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NMDA receptor antagonism disrupts the acquisition and retention of the Context Preexposure Facilitation Effect in adolescent rats

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Abstract

The context preexposure facilitation effect (CPFE) is a contextual fear conditioning paradigm in which learning about the context, acquiring the context-shock association, and retrieving/expressing contextual fear are temporally dissociated. The current study investigated the involvement of NMDA receptors in contextual fear acquisition, retention, and expression across all phases of the CPFE in adolescent rats. In Experiment 1 systemic injections of 0.1 mg/kg MK-801, a non-competitive NMDA receptor antagonist, given before multiple context preexposure disrupted the acquisition of a context representation. In Experiment 2, pre-training MK-801 disrupted both immediate acquisition of contextual fear measured by postshock freezing, as well as retention test freezing 24 hours later. Experiment 3 showed that expression of contextual fear via a 24hr retention freezing test does *not* depend on NMDA receptors, indicating that MK-801 disrupts learning rather than performance of freezing behavior. In Experiment 4, consolidation of contextual information was partially disrupted by post-preexposure MK-801 whereas consolidation of contextual fear was not disrupted by post-training MK-801. Finally, Experiment 5 employed a dose-response design and found that a pre-training dose of 0.1 mg/kg MK-801 disrupted both postshock and retention test freezing while lower pre-training doses of MK-801 (0.025 or 0.05 mg/kg) only disrupted retention freezing. This is the first study to distinguish the role of NMDA receptors in acquisition (post-shock freezing), retention, expression, and consolidation of context vs. context-shock learning using the CPFE paradigm in adolescent rats. The findings provide a foundation for similar developmental studies examining these effects from early ontogeny through adulthood.

Keywords

NMDA receptors; systemic MK-801; postshock; retention; CPFE; dose-response NMDA receptor involvement in the CPFE

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1. Introduction

Pavlovian fear conditioning, particularly contextual fear conditioning, has become a topic of immense interest in the neurobiology and ontogeny of learning [1–9]. During a standard contextual fear conditioning procedure, rats are placed into a chamber and given a delay period to explore the context before experiencing a brief foot-shock. The rats can then be probed for contextual fear learning in a postshock freezing test immediately after the shock or in a retention test after a delay period, displaying a species-typical freezing response after acquiring the context-shock association [10, 11]. Because learning of the context and the context-shock association occurs in the same session, it is difficult to distinguish the contributions of different processes and neural circuits to learning and memory. For example, contextual fear learning in this procedure can be supported by a dominant hippocampal system in which the features of a context are bound into a conjunctive representation, or by a system that does not depend on the hippocampus and selectively attends to individual features of the context [4, 12, 13]. A variant of contextual fear conditioning called the *context preexposure facilitation effect* (CPFE) cannot be learned based on individual features of the context [13]. The CPFE temporally separates learning about the context, acquiring the context-shock association, and expressing contextual fear into three distinct phases [12]. Our lab is using this paradigm to examine the ontogeny and neurobiology of contextual fear learning. We have shown that the CPFE emerges between postnatal day (PD) 17 and 24 in the rat [14], at which point it depends on hippocampal NMDA receptors and on conjunctive-rather than feature-based context representations in developing rats [15]. We have also shown that the CPFE engages plasticity and drives immediate early gene expression in the hippocampus, amygdala, and prefrontal cortex in adolescent rats [5, 6].

One question that has not yet been examined in developing rats is the role of NMDA receptors on the training day of the CPFE protocol. In adult rats, NMDA glutamate-type receptor plasticity in the hippocampus is only required during the acquisition of a context representation on the context preexposure day [16, 17]. We have found that systemic injections or intra-dorsal-hippocampal infusions of NMDA receptor antagonists also block acquisition of the context representation in juvenile rats [14, 18]. In adult rats, NMDA receptor plasticity in the basolateral amygdala is needed to acquire a context-shock representation on the training day of the CPFE [16].

