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

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Abstract

In standard contextual fear conditioning (sCFC), learning of the context and formation of the context-shock association occur in the same training session whereas in the context preexposure facilitation effect (CPFE) learning the context (preexposure) and the context-shock association (training) are separated by 24 hours. In both procedures conditioned freezing can be measured immediately (post-shock test) or during a 24-hour retention test. 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; 1]. 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; 2]. Experiment 1 extended this literature from adult to adolescent rats and to the role of BLA activity and plasticity in post-shock freezing during the CPFE. 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 disrupted 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 plasticity 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.

Keywords

amygdala; contextual fear conditioning; context preexposure facilitation effect; ontogeny; spatial learning

1. Introduction

Contextual fear conditioning is a useful paradigm for studying the neurobiology of learning and memory in developing and adult rats (e.g., [3, 4, 5,6]). Of the many variants of

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contextual fear conditioning paradigms, this study focuses on two: standard contextual fear conditioning (sCFC) and the context preexposure facilitation effect (CPFE). Standard contextual fear conditioning involves a 3-min exposure to a context followed by a brief foot-shock. This paradigm requires learning of the context and acquiring a context-shock association within a single trial, limiting separate analysis of these two components. The CPFE overcomes this by separating the procedure into three phases: preexposure, training, and testing. The preexposure day involves learning the context, and the training day involves association of the retrieved context representation with immediate foot-shock. In either variant, successful acquisition of the context-shock association can be measured immediately following the shock in a post-shock freezing test or assessed 24 hours later in a retention freezing test.

A longstanding theory of contextual fear conditioning [4,7,3] holds that the hippocampus is necessary for learning of the context representation and that information about the context is projected from the hippocampus to the amygdala where it converges with information about the shock to form the context-shock association. Neural activity reflecting the context-shock association is then projected from the amygdala to the ventral periaqueductal gray (PAG) which generates the freezing response. This fear conditioning model 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, which raises the possibility that “elemental” context cues can mediate contextual fear when the hippocampus is not functional [8,9]. In contrast, in the CPFE, the context representation is first learned incidentally (without reinforcement) and consolidated over 24 hr before it is retrieved via pattern completion and associated with immediate shock on the training day [10]. Thus, sCFC can be learned based on either “elemental” or “configural” context representations whereas the CPFE requires configural context learning [10,11]. Previous studies have confirmed that the hippocampus to be involved in all three phases of the CPFE [9]. The question of whether the basolateral amygdala (BLA) plays a similar role in these two variants of contextual fear conditioning has received little attention. As with sCFC, NMDAR-plasticity in the BLA on the training day of the CPFE is necessary for 24-hour retention [2]. But, few, if any, studies directly 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 causal 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 examined the role of activity and plasticity within the BLA in contextual fear conditioning in adolescent rats. Experiment 1 examined post-shock and retention freezing following microinfusions of the GABA_A agonist muscimol into the BLA prior to the training day of the CPFE. Experiment 2 examined post-shock and retention freezing in both the CPFE and sCFC following training day infusions of the NMDA receptor

antagonist APV into the BLA. In an effort to examine retention test freezing without prior influences from the post-shock freezing test, Experiment 3 removed the post-shock freezing test and examined retention test freezing only and replicated the findings from Experiment 2. Together, Experiments 2 and 3 offered a within-subjects and between-subjects comparison of post-shock vs. retention test freezing in adolescent rats trained on either sCFC or the CPFE.

2. Materials and Methods

2.1. Subjects

Animal husbandry was as described in our previous reports [12,13]. All experiments used adolescent (PD31-33) Long-Evans rats bred at the Office of Laboratory Animal Medicine at the University of Delaware. In Experiment 1, there were a total of 40 rats (21 females and 19 males) derived from 9 separate litters. In Experiment 2, there were a total of 130 rats (63 females and 67 males) derived from 24 litters. In Experiment 3, there were a total of 56 rats (27 females and 29 males) derived from 14 separate litters. 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.

2.2. Stereotaxic Surgery

Animal surgeries reflected a modification of surgical implantation of intracranial injection cannulas in juvenile and adolescent rats that has been previously described for hippocampus and medial prefrontal cortex (mPFC) in our lab [5,13,14]. 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 [5,13]. 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 μ l 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).

