access to water and rat chow for the remainder of the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

Stereotaxic Surgery

Surgery was a modification of surgical implantation of intracranial injection cannulas in juvenile and adolescent rats that has been previously described for intra-hippocampal and intra-medial prefrontal cortex (mPFC) cannula in our lab (Robinson-Drummer et al., 2016; Schiffino, Murawski, Rosen, & Stanton, 2011; Jablonski, Watson, & Stanton, 2010). Rats were obtained from post-weaning group housing on the morning of PD29 and anesthetized with a primary injection volume of 1 mg/kg of an 85:15 ketamine/xylazine drug mixture prior to surgery, with small supplemental doses given as needed. Guide cannulas (Plastics One, Roanoke, VA) were bilaterally implanted to terminate in the BLA using the following coordinates: anteroposterior (AP) +3.6 mm, mediolateral (ML), ±5.3 mm relative to interaural midline, and dorsoventral (DV), -7.0 mm relative to the top of the skull. Cannula were fixed in place on the skull using dental acrylic and curved "skull hooks" as previously reported (Robinson-Drummer et al., 2016; Schiffino et al., 2011). Following surgery, a dummy

injector extending the same length as the drug injector tips and dust caps were inserted in the guide cannula to reduce occlusion of the guide cannula. Rats were allowed to recover in individual clear cages with electric heating pads placed under half of the cage floor. Twenty-four hours following surgery (PD30), animals were infused with 0.25 µl of the vehicle phosphate buffered saline (PBS) in both hemispheres to reduce occlusion in the guide cannula and to acclimate the animals to being handled during infusions before the start of behavioral procedures the following day (PD31).

Drug Infusion

Infusions were as described in previous reports (e.g., Heroux et al., 2016) with modifications in the infusion site and rate (see below). Microinjections of the vehicle PBS or the $GABA_A$ receptor agonist muscimol (Sigma-Aldrich, St. Louis, MO) were administered approximately 15 minutes prior to behavioral procedures on PD32. Animals were gently held by hand while PBS or muscimol (2 µg/µl dissolved in PBS) was infused into both hemispheres at a rate of 0.125 µl per minute for two minutes, resulting in a final infusion volume of 0.25 µl and a final dose of 0.5 µg per side for each animal. This dose was chosen because of its efficacy to disrupt BLA functioning prior to training day of sCFC (Helmstetter & Bellgowan, 1994; Huff et. al, 2018) as well as its ability to disrupt hippocampal functioning when given prior to any phase of the CPFE (Matus-Amat et al., 2004). Injector tips were left in the guide cannula for one-minute following infusion to allow sufficient diffusion of the drug. Animals were

returned to their home-cage for approximately 15 minutes until the start of behavioral testing.

Apparatus and Stimuli

The apparatus and stimuli used have been previously described (Heroux et al., 2016; Murawski & Stanton, 2010; Robinson-Drummer et al., 2016). Fear conditioning occurred in four Plexiglas chambers measuring 16.5 cm × 12.1 cm × 21.6 cm which were arranged in a 2 × 2 formation on a Plexiglas stand within a fume hood to provide ambient light and background noise (Context A). Each chamber had a grid floor made of 9 stainless steel bars (11.5 cm from the top of the chamber), 0.5 cm in diameter and spaced 1.25 cm apart. The alternate context (Context B) consisted of the same Plexiglas chambers with a convex wire mesh insert that covered the back wall and floor of the chamber and a white paper sleeve that covered the outside walls of the chamber. The 2s 1.5 mA footshock unconditioned stimulus (US) was delivered using a shock scrambler (Med Associates, Georgia, VT ENV-414S) connected to the grid floor of the chamber. The fear chambers were cleaned with 5% ammonium hydroxide solution prior to each load of experimental animals. Videos of each session (preexposure, training, testing) were recorded using Freeze Frame 3.0 software (Actimetrics, Wilmette IL) with freezing defined as a bout of 0.75 s or longer without a change in video pixilation.

Context Preexposure Facilitation Effect

The multiple preexposure CPFE behavioral procedure has been described previously (Dokovna, Jablonski, & Stanton, 2013; Heroux et al., 2016; Robinson-Drummer et al., 2016, Figure 2). The CPFE procedure took place over the course of