The present study sought to examine the role of NMDA receptors across all phases of the CPFE in adolescent rats, particularly examining the role of NMDA receptors in the acquisition and retention of the context-shock association on the training day. We examined adolescent rats as a point of comparison with adult rats that could help guide future studies across earlier stages of ontogeny. One limitation of previous research about the role of NMDA receptors in the CPFE in both adult and developing animals is the lack of an immediate measure for the acquisition of contextual fear. The present study is the first to address this limitation by including postshock freezing tests as a measure of the immediate acquisition of contextual fear, allowing for better temporal characterization of the role of NMDA receptors in the acquisition and retention of the CPFE in developing animals. The first three experiments examined the role of NMDA receptors on the preexposure

(Experiment 1), training (Experiment 2A and 2B), and testing (Experiment 3) day of the CPFE. In addition, Experiment 4 examined the effects of NMDA receptor antagonism on the consolidation of contextual information and contextual fear, allowing us for the first time to separate acquisition vs. consolidation effects in the previous experiments. Finally, Experiment 5 employed a dose-response design and reexamined the effect of pre-training NMDA receptor antagonist injections on postshock and retention test freezing in adolescent rats.

2. Experiment 1

Our lab has previously demonstrated that both systemic injections and DHPC infusions of MK-801 before a single context preexposure procedure disrupts the CPFE by blocking the acquisition of a context representation in adolescent rats [14, 18]. The purpose of Experiment 1 was to extend these findings to the role of NMDA receptors in the acquisition of a context representation during a multiple context preexposure protocol, which acts to strengthen the acquired representation of the context [19]. Therefore, animals were given either Saline or the non-competitive NMDA receptor antagonist MK-801 prior to multiple context preexposure and retention test freezing after training was analyzed.

2.1. Methods

2.1.1 Subjects—Animal husbandry was as described in our previous reports [19]. Subjects for Experiment 1 were 48 Long Evans rats (24 females and 24 males), derived from 12 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Time-mated females were housed with breeder males overnight and were examined for an ejaculatory plug the following day and, if found, 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 postnatal day (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 in 45 cm × 24 cm × 17 cm cages. On PD29 animals were individually housed 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 subjects were treated in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

2.1.2. Apparatus and stimuli—The apparatus and stimuli used have been previously described [20, 21]. 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 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 2 second 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. Videos of each session (preexposure, training, testing) were recorded using FreezeFrame 3.0 software (Actimetrics, Wilmette IL) with freezing defined as a bout of 0.75 s or longer without a change in video pixilation.

2.1.3. Design and procedure—The CPFE procedure took place over the course of 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). Both preexposure groups underwent a multiple preexposure protocol described previously [19, 22]. Multiple preexposure consisted of one initial 5 minute exposure to the chamber followed by five 1 minute exposures with a 1 minute interval between exposures. Animals were placed in transport boxes on a cart inside the training room during the 1 minute inter-trial interval. Animals were assigned to receive either 1 ml/kg 0.9% sterile saline (*Saline* condition) or 1 ml/kg of 0.1 mg/ml MK-801 solution (purchased from Tocris; *MK-801* condition) prior to the preexposure session on the first day. All injections were given intraperitoneally in this and subsequent experiments. This MK-801 dose was chosen because of previous work in weanling rats on contextual fear learning of the CPFE in our laboratory [18]. Load order and composition was counterbalanced across the preexposure variable (Pre vs. Alt Pre), drug (Saline vs. MK-801), and sex (Male vs. Female) for all three days of the CPFE.

On the first day of the behavioral protocol, PD31 animals were weighed and wheeled on a cart into a separate room and received either an injection of 1 ml/kg 0.9% sterile saline or 1 ml/kg of a 0.1 mg/ml MK-801 solution 30 minutes (± 5 minutes) before the preexposure session. The animals were wheeled back into the colony room until the preexposure session, and then placed 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. The rats were brought over and remained in a hallway adjacent to the testing room for <5 min while the fear chambers were cleaned with 5% ammonium hydroxide solution. The weighing, cleaning, and transport protocol was consistent across all experimental sessions and days. Pre animals were brought over and placed in Context A for the multiple preexposure, whereas animals in the Alt Pre group underwent multiple preexposure in the alternate context (Context B).