2.3. Drug Infusion

Infusions were as described in previous reports [15] with modifications in the infusion site and rate (see below). In Experiment 1, microinjections of the vehicle PBS or the GABA_A receptor agonist muscimol (Sigma-Aldrich, St. Louis, MO; 2 μ g/ μ l dissolved in PBS) were administered approximately 15 minutes prior to behavioral procedures on PD32. In Experiments 2 and 3, microinjections of the vehicle PBS or the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (APV) (Tocris, Ellisville, MO; 5 μ g/ μ l dissolved in PBS) were administered immediately prior to behavioral procedures on PD31 (sCFC) or PD32 (CPFE). In all experiments, animals were gently held while PBS or drug was infused into both hemispheres at a rate of 0.125 μ l per minute for two minutes, resulting in a final infusion volume of 0.25 μ l per side for each animal. This dose of muscimol was chosen because of its efficacy to disrupt BLA functioning prior to the training day of sCFC [16,17] as well as its ability to disrupt hippocampal or prefrontal functioning when given prior to any phase of the CPFE [9,15]. The dose of APV was chosen because it disrupts fear conditioning [18]. 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 (Experiment 1) or 2 minutes (Experiment 2 and 3) prior to start of behavioral testing.

2.4. Apparatus and Stimuli

The apparatus and stimuli used have been previously described [12,13,19]. Fear conditioning occurred in four Plexiglas chambers measuring 16.5 cm $\times$ 12.1 cm $\times$ 21.6 cm which were arranged in a 2 $\times$ 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. Footshock 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 as previously described [13].

2.5. Procedures

2.5.1. Context Preexposure Facilitation Effect—The CPFE procedure has been described previously [12,13,20]. The CPFE procedure took place over the course of three days from PD31 to PD33 ($\pm$ 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 [19]). 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 [10]. A multiple-preexposure protocol was used [20] and 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 and were then returned to their transport cages and were taken back to their home-cages (Experiments 1 and 2) or were immediately removed from the chambers and returned to their home cages following the shock (Experiment 3). Twenty-four hours later (PD33), animals were again tested in Context A for 5-minutes. Testing consisted of a 5 min exposure to the chamber with no additional exposure to the unconditioned stimulus.

2.5.2. Standard Contextual Fear Conditioning—The sCFC procedure has been described previously [15,21]. 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; [22]). 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.

2.6. 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. For Experiment 1, out of 40 surgeries (see Figure 1B 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). For Experiment 2, out of 149 surgeries (see Figure 2A for placements), there were a total of 25 misplaced cannula with the distribution as follows: CPFE-Alt-Pre (3), CPFE-Pre-APV (5), CPFE-Pre-PBS (5), sCFC-Delayed-APV (3), sCFC-Delayed-PBS (4), and sCFC-Imm. Shock (5). For Experiment 3, out of 56 surgeries (see Figure 3A 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).

2.7. Data Analysis

Data processing procedures have been described previously [12,13]. 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, in which animals from Alt-Pre conditions for each drug were pooled, was used as reported previously [12,13]. Data from animals in this 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 ($ps > .05$). This reduces animal use and simplifies the experimental design. There were also no main effects or interactions involving sex across any of the experiments ($ps > .05$), so the data were collapsed across this variable, except in one instance that is reported below (Experiment 2).

Freezing behavior was analyzed with a Condition (*PBS, Muscimol or APV, Pooled-Alt-Pre*) $\times$ Phase (*Post-shock, Retention*) between-within ANOVA with Phase being the repeated measure. Pair-wise contrasts were performed with planned comparison tests. As in previous reports [13,15] a data point was excluded from analysis as an outlier if it had a score ± 1.96 standard deviations from its group mean, however, the average Z-score for removed outliers across all experiments was 4.87 ± 0.82 . The Experiment 1 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). The Experiment 2 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).. The Experiment 3 outliers were distributed as follows: CPFE-APV (2), CPFE-PBS (1), and sCFC-PBS (1).