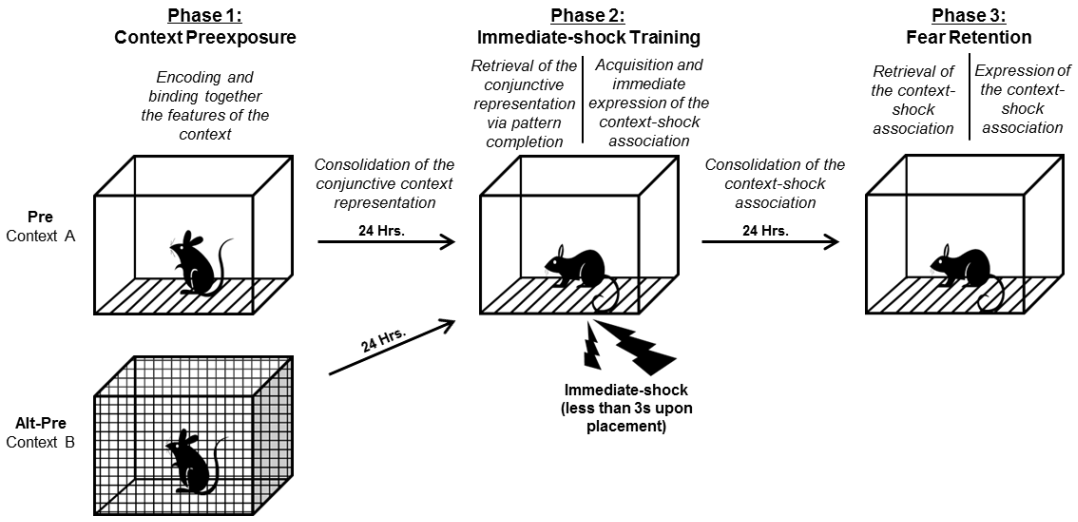

Figure 2 A schematic illustration of the CPFE across all three phases (preexposure, training, and testing). Context preexposure involves learning of the context and forming a conjunctive context representation. Immediate shock training involves retrieval of the context representation, formation of a context-shock association, and expression of the context-shock association (experiments 1 and 2 only). Fear retention involves retrieval and expression of the context-shock association.

three days from PD31 to PD33 (±1 day). Animals were assigned to either preexposure (Pre condition) or alternate preexposure (Alt Pre condition). Animals in the preexposure group were preexposed to the training context (Context A), and animals in the Alt Pre group were preexposed to the alternate context (Context B, as described

by Murawski & Stanton, 2010). Animals preexposed to an alternate context (Context B) on the first day of the CPFE serve as non-associative behavioral controls as they fail to acquire a context representation needed to be retrieved and associated with shock on the training day (Rudy, 2009). Multiple preexposure consisted of one initial 5 min exposure to the chamber, followed by five 1 min exposures, with a 1 min interval between exposures. Animals were placed in transport boxes on a cart inside the training room during the 1 min inter-trial interval.

On PD31, animals were weighed and carted to the behavioral testing room in transport cages of clear Lexan (11 cm × 11 cm × 18 cm) covered on all sides with orange construction paper to obscure visual cues during transport. Pre animals were placed in Context A for the multiple preexposure, whereas animals in the Alt Pre group underwent multiple preexposure in the alternate context (Context B). On PD32, rats were carried in pairs of two into the testing room in their transport cages, placed in their respective training chamber, and given two immediate 1.5 mA 2s footshocks separated by 1s in Context A. Animals remained in the chambers for a 3-minute post-shock freezing test immediately following the footshocks then returned to their transport cages and were taken back to their home-cages. On PD33, animals were tested in Context A for 5-mintues. Testing consisted of a 5 min exposure to the chamber with no additional exposure to the unconditioned stimulus. Drug infusions of PBS or muscimol took place 15 min before immediate shock training on PD32.

Histology

Within 24-48 hours of behavioral testing, rats were sacrificed by rapid decapitation. Brains were removed and frozen in -45 °C isopentane and then stored at -80 °C until being sectioned on a cryostat. Coronal sections of approximately 40 μm were taken throughout the entirety of the cannula tracts visible in the brain tissue. The 40 μm coronal slices were mounted on charged microscope slides and stained with Neutral Red (1%). Slides were photo-captured and analyzed to confirm the placement of the cannula injector tip in the BLA. Out of 40 surgeries (see Figure 3A for placements), there were a total of 6 misplaced cannula with the distribution as follows: Alt Pre (1), Pre-Musc (3), and Pre-PBS (2).

Data Analysis

Data processing procedures have been described previously (Heroux et al., 2016; Robinson-Drummer et al., 2016). A human observer blind to the experimental groups verified the freezing threshold setting with Freeze View 3.0 (Actimetrics, Wilmette IL). The software program computes a "motion index" that was adjusted to set a freezing threshold separately for each animal (per software instructions) by a blind observer who verified from the video record whether or not small movements were scored as freezing. Once set, the threshold did not change during a session. We have validated this procedure against other scoring methods (e.g., hand scoring of video records by two blind observers). Freezing behavior was scored as the total

percent time spent freezing (defined as the cessation of all movement except breathing) in each respective session bin (context exposure, pre-shock freezing, post-shock freezing, and a 24hr retention test).

Once percent freezing was reliably determined, the data were imported into STATISTICA 64 data analysis software and freezing behavior was analyzed with a series of ANOVAs. Statistical significance was set to $p < .05$. A "Pooled Alt-Pre" condition was used as reported previously (Heroux et al., 2016; Robinson-Drummer et al., 2016). Data from animals in the non-associative alternate preexposure group were collapsed across drug condition as freezing was uniformly low and there were no significant differences between control animals given either drug (*p*s > .05). This reduces animal use and simplifies the experimental design. There were also no main effects or interactions involving sex across any of the experiments (*p*s > .05), so the data were collapsed across this variable.

Freezing behavior was analyzed with a Drug (*PBS, Muscimol, Pooled-Alt-Pre*) × Phase (*Post-shock, Retention*) repeated measures ANOVA with Phase being the repeated measure. Post-hoc contrasts were performed with planned comparison tests. A rat was excluded from analysis as an outlier if it had a score ±1.96 standard deviations from its group mean. The outliers were distributed as follows: Alt-Pre Post-shock (1), Pre-MUSC Post-shock (1), Pre-MUSC Retention (1), Pre-PBS Post-shock (1), and Pre-PBS Retention (1).