Twenty-four hours later, on the training day of the behavioral protocol, animals from all groups were trained with an immediate 1.5 mA 2-s footshock in Context A. Rats were carried into the testing room one at a time, placed in their respective training chamber, and received an immediate footshock. Animals were immediately removed from the chambers following the footshock, returned to their transport cages, and taken back to their homecages. Twenty-four hours later, all animals were tested in Context A for 5 minutes in the same chamber they had been trained in. Testing consisted of a 5 minute exposure to the chamber with no unconditioned stimulus presented.

2.1.4. Data and statistical analysis—A human observer blind to the experimental groups verified the freezing threshold setting with FreezeView 3.0 (Actimetrics, Wilmette IL) by sorting the session and adjusting the threshold if necessary to ensure that small movements were not recorded as freezing. Freezing behavior was scored as the total percent time spent freezing (defined as the cessation of all movement except breathing) over a 5-min session for the testing session.

Once percent freezing was reliably determined, the data were imported into STATISTICA 64 data analysis software, and freezing behavior was analyzed with a 2 (Preexposure group; *Pre* vs. *Alt Pre*) × 2 (Drug treatment; *Saline* vs. *MK-801*) × 2 (Sex; male vs. female) factorial ANOVA. Post-hoc contrasts were performed with Newman-Keuls tests. A rat was excluded from analysis as an outlier if in a given group it had a score ± 2 standard deviations from the group mean. Three rats were removed from analysis, one from each of the following groups: Pre-MK-801, Alt-Pre-Saline, and Alt-Pre-MK-801. Behavioral analysis was conducted on the remaining 45 animals distributed as follows: Pre-Saline (n= 11), Pre-MK-801 (n= 11), Alt-Pre-Saline (n= 12), Alt-Pre-MK-801 (n= 11).

2.2. Results and discussion

The results of Experiment 1 appear in Fig. 1. ANOVA indicated no main effect or interaction of Sex (*p*s > .2) so the data were collapsed across this variable and analyzed via 2 (Drug; *Saline* vs. *MK-801*) × 2 (Preexposure; *Pre* vs. *Alt Pre*) factorial ANOVA. Animals that received MK-801 before the multiple preexposure session displayed significantly less freezing than Saline control animals. A factorial ANOVA revealed a main effect of Preexposure [$F(1,41) = 13.54$, $p < .001$] and Drug [$F(1,41) = 15.67$, $p < .001$], and a significant Preexposure × Drug interaction [$F(1,41) = 8.97$, $p < .001$]. Newman-Keuls tests revealed that the Saline Pre group froze significantly higher than all three other groups ($p < .001$) which did not differ in any other comparison (*p*s > .6). This experiment shows that acquisition of a context representation during the multiple context preexposure procedure in the CPFE is dependent on NMDA receptor functioning in adolescent rats. This confirms our previous findings which used a single context preexposure procedure [14, 18].

3. Experiment 2

The purpose of Experiment 2 was to examine the involvement of NMDA receptors in acquisition of fear on the training day of the CPFE in developing animals. Previous research using adult rats has shown that pre-training infusion of the competitive NMDA antagonist APV into the BLA on the training day of the CPFE disrupts the acquisition of the context-shock association measured by a retention freezing test 24 hours later [17]. Experiment 2 examined the disruptive effects of pretraining MK-801 administration by measuring freezing immediately after training using a postshock freezing test (Experiment 2A) or after a long-term (24 hour) retention interval (Experiment 2B). This allows for more accurate temporal assessment of NMDA receptor involvement on the training day of the CPFE relative to studies that examine 24-hr retention without assessing post-shock freezing. We predicted that MK-801 would disrupt both acquisition and retention of context fear relative to saline control groups.