3. Experiment 1

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

3.1. Results

After outliers were excluded, 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 1 depicts a cannula tract from a representative brain (Panel A), cannula placements (Panel B) and freezing behavior (Panel C). The between-within ANOVAs indicated no main effect or interaction involving Sex ($p > .119$) so the data were collapsed across this variable and analyzed via a 3 (Condition; *Pooled Alt. Pre vs. Pre-PBS vs. Pre-Muscimol*) $\times$ 2 (Phase; *Post-shock vs. Retention*) between-within ANOVA. The ANOVA revealed a main effect of Condition [$F(2,26) = 14.926, p < 0.001$] and a significant Phase $\times$ Condition interaction [$F(2,26) = 4.907, p < 0.016$]. There was a significant disruption in freezing behavior in the Muscimol group when compared to the PBS group on both post-shock ($p < 0.001$) and retention ($p < 0.015$) freezing tests. The PBS group also showed a significant difference from the pooled-Alt-Pre group on both post-shock ($p < 0.001$) and retention ($p < 0.015$) freezing tests while the Muscimol groups did not ($p > 0.66$). Freezing declined somewhat across the post-shock and retention freezing tests within the PBS group ($p < 0.001$). Overall, these results indicate that inactivation of the BLA prior to immediate-shock training of the CPFE disrupts both the acquisition (post-shock freezing) and retention of contextual fear.

4. Experiment 2

This experiment examined the specific role of NMDA receptor plasticity in the BLA in the acquisition and retention of contextual fear during the CPFE. It also asked whether the NMDARs in the BLA are playing a similar role across variants of contextual fear conditioning (sCFC vs. the CPFE).

4.1. Results

After outliers were removed, behavioral analysis was conducted on 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 2 depicts cannula placements (Panel A) and freezing behavior (Panels B and C). Results from each paradigm (CPFE and sCFC) were analyzed separately. The sCFC between-within ANOVA revealed no main effect or interaction of sex ($p > 0.217$) 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*) between-within ANOVA, and revealed a main effect of Condition [$F(2,37) = 7.668, p < 0.002$], a main effect of Phase [$F(1,37) = 16.452, p < 0.001$], 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 ($p > 0.506$), but did reveal reduced retention freezing in the APV group versus the PBS group [$F(1,37) = 4.253, p < 0.047$]. There was a significant difference in post-shock freezing between the Pooled-Imm.-Shock and both the APV group [$F(1,37) = 8.749, p < 0.006$] and PBS groups [$F(1,37) = 11.825, p < 0.002$]. In contrast, retention freezing revealed a significant difference between the Pooled-Imm.-Shock and the PBS group [$F(1,37) = 5.414, p < 0.026$], but not the Pooled-Imm.-Shock and the APV group ($p > 0.506$). Overall, these data show that NMDA receptors disrupt retention but not acquisition of contextual fear in sCFC in adolescent rats.

The CPFE ANOVA indicated a main effect of sex ($p > 0.035$) with the males freezing higher (14.03%) than the females (8.75%), but no interactions involving 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 collapsed 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*) ANOVA. The CPFE ANOVA revealed a main effect of Condition [$F(2,50) = 12.647, p < 0.001$], a main effect of Phase [$F(1,50) = 7.993, p < 0.007$], but no Condition x Phase interaction ($p > 0.083$). Planned comparisons revealed a decrease in Post-shock [$F(1,50) = 6.316, p < 0.015$] and Retention [$F(1,50) = 7.750, p < 0.008$] 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 [$F(1,50) = 15.508, p < 0.001$] and Retention [$F(1,50) = 15.474, p < 0.001$] 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 the CPFE and sCFC.

5. Experiment 3

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 replicate and extend the results from Experiment 2 by measuring retention test freezing but not post-shock freezing.

5.1. Results

Behavioral analysis was conducted on 44 animals distributed as follows: CPFE-APV (N=11), CPFE-PBS (N=11), sCFC-APV (N=12), and sCFC-PBS (N=10). Figure 3 depicts cannula placements (Panel A) and freezing behavior (Panels B and C). Results from each paradigm (CPFE and sCFC) were analyzed via two-tailed independent-samples t-test (APV

vs SAL). The CPFE t-test revealed a significant effect of Drug [$t(12) = -2.17, p < 0.025$], indicating that there was a significant disruption in freezing behavior of the APV group when compared to the PBS group. The sCFC t-test also revealed a significant effect of Drug [$t(9) = -2.25, p < 0.026$], 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.