Results

Behavioral analysis was conducted on the remaining 34 animals distributed as follows: Pooled Alt-Pre (N=13), Pre-Muscimol (N=10), and Pre-PBS (N=11). Figure 3 depicts cannula placements (Panel A) and freezing behavior (Panel B). The repeated measures ANOVAs indicated no main effect or interaction of Sex (*p*s > .119) so the data were collapsed across this variable and analyzed via a 3 (Drug; *Pooled Alt. Pre vs. Pre-PBS vs. Pre-Muscimol*) x 2 (Phase; *Post-shock vs. Retention*) repeated measures ANOVA. The ANOVA revealed a main effect of Drug [$F(2,26) = 14.926$, $p = 0.000048$] and a significant Phase x Drug interaction [$F(2,26) = 4.907$, $p = 0.016$]. There was a significant disruption in freezing behavior of the Muscimol group when compared to the PBS group on both post-shock ($p = 0.00015$) and retention ($p = 0.014$) freezing tests. The PBS group also showed a significant difference from the pooled-alt-pre group on both post-shock ($p = 0.00017$) and retention ($p = 0.014$) freezing tests while the Muscimol groups did not (*p*s > 0.66). Freezing declined somewhat across the post-shock and retention freezing tests within the PBS group ($p = 0.0094$). Overall, these results indicate that inactivation of the BLA prior to training day of the CPFE disrupts both the acquisition (post-shock freezing) and retention of contextual fear.

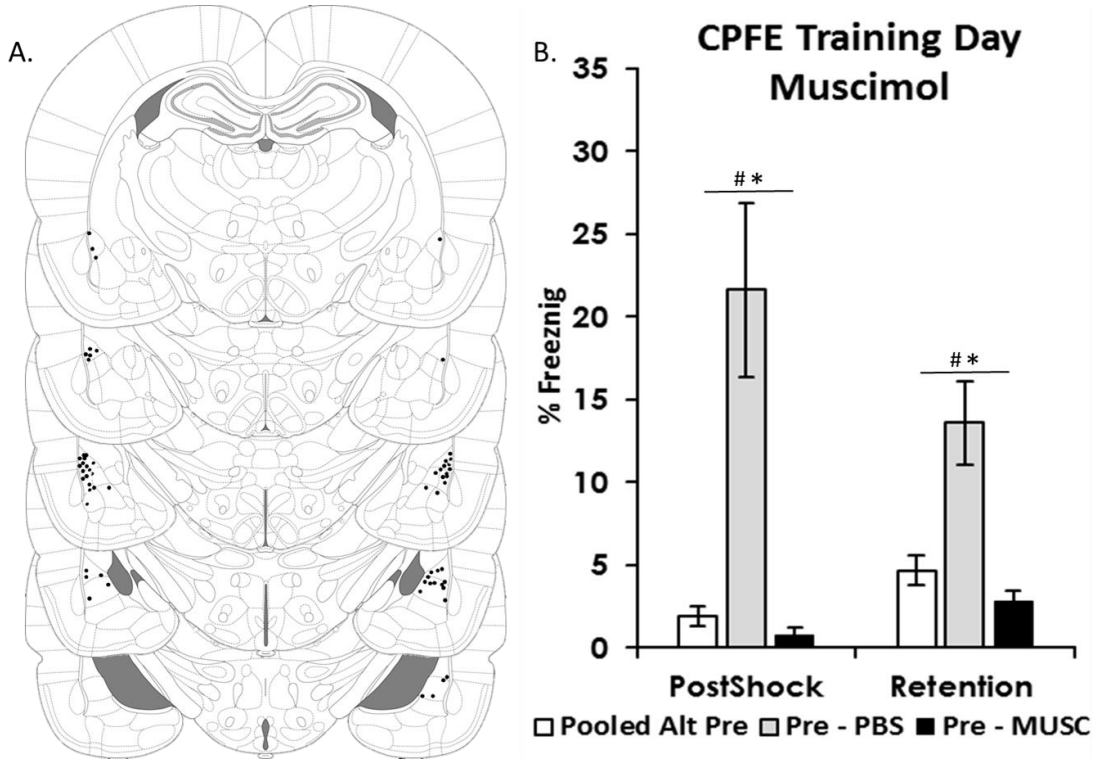

Figure 3 (A) A diagram of the histological analysis confirming cannula placement into the basolateral amygdala. The slices represent -2.40mm, -2.76mm, -3.12mm, -3.48, and -3.72mm from bregma (from top to bottom). A "hit" was classified by the center of the guide cannula tract being in between Plate 50 (Bregma -2.04mm) to Plate 65 (Bregma -3.84mm) of Paxinos & Watson (2007). Cannula misses are not shown. (B) A graphical representation of the Phase by Condition interaction seen in percentage freezing. A significant different ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group is represented with a *. A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-MUSC group is represented with a #.

Chapter 3

EXPERIMENT 2: INVOLVMENT OF BLA NMDA RECEPTORS IN ADOLESCENT CONTEXTUAL FEAR CONDITIONING

Introduction

This experiment sought to further explore the role of the BLA by examining the specific role NMDA receptor plasticity plays on the acquisition and retention of contextual fear during the CPFE. It also asked whether the BLA NMDA receptors are playing a similar role in different variants of contextual fear conditioning by directly comparing the CPFE with sCFC.

Materials and Methods

Subjects

Animal husbandry was as described in Experiment 1. There were a total of 149 adolescent Long Evans rats (75 females and 74 males) derived from 24 separate litters bred at the Office of Laboratory Animal Medicine at the University of Delaware.

Stereotaxic Surgery

Surgical protocols were the same as in Experiment 1.

Drug Infusions

Microinjections of the vehicle PBS or the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (APV) (Tocris, Ellisville, MO) were administered immediately prior to behavioral procedures on PD31 (sCFC) or PD32 (CPFE). Animals were gently held by hand while PBS or APV (5 μg/μl dissolved in PBS) was infused into both hemispheres at a rate of 0.125 μl per minute for two minutes,

resulting in a final infusion volume of 0.25 μl and a final dose of 1.25 μg per side for each animal. This dose was chosen in part because it has been shown to be effective in disrupting fear conditioning previously (Miserendino et. al., 1990). Injector tips were left in the guide cannula for one minute following infusion to allow sufficient diffusion of the drug. Animals were returned to their home-cage for approximately 2 minutes until the start of behavioral testing.

Apparatus and Stimuli

The apparatus and stimuli used were the same as described in Experiment 1.

Context Preexposure Facilitation Effect

The multiple preexposure CPFE behavioral procedure was the same as described in Experiment 1. Drug infusions of PBS or APV took place immediately before immediate shock training on PD32.

Standard Contextual Fear Conditioning

The sCFC procedure has been described previously (Heroux et al., 2017; Schreiber et al., 2014). The sCFC procedure took place over the course of two days from PD31 to PD32 (±1 day). All chambers, stimuli, and drug infusion protocols used were identical to the ones used in Context A for the CPFE experiments (see *Apparatus and stimuli* and *Drug infusion*). On PD31, animals were assigned to one of two behavior conditions: Delayed Shock or Immediate Shock (Imm. Shock). Animals in the Delayed Shock condition received three minutes of context exposure in Context A, followed by two 1.5 mA 2s footshocks separated by 1s. Subsequently, animals

received 3 additional minutes of exposure to the chamber with no additional shock presentations (post-shock freezing test). Animals in the Imm. Shock condition were given two immediate foot-shocks then remained in the context for 3 minutes for a post-shock freezing test. This groups served as behavioral controls for the delayed-shock condition as the placement-to-shock interval was under 5 sec resulting in the immediate shock deficit (i.e. an inability to form a context-shock association due to insufficient time to process the context; Fanselow, 1990). On PD32, rats were tested in Context A for 5 min in the same chamber they had been trained in with no additional presentations of the unconditioned stimulus.

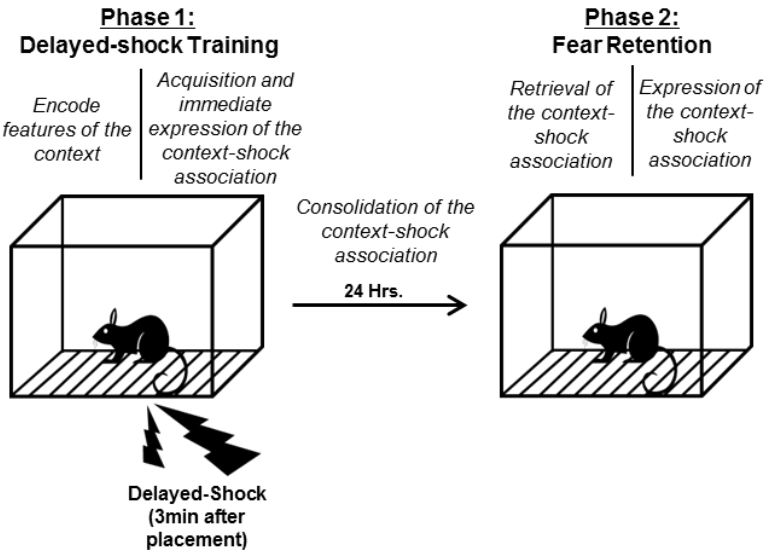

Figure 4 Schematic of standard contextual fear conditioning. Delayed-shock training involves learning the context and forming a context-shock association. Fear retention involves retrieving the context-shock association and expression of a freeze response as measured by freezing. Not shown is immediate-shock training in which animals are unable to form context-shock association due to no prior exposure to the context.

Histology

Protocols were the same as in Experiment 1. Out of 149 surgeries (see Figure 5A for placements), there were a total of 44 misplaced cannula with the distribution as follows: CPFE-Alt-Pre (10), CPFE-Pre-APV (11), CPFE-Pre-PBS (8), sCFC-Delayed-APV (5), sCFC-Delayed-PBS (5), and sCFC-Imm. Shock (5).

Data Analysis

Data analysis procedures were the same as in Experiment 1. Freezing behavior was analyzed with a Drug (*PBS, APV, Pooled-Alt-Pre*) × Phase (*Post-shock, Retention*) repeated measures ANOVA for the CPFE group and a Drug (*PBS, APV, Pooled-Imm. Shock*) × Phase (*Post-shock, Retention*) repeated measures ANOVA for the sCFC group with Phase being the repeated measure in both. Post-hoc contrasts were performed with planned comparison tests. A rat was excluded from analysis as an outlier if it had a score ±1.96 standard deviations from its group mean. The outliers were distributed as follows: CPFE-Alt-Pre-Post-shock (1), CPFE-Alt-Pre-Retention (1), CPFE-Pre-APV Post-shock (2) Retention (1), CPFE-Pre-PBS Post-shock (2) Retention (2), sCFC-Delayed-APV Post-shock (1) Retention (1), sCFC-Delayed-PBS Post-shock (2) Retention (2), sCFC-Imm-Shock-Post-Shock (2), and sCFC-Imm-shock-Retention (1).

Results

Behavioral analysis was conducted on the remaining 118 animals distributed as follows: CPFE-APV (N=18), CPFE-PBS (N=22), CPFE-Pooled-Alt-Pre (N=16), sCFC-APV (N=19), sCFC-PBS (N=16), and sCFC-Pooled-Imm-Shock (N=11). Figure 5 depicts cannula placements (Panel A) and freezing behavior (Panels B and C). Results from each paradigm (CPFE and sCFC) were analyzed separately in repeated-measures ANOVAs. The sCFC repeated-measures ANOVA revealed no main effect or interaction of sex (ps > 0.217) so data were collapsed across this variable and analyzed via a 3 (Condition; *Delayed-APV vs. Delayed-PBS vs. Pooled Imm-Shock*) x 2 (Phase; *Post-Shock vs. Retention*) repeated measures ANOVA. The sCFC ANOVA revealed a main effect of Condition [$F(2,37) = 7.668$, $p = 0.0016$], a main effect of Phase [$F(1,37) = 16.452$, $p = 0.00025$], and a Phase x Condition interaction [$F(2,37) = 3.430$, $p = 0.043$]. Planned comparisons revealed no difference in Post-Shock freezing levels between the APV and the PBS groups ($p = 0.507$), but did reveal a knock down in APV retention freezing compared to the PBS group [$F(1,37) = 4.253$, $p = 0.0463$]. There was a significant difference between the Pooled Imm. Shock and the APV group [$F(1,37) = 8.749$, $p = 0.0054$] and Pooled Imm. Shock and the PBS group [$F(1,37) = 11.825$, $p = 0.0015$] in Post-Shock freezing. Whereas, there was a significant difference between the Pooled Imm. Shock and the PBS group [$F(1,37) = 5.414$, $p = 0.0256$] in retention freezing, but there was no difference between the Pooled Imm. Shock and the APV group ($p > 0.506$). Overall,

this shows that NMDA receptors disrupt retention but not acquisition of contextual fear in sCFC.

The CPFE repeated measures ANOVA indicated a main effect of sex (p = 0.0356) with the males freezing higher (14.03%) than the females (8.75%), but no interactions of sex (ps > 0.159). Due to the lack of interactions of sex with the variables of interest, along with lack of replication of this sex effect in Experiment 3, the data were collapse across this variable and analyzed via a 3 (Condition; *Pooled Imm. Shock vs. Delayed-PBS vs. Delayed-APV*) x 2 (Phase; *Post-Shock vs. Retention*) repeated measures ANOVA. The CPFE ANOVA revealed a main effect of Condition [$F(2,50) = 12.647$, $p = 0.000036$], a main effect of Phase [$F(1,50) = 7.993$, $p = 0.0067$], but no Condition x Phase interaction ($p = 0.084$). Planned comparisons revealed a decrease in Post-shock [$F(1,50) = 6.316$, $p = 0.0152$] and Retention [$F(1,50) = 7.750$, $p = 0.0076$] freezing in the APV group when compared to the PBS group. They also revealed no differences between the Pooled Alt-Pre and the APV group in either Post-shock ($p > 0.164$) or Retention ($p > 0.255$) freezing. However, the PBS group did significantly differ from the Pooled Alt-Pre group on both Post-shock [$F(1,50) = 15.508$, $p = 0.00026$] and Retention [$F(1,50) = 15.474$, $p = 0.00026$] freezing. Overall, this suggests that NMDA receptors are involved in both the acquisition and retention of contextual fear in the CPFE. Taken together with the results from sCFC, this suggests that NMDA receptors are differentially involved in the acquisition of contextual fear across CPFE and sCFC.

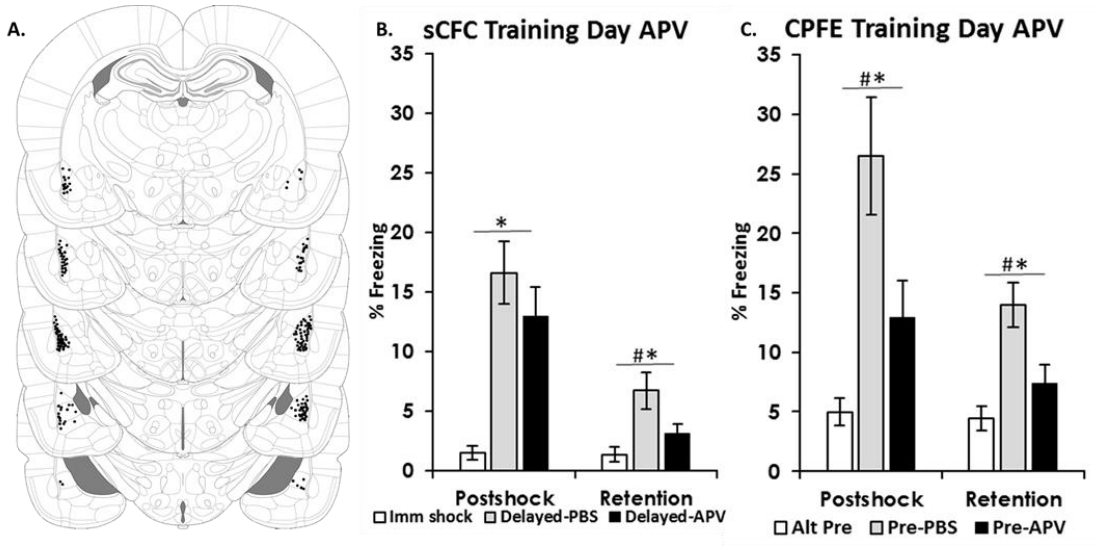

Figure 5 (A) A diagram of the histological analysis confirming cannula placement into the basolateral amygdala. The slices represent -2.40mm, -2.76mm, -3.12mm, -3.48, and -3.72mm from bregma (from top to bottom). A "hit" was classified by the center of the guide cannula tract being in between Plate 50 (Bregma -2.04mm) to Plate 65 (Bregma -3.84mm) of Paxinos & Watson (2007). Cannula misses were not included. (B) A graphical representation of the Phase by Condition interaction seen in freezing percentages during the sCFC procedure. A significant different ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group is represented with a *. A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-MUSC group is represented with a #. (C) A graphical representation of the Phase by Condition interaction seen in freezing percentages during the CPFE procedure. A significant different ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group is represented with a *. A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-MUSC group is represented with a #.

Chapter 4

EXPERIMENT 3: INVOLVEMENT OF BLA NMDA RECEPTORS IN THE RETENTION OF ADOLESCENT CONTEXTUAL FEAR CONDITIONING

Introduction

In the previous experiment, retention-test freezing in the sCFC group was unusually low relative to what is reported in the literature and in previous studies from our lab. In these studies, the post-shock freezing test is typically omitted, raising the possibility that inclusion of this test depresses retention performance. This experiment sought to further explore the results from Experiment 2 by measuring only retention test freezing without post-shock freezing.

Materials and Methods

Subjects

Animal husbandry was as described in Experiment 1. There were a total of 56 adolescent Long Evans rats (27 females and 29 males) derived from 14 separate litters bred at the Office of Laboratory Animal Medicine at the University of Delaware.

Stereotaxic Surgery

Surgical protocols were the same as in Experiment 1.

Drug Infusions

Drug Infusion protocols were the same as Experiment 2.

Apparatus and Stimuli

The apparatus and stimuli used are the same as described in Experiment 1.

Context Preexposure Facilitation Effect

The multiple preexposure CPFE behavioral procedure was the same as described in Experiment 1 except there were no alternate pre-exposed controls. Drug infusions of PBS or APV took place immediately before immediate shock training on PD32.

Standard Contextual Fear Conditioning

The sCFC procedure was the same as Experiment 2 except on training day animals did not receive the 3-minute post-shock test and were instead removed immediately following the shock and returned to their home cages. There were also no immediate shock control animals run in experiment 3.

Histology

Protocols were the same as in Experiment 1. Out of 56 surgeries (see Figure 6A for placements), there were a total of 8 misplaced cannula with the distribution as follows: CPFE-Pre-APV (1), CPFE-Pre-PBS (2), sCFC-Delayed-APV (2), and sCFC-Delayed-PBS (3).

Data Analysis

Data processing procedures were the same as in Experiment 1. Freezing behavior was analyzed with a Drug (*PBS, APV*) × Phase (*Retention*) one-way ANOVA. A rat was excluded from analysis as an outlier if it had a score ±1.96

standard deviations from its group mean. The outliers were distributed as follows: CPFE-APV (2), CPFE-PBS (1), and sCFC-PBS (1).

Results

Behavioral analysis was conducted on the remaining 44 animals distributed as follows: CPFE-APV (N=11), CPFE-PBS (N=11), sCFC-APV (N=12), and sCFC-PBS (N=10). Figure 6 depicts cannula placements (Panel A) and freezing behavior (Panels B and C). Results from each paradigm (CPFE and sCFC) were analyzed separately in one-way ANOVAs. The factorial ANOVAs revealed no main effect or interaction of sex (ps > 0.522) so data were collapsed across this variable and analyzed via a (Drug; *APV vs. PBS*) x (Phase; *Retention*) one-way ANOVA for each of the paradigms. The CPFE ANOVA revealed a main effect of Drug [$F(1,20) = 4.715$, $p = 0.042$], indicating that there was a significant disruption in freezing behavior of the APV group when compared to the PBS group. The sCFC ANOVA also revealed a main effect of Drug [$F(1,20) = 6.086$, $p = 0.0228$], indicating a significant disruption in freezing behavior of the APV group when compared to the PBS group. Overall, these results indicate that disruption of NMDA receptors within the BLA prior to training day of both the CPFE and sCFC disrupts retention of contextual fear.

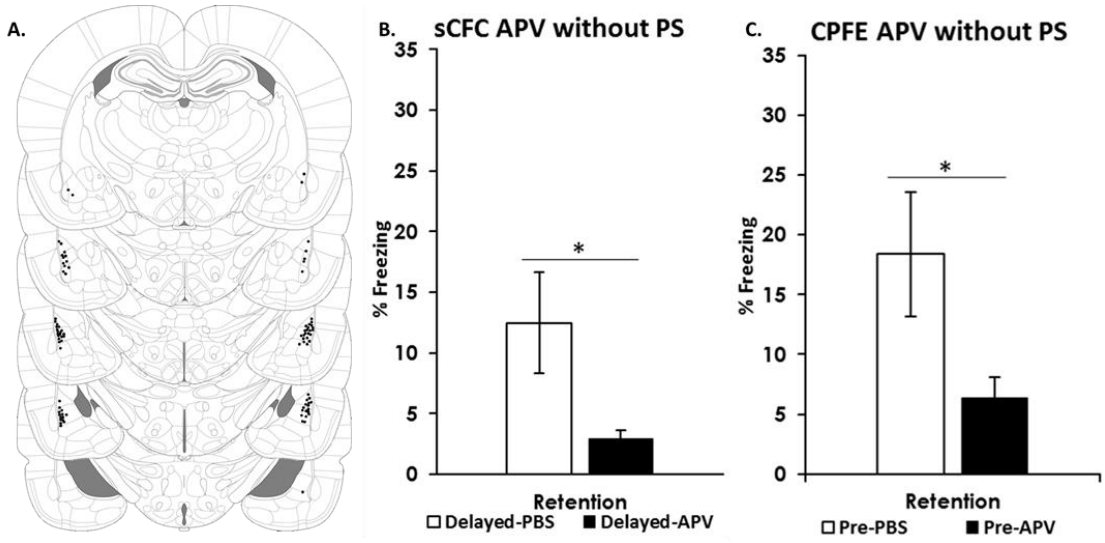

Figure 6 (A) A diagram of the histological analysis confirming cannula placement into the basolateral amygdala. The slices represent -2.40mm, -2.76mm, -3.12mm, -3.48, and -3.72mm from bregma (from top to bottom). A "hit" was classified by the center of the guide cannula tract being in between Plate 50 (Bregma -2.04mm) to Plate 65 (Bregma -3.84mm) of Paxinos & Watson (2007). Cannula misses were not included. (B) A graphical representation of the Phase by Condition interaction seen in freezing percentages during the sCFC procedure. A significant different ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group is represented with a *. A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-APV group is represented with a #. (C) A graphical representation of the Phase by Condition interaction seen in freezing percentages during the CPFE procedure. A significant different ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group is represented with a *. A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-APV group is represented with a #.

Chapter 5

DISCUSSION

The role of the basolateral amygdala in adolescent fear conditioning was explored using standard contextual fear conditioning and the context preexposure facilitation effect. Experiment 1 found that inactivation of the BLA prior to training day of the CPFE disrupted both post-shock and retention freezing. Experiment 2 found that disruption of BLA NMDA receptors prior to training day disrupted both post-shock and retention freezing during the CPFE, but only disrupted retention freezing during sCFC. Experiment 3 found that disruption of BLA NMDA receptors prior to training day of the CPFE and of sCFC impairs retention freezing when post-shock freezing is not measured. Taken together the results suggest an important role of the BLA in adolescent contextual fear conditioning. Interestingly, it also suggests that the BLA plays a different role in the acquisition of contextual fear in sCFC and the CPFE.

The involvement of the BLA in contextual fear conditioning is indisputable, however the specific role it plays within the different components of contextual fear conditioning is not fully understood. The CPFE offers a unique advantage for analyzing its contribution to the individual components of contextual fear conditioning yet is rarely used in the current literature. A study that utilized this paradigm in adult rats showed plasticity in the BLA to be involved in the 24-hr retention of the context-shock association, but not in the acquisition of a contextual representation nor in the expression of contextual fear (Huff & Rudy, 2004; Matus-Amat et. al., 2007). This is

consistent with the current results showing that the BLA is not only involved in the *acquisition* of the context-shock association (Matus-Amat et al., 2007 only measured retention), but specifically that NMDA receptor plasticity within the BLA is involved. The current results also extend the previous study to examine short-term contextual fear (post-shock freezing). The results from the post-shock freezing measure suggest that the plasticity within the BLA is involved in both immediate acquisition and 24-hr retention of the context-shock association during the CPFE.

While studies utilizing the CPFE are sparse, there are many studies that examine the role of the BLA in sCFC. These studies have shown that lesions (Kim, Rison, & Fanselow, 1993; Maren, 1999; Maren, Aharonov & Fanselow, 1996; Goosens & Maren, 2001), inactivation via muscimol (Huff et. al., 2018; Ponnusamy, Poulos, & Fanselow, 2007; Helmstetter & Bellgowan, 1994; Muller et. al., 1997), and disruption of plasticity within the BLA (Fanselow and Kim, 1994; Maren et. al., 1996; Malkani & Rosen, 2001; Kim et. al., 1991) prior to training day of sCFC impairs retention of contextual fear. This impairment can be overcome by administering overtraining after lesions or inactivation of the BLA, suggesting a possible BLA-independent mechanism of contextual fear (Maren, 1999; Ponnusamy, Poulos, & Fanselow, 2007). Findings concerning the role of the BLA in post-shock freezing are less consistent. Some previous studies of contextual fear conditioning have shown that NMDA receptor antagonism does not disrupt post-shock freezing, suggesting a lack of a role in the immediate acquisition of contextual fear (Malkani & Rosen, 2001; Kim et. al., 1992), whereas other studies have shown that NMDAR antagonists do

disrupt post-shock freezing (Fanselow & Kim, 1994; Maren et. al, 1996). Furthermore, it has been shown that disruption of NMDA receptor plasticity during sCFC also influences the expression of early growth response gene 1 (EGR-1), an immediate early gene (IEG) that is expressed following fear conditioning (Malkani & Rosen, 2001). Specially, when APV is administered into the ventricles prior to training day of sCFC it not only disrupts retention freezing, but also disrupts the expression of EGR-1 in the amygdala (Malkani & Rosen, 2001).

There are many reasons for these discrepant findings in the literature. It is possible that learning following a shock is initially NMDA-independent but becomes more NMDA-dependent over time. The studies finding a disruption in post-shock freezing used multiple shocks presented at 20 sec intervals and measured freezing after the final shock (Maren et. al., 1996). If NMDA-dependence is a function of time from the beginning of the learning experience (i.e. after the first shock) then their measure of post-shock freezing is delayed compared to the post-shock freezing measure in the current study as well as the other studies with results similar to ours. This would also explain why retention freezing is more susceptible to BLA manipulations. Retention freezing typically occurs 24 hours after the first learning event, which according to the above hypothesis, would make it more NMDA-dependent. This is further supported by the fact that, following APV micro-infusions, freezing is always higher during post-shock freezing when compared to retention freezing. Another possible explanation for the difference in findings is the levels of freezing. The current studies are examining freezing during development, which

produces lower freezing levels when compared to adults. The lack of effect in post-shock freezing may be due to low levels of freezing in our PBS group when compared to that of the ACSF group in the Maren et. al. (1996) study. However, this is most likely not the case due to evidence from other studies in adults that show similar result but have much higher freezing levels (Kim et. al., 1992; Malkani & Rosen, 2001). Another possibility is that there is a BLA-independent pathway that can regulate contextual fear conditioning (Ponnusamy, Poulos, & Fanselow, 2007). It has been suggested previously that these alternative pathways are less efficient than the amygdala pathways and as such are only active when the amygdala pathway is compromised (Ponnusamy, Poulos, & Fanselow, 2007). This view suggests that this compensatory amygdala-independent pathway is only active if the amygdala is "off-line." However, it is possible that this mechanism is involved in the initial amygdala-independent fear-response as well. A possible alternate brain structure that has been suggested to mediate this learning is the bed nucleus of the stria terminalis (BNST, Ponnusamy, Poulos, & Fanselow, 2007). This structure receives projections from the hippocampus (which forms the context representation) and send projects to the PAG (which is involved in the production of the freezing response). Further studies of the involvement of BNST is required to test this hypothesis. It is also important to note that the differential involvement of NMDA receptors in post-shock and retention freezing is further evidence that there is a difference between these measures. Post-shock freezing is a measure of the acquisition of contextual fear, while retention freezing is a measure of the consolidation and/or the retrieval of contextual fear.

BLA plasticity is involved differentially in sCFC and the CPFE, at least with respect to post-shock freezing. There are a few possible explanations for the differential effects of NMDA antagonism on post-shock freezing. First, the levels of post-shock freezing in the CPFE are slightly higher than in sCFC making the CPFE less susceptible to ”floor effects.” However, it is unlikely this is the main cause of the different outcomes because there is little difference in freezing across these paradigms in the present study. Second, NMDA plasticity may be differentially involved in associating a retrieved context with a shock versus associating a perceived context with a shock. This could be because a retrieved context representation is weaker than a context representation that is being perceived. Third, state-dependent learning effects associated with drug cues might play a role. In the CPFE, “stimulus effects” of APV administration on the training day might produce generalization decrement that interferes with retrieval of the representation of the pre-exposed context (acquired off the drug) whereas, in sCFC, this would not happen because context learning and context-shock learning both occur in the presence of APV “drug cues.” We have ruled out “state-dependent” learning effects for systemically administered NMDA antagonists during the CPFE (Heroux et al., 2016) and such effects are unlikely to explain other findings in the present study. However, further experimental tests of state-dependent learning could be performed in future studies. Finally, it is also possible that a context representation that is being retrieved reaches the BLA from different brain regions or via different pathways than a context representation that is being actively perceived. The CPFE is thought to depend necessarily on hippocampal

projections whereas other projections (conveying "elemental" features of context) might operate in sCFC (Rudy, 2009).

It has been shown that there are discontinuities in the development of contextual fear conditioning in mice, in that preadolescences and adults show normal levels of freezing but adolescents are impaired (King et. al., 2013). There are also reports of an attenuation in fear extinction learning during adolescence (McCallum, Kim & Richardson, 2010; Pattwell et. al., 2012).However, the current study found no developmental differences in contextual fear conditioning or the role of the BLA in adolescents compared to adults. In both adolescents and adulthood, BLA plasticity is involved in the retention of the context shock association in both the CPFE and sCFC. Whether the BLA plays a similar role in pre-weanling and juvenile rats, when contextual fear conditioning is first emerging ontogenetically, is an interesting question that requires further study. Studies of IEG expression in the amygdala during both sCFC and the CPFE might help inform this question (Robinson-Drummer et al., 2018).

The current set of experiments show support for the involvement of the BLA in adolescent contextual fear conditioning. Specifically, neural activity in the BLA on training day of the CPFE is required to form a context-shock association and retain it over 24 hours. This is also true of ***plasticity*** within the BLA. However, plasticity within the BLA on the training day of sCFC is not involved in the initial acquisition of the context-shock association but it is required for retention of the context-shock association.

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APPROVAL FOR THE USE OF ANIMAL SUBJECTS

UNIVERSITY OF DELAWARE | Institutional Animal Care and Use Committee (IACUC)

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From: Gwen Talham, DVM, Director, Animal Care Program

Subject: IACUC approval for Lauren Miller

Date: 4/23/2018

Lauren Miller was approved by the IACUC to work with animals on Mark Stanton's protocol #1104 "Rodent Models of Cognitive Development: Eyeblink and Fear Conditioning and T-maze Learning". Please contact me at 831-2980 or gtalham@udel.edu with any additional questions.