4. Experiment 2A

4.1. Methods

4.1.1 Subjects—Subjects for Experiment 2A were 47 Long Evans rats (23 females and 24 males), derived from 6 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Rats were bred, culled, reared, etc. as described previously (See Section 2.1.1.). No more than 1 same-sex littermate was assigned to a given experimental condition.

4.1.2. Apparatus, design, and behavioral procedure—The apparatus, preexposure context (Context A and Context B), and training context were the same as in Experiment 1. Context preexposure (on PD31) and training (on PD32) followed similar procedures to Experiment 1, except that animals received a systemic injection of 0.1 mg/kg MK-801 or Saline prior to training (rather than prior to preexposure).

4.1.4. Data and statistical analysis—Data analysis and statistical tests were performed using the same programs and methods as Experiment 1. Freezing behavior was analyzed with a 2 (Preexposure group; *Pre* vs. *Alt Pre*) × 2 (Drug treatment; *Saline* vs. *MK-801*) × 2 (Sex; male vs. female) factorial ANOVA. Post-hoc contrasts were performed with Newman-Keuls tests. Four rats were removed from analysis after being identified as outliers, one from each of the following groups: Pre-Saline, Pre-MK-801, Alt-Pre-Saline, and Alt-Pre-MK-801. Behavioral analysis was conducted on the remaining 43 animals distributed as follows: Pre-Saline (n=11), Pre-MK-801 (n=11), Alt-Pre-Saline (n=10), Alt-Pre-MK-801 (n=11).

4.2. Results and discussion

The results of Experiment 2A appear in Fig. 2. ANOVA indicated no main effect or interaction of Sex (*p*s > .2) so the data were collapsed across this variable and analyzed via 2 (Drug; *Saline* vs. *MK-801*) × 2 (Preexposure; *Pre* vs. *Alt Pre*) factorial ANOVA. Animals in the Pre-Saline group froze more on the retention day than any other group (Pre-MK-801, Alt Pre-Saline, and Alt Pre-MK-801). A factorial ANOVA revealed a main effect of Preexposure [$F(1,39) = 28.97$, $p < .001$] and Drug [$F(1, 39) = 11.02$, $p < .005$], and a significant Preexposure × Drug interaction [$F(1,39) = 11.73$, $p < .005$]. A Newman-Keuls post hoc test revealed that the Saline Pre group froze significantly more than all three other groups ($p < .001$) which did not differ (*ps* > .15).

This experiment shows that NMDA receptor antagonism on the training day disrupts freezing behavior on the retention day of the CPFE. Freezing behavior in the Alt Pre control group did not differ across drug, and there was no difference between animals in the Pre-MK-801 group and both non-associative, alternate-preexposure controls. It is unclear whether MK-801 disrupts the acquisition of the context-shock association as there was no group that was tested immediately after the shock. Accordingly, Experiment 2B reexamined the effect of pre-training MK-801 by assessing contextual fear expression directly after the training experience during a postshock freezing test.

4. Experiment 2B

5.1. Methods

5.1.1. Subjects—Subjects for Experiment 2B were 50 Long Evans rats (25 females and 25 males), derived from 11 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Rats were bred, culled, reared, etc. as described previously (See Section 2.1.1.). No more than 1 same-sex littermate was assigned to a given experimental condition.

5.1.2. Apparatus, design, and behavioral procedure—The apparatus, preexposure context (Context A and Context B), and training context were the same as in Experiment 2A. Context preexposure (on PD31) and training (on PD32) followed similar procedures to Experiment 2A, except that instead of being taken out after the immediate shock on the training day, animals received a 3 minute postshock freezing test that consisted of no additional presentations of the unconditioned stimuli. The animals were not brought back twenty-four hours later for retention testing as in Experiment 1.

5.1.3. Data and statistical analysis—Data analysis and statistical tests were performed using the same programs and methods as previous experiments. Freezing behavior was analyzed with a 2 (Preexposure group; *Pre* vs. *Alt Pre*) × 2 (Drug treatment; *Saline* vs. *MK-801*) × 2 (Sex; male vs. female) factorial ANOVA. Two rats were removed from analysis due to corrupt data videos rendering them unable to be scored, one from each of the following groups: Pre-MK-801 and Alt Pre-MK-801. Scores of 5 rats were removed from analysis as outliers, one from each of the following groups: Pre-Saline, Pre-MK-801, Alt Pre-MK-801, and two animals were removed from the Alt Pre-Saline group. Behavioral analysis was conducted on the remaining 43 animals distributed as follows: Pre-Saline (n=11), Pre-MK-801 (n=9), Alt-Pre-Saline (n=11), Alt-Pre-MK-801 (n=12).

5.2. Results and discussion

5.2.1. Behavioral measures—The results of Experiment 2B appear in Fig. 3. Similar to the Experiment 2A, ANOVA results indicated no main effect or interaction of Sex (*p*s > .15), thus the data are collapsed across this variable and analyzed via 2 (Drug; *Saline* vs. *MK-801*) × 2 (Preexposure; *Pre* vs. *Alt Pre*) ANOVA. The pattern of results was very similar to the previous experiment with the Pre-Saline group demonstrating greater amounts of postshock freezing than the other three groups. ANOVA revealed main effects of Preexposure [$F(1,39) = 19.78$, $p < .001$] and Drug [$F(1, 39) = 9.74$, $p < .005$], and a significant Preexposure × Drug interaction [$F(1,39) = 5.20$, $p < .05$]. Post hoc tests revealed that the Saline Pre group froze significantly more than all three other groups ($p < .001$) which did not differ among themselves (*ps* > .30).

This experiment shows that MK-801 induced NMDA receptor antagonism on the training day of the CPFE disrupts freezing behavior on the training day measured by a postshock freezing test. This effect was not attributable to the US alone as the non-associative Alt-Pre controls did not freeze at comparable levels to the Pre-Saline group in response to an immediate shock ($p > .57$). Although animals that received MK-801 displayed a significant

disruption of postshock freezing, it is unclear whether or not this disruption reflects learning or a drug performance effect. This possibility was assessed in the next experiment.

6. Experiment 3

Previous research using adult rats has shown that DHPC and BLA NMDA receptors are not required for the expression of contextual fear in the CPFE using adult rats [17]. The purpose of Experiment 3 was to extend this finding in adolescent rats and to examine any drug "performance" effects of systemically administered MK-801. If the expression of contextual fear during a retention freezing test in the CPFE is not dependent on NMDA receptors, then injecting animals with MK-801 before a retention test provides an opportunity to examine possible drug effects such as increased locomotor activity or state dependency (failure to retrieve fear caused by a change in drug state). As such, Experiment 3 utilized pre-retention systemic injections of 0.1 mg/kg or Saline to examine the involvement of NMDA receptors in the expression of contextual fear acquired during the CPFE protocol. There was no significant difference between animals given MK-801 or Saline before the retention freezing test.

6.1. Methods

6.1.1 Subjects—Subjects for Experiment 3 were 24 Long Evans rats (11 females and 13 males), derived from 6 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Rats were bred, culled, reared, etc. as described previously (See Section 2.1.1.). No more than 1 same-sex littermate was assigned to a given experimental condition.

6.1.2. Apparatus, design and behavioral procedure—The apparatus, preexposure context (Context A and Context B), and training context were the same as in Experiment 1 and 2A. Context preexposure (on PD31) and training (on PD32) followed similar procedures to Experiment 1 and 2A, except that animals received a pre-test (retention test) systemic injection of 0.1 mg/kg MK-801 or Saline prior to the retention test (instead of before preexposure or training). In addition, no Alt-Pre groups were included in this study.

6.1.3. Data and statistical analysis—Data analysis and statistical tests were performed using the same programs and methods as previous. Freezing behavior was analyzed with a 2 (Drug treatment; *Saline* vs. *MK-801*) × 2 (Sex; male vs. female) factorial ANOVA. Two outlier scores were removed from analysis, one each from the Saline and MK-801 groups. Behavioral analysis was conducted on the remaining 22 animals distributed as follows: Saline (n=11), MK-801 (n=11).

6.2. Results and discussion

The results of Experiment 3 appear in Fig. 4. Similar to the previous experiments, ANOVA indicated no main effect or interaction of Sex (*p*s > .07), thus the data are collapsed across this variable and analyzed via two-tailed independent-samples t-test (MK-801 vs. Saline). The t-test revealed no significant difference between animals given MK-801 or Saline before the retention freezing test [$t(20) = .11$, $p > .90$]. This finding not only replicates the

previous findings in adult rats that NMDA receptors are not required for the retention day of the CPFE, but it also suggests that freezing deficits seen after MK-801 administration reflect learning and not a performance effect.

7. Experiment 4

One possible explanation for the disruption of retention freezing observed by MK-801 administration before multiple context preexposure or training could potentially be an effect on the consolidation of contextual information and contextual fear. Experiment 2B showed that pre-training MK-801 disrupts the acquisition of a context-shock association measured by a postshock freezing test, but this doesn't control for any possible consolidation effects that would reflect persisting effects of pre-training drug administration into the period following the training episode. Experiment 4 examined the effects of post-preexposure and post-training injections of MK-801 on the consolidation of contextual information and contextual fear to determine whether post-training drug effects are sufficient to account for the effects of pretraining drug injections reported in Experiments 1 and 2.

7.1. Methods

7.1.1. Subjects—Subjects for Experiment 4 were 72 Long Evans rats (40 females and 32 males), derived from 13 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Rats were bred, culled, reared, assigned to groups, etc. as described previously (See Section 2.1.1.).

7.1.2. Apparatus, design, and behavioral procedure—The apparatus and contexts used were the same as previous experiments. The behavioral protocol consisted of context preexposure (PD31), training (PD32), and testing (PD33). Unlike the previous experiments, Experiment 4 utilized post-preexposure and post-training drug injections to separate drug effects on acquisition vs. consolidation of the respective learning events. The preexposure day consisted of multiple preexposure in Context A (*Pre* condition) or Context B (*Alt Pre* condition) with some animals receiving an i.p. injection of either 1 ml/kg of 0.9% sterile saline or 0.1 mg/kg MK-801 immediately upon returning to the colony room after multiple context exposure (*Post-Pre* condition). The purpose of the timing of this injection was to target the consolidation of the contextual information the animal learned about during multiple preexposure. The training day consisted of animals being placed into Context A and receiving an immediate footshock followed by a 1 minute postshock freezing test with no additional presentations of the US. A group of animals received either Saline or 0.1 mg/kg MK-801 upon returning to the colony room after training (*Post-Train* condition). The purpose of the timing of this injection was to target the consolidation of the context-shock association acquired during the training session. The animals were brought back twenty-four hours later for retention testing as in Experiment 1 and 3. To simplify the experimental design, a number of *Alt-Pre* animals were selected from each group and pooled to form a final group (Group *Pooled-Alt-Pre*). This was justified because freezing was uniformly low regardless of experimental condition in the Alt-Pre groups (confirmed by a preliminary ANOVA [$F(1,13) = .058$, $p > .81$]). Creating a Pooled-Alt-Pre group thereby reduced animal use and made the design more manageable.

7.1.3. Data and statistical analysis—Data analysis and statistical tests were performed using the same programs and methods as in previous experiments. Postshock freezing behavior was analyzed with a 2 (Sex; *male vs. female*) × 2 (Drug; *Saline vs. MK-801*) factorial ANOVA. Two outlying scores were removed from analysis, one each from the following two groups: Pooled-Alt-Pre-Saline and Saline-Post-Pre. Behavioral analysis was conducted on the remaining 38 animals distributed as follows: Saline-Post-Pre (n=15), MK-801-Post-Pre (n=16), and Pooled-Alt-Pre (n=7). The Pooled-Alt-Pre group was derived by combining the following groups: Alt-Pre-Saline-Post-Pre (n=4), Alt-Pre-MK-801-Post-Pre (n=3).

Retention test freezing behavior was also analyzed with a 2 (Sex; *male vs. female*) × 2 (Consolidation Target; *Post-Pre vs. Post-Train*) × 2 (Drug; *Saline vs. MK-801*) factorial ANOVA. Data from 6 rats were removed as outliers, one from each of the following groups: Saline-Post-Pre, Saline-Post-Train, MK-801-Post-Train, Pooled-Alt-Pre, and two rats were removed from MK-801-Post-Pre. Behavioral analysis was conducted on the remaining 66 animals distributed as follows: Saline-Post-Pre (n=15), MK-801-Post-Pre (n=14), Saline-Post-Train (n=11), MK-801-Post-Train (n=11), and Pooled-Alt-Pre (n=15). The Pooled-Alt-Pre group was derived from combining the following groups: Alt-Pre-Saline-Post-Pre (n=4), Alt-Pre-MK-801-Post-Pre (n=4), Alt-Pre-Saline-Post-Train (n=4), Alt-Pre-MK-801-Post-Train (n=3).

7.2. Results and discussion

The results of the postshock freezing test in Experiment 4 appear in Fig. 5A. ANOVA indicated no main effect or interaction of Sex (*p*s > .5) for either the postshock freezing or retention tests, so all of the data were collapsed across this variable. Postshock freezing was analyzed with a one-way ANOVA (Drug; *Saline, MK-801, Pooled Alt Pre*), which revealed a main effect of Drug [$F(2,43) = 31.16$, $p < .001$]. Post hoc tests showed that animals that received post-preexposure injections of Saline froze significantly higher than animals that received post-preexposure injections of 0.1 mg/kg MK-801 ($p < .001$). Both Saline and MK-801 animals froze significantly more than the Pooled-Alt-Pre group ($p < .001$), indicating that context consolidation was only partially disrupted in the MK-801-Post-Pre group, as measured by postshock freezing.

The results of the retention freezing test in Experiment 4 appear in Fig. 5B. Retention test freezing was analyzed via a 2 (Drug, *MK-801 vs. Saline*) × 2 (Consolidation Target, *Post-Pre, Post-Train*) factorial ANOVA. ANOVA revealed no significant main effect of Drug [$F(1,47) = 1.68$, $p > .2$] and Consolidation Target [$F(1,47) = 3.17$, $p > .08$] or interaction between the two [$F(1,47) = .87$, $p > .35$]. To compare all four groups against the Pooled-Alt-Pre control group, a one-way ANOVA on the 5 groups was performed, followed by a Dunnett's test that contrasted the 4 Pre groups with the Alt-Pre group. This ANOVA revealed a main effect of Treatment Group [$F(4,61) = 6.28$, $p < .001$] . The Dunnett's test revealed that all groups except for the MK-801-Post-Pre group froze significantly more than the Pooled-Alt-Pre behavioral control group ($p < .01$).

This experiment demonstrates that the consolidation of contextual information on the preexposure day is partially NMDA receptor dependent. In contrast, consolidation of the

context-shock association on the training day of the CPFE does not depend on NMDA receptors. Therefore, in the previous experiments, giving animals MK-801 injections prior to training suggests disruption of the acquisition but not consolidation of the context-shock association in the CPFE.

8. Experiment 5

Experiment 2A and 2B separately demonstrated that the acquisition of contextual fear measured by a postshock freezing test or retention test is NMDA receptor dependent, supported by the finding that pre-training MK-801 administration disrupts both postshock and retention test freezing. Experiment 5 not only reexamined this effect by combining postshock and retention freezing tests within the same CPFE experimental design, but also employed a dose-response design to explore any differential involvement of NMDA receptors in postshock and retention freezing and also to examine any possible nonspecific drug effects occurring at high doses.

8.1. Methods

8.1.1 Subjects—Subjects for Experiment 5 were 63 Long Evans rats (28 females and 31 males), derived from 10 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Rats were bred, culled, reared, etc. as described previously (See Section 2.1.1.). No more