6. 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 the training day of the CPFE disrupted both post-shock and retention freezing. Experiment 2 found that disruption of BLA NMDA receptors prior to training 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, these results suggest a necessary role of the BLA across variants of contextual fear conditioning in adolescent rats. Interestingly, it also suggests that the NMDA receptor plasticity within 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 well documented, but 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 the neural mechanisms of individual components of contextual fear conditioning despite it being rarely used in the current literature. A study that utilized this paradigm in adult rats showed NMDA receptor 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 [2,23]. The present study extends the finding of impaired 24-hr retention to adolescent rats and also shows that NMDA receptor plasticity within the BLA is necessary for *acquisition* of the context-shock association (Matus-Amat et al [2] only measured retention). Thus, NMDA receptor 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 of the role of the BLA in sCFC. These studies have shown that lesions [24, 25, 26, 27] inactivation via muscimol [16, 17, 28, 29] and disruption of NMDA receptor plasticity within the BLA [1, 30, 31, 32] prior to training impairs 24-hr retention of contextual fear. This impairment can be overcome by administering overtraining after lesions or inactivation of the BLA, suggesting a possible, secondary BLA-independent mechanism of 24-hr retention of contextual fear [25, 28]. Findings concerning the role of the BLA in post-shock freezing are less consistent. Some studies 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 [31,33], whereas other studies have shown that NMDA antagonism does disrupt post-shock freezing [1,30]. Furthermore, NMDA receptor antagonism during sCFC also reduces the expression of early

growth response gene 1 (EGR-1) following fear conditioning [31]. Specially, intraventricular APV prior to sCFC training spares post-shock freezing but disrupts post-training EGR-1 expression in the amygdala and 24-hr retention of freezing [31].

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 [1]. 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. Another possible explanation for the difference in findings is the levels of freezing. The lack of effect on 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. [1] study. However, this is not supported by other studies in adult rats that show similar results to ours but have much higher freezing levels [31,33]. Another possibility is that there is an alternate neural pathway that mediates post-shock freezing when NMDA receptor plasticity within the BLA is disrupted. For example, the bed nucleus of the stria terminalis (BNST) could receive context information via projections from the hippocampus and elicit the freezing response via projections to the PAG [28]. Further study of the role of BNST in post-shock freezing during sCFC is required to test this hypothesis.

NMDA receptor plasticity within the BLA is differentially involved in post-shock freezing in sCFC and the CPFE, with NMDAR-antagonism disrupting the CPFE but not sCFC. There are a few possible explanations for this dissociation. First, NMDA receptor 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 actively being perceived. Second, 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 [12] and we have not observed these effects with microinfusions studies of other brain structures that are important for the CPFE [34, 35]. However, further experimental tests of state-dependent learning could be performed in future studies. Finally, it is also possible that a context information that is being retrieved on the training day of the CPFE reaches the BLA from different brain regions or via different pathways than a context representation that is being actively perceived during sCFC. The CPFE is thought to depend necessarily on hippocampal projections whereas other projections (conveying “elemental” features of context) might operate in sCFC [10].

The present study extends previous research on adult rats to the early adolescent period. 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 [36]. There are also reports of an attenuation in fear extinction learning during adolescence [37, 38]. 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 adolescent and adult rats, NMDA receptor plasticity within the BLA 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.

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Highlights

- In the CPFE, encoding and retention of contextual fear depends on BLA NMDARs.
- In standard context conditioning, only retention of fear depends on BLA NMDARs.
- Adolescents and adults share common mechanisms of contextual fear conditioning.

Figure 1.

(A) Image of a coronal section of a rat brain showing representative cannula placements (B) 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)[39]. Cannula misses are not shown. (C) A graphical representation of the Phase by Condition interaction seen in percentage freezing. A significant difference ($p < 0.05$) between the Pre-PBS group and the pooled Alt-Pre group is represented with a * (Alt-Pre condition pooled across drug). A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-MUSC group is represented with a #.

Figure 2.

(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)[39]. 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 difference ($p < 0.05$) 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 difference ($p < 0.05$) between the Pre-PBS group and the Pooled Alt-Pre group is represented with a * (Alt-Pre condition pooled across drug). A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-APV group is represented with a #.

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)[39]. 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 difference ($p < 0.05$) between the Pre-PBS group and the pooled alt-pre group (animals in the alt-pre condition pooled across drug) 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 difference ($p < 0.05$) between the Pre-PBS group and the Pooled Alt-Pre group is represented with a * (Alt-Pre condition pooled across drug). A significant difference ($p < 0.05$) between the Pre-PBS and the Pre-APV group is represented with a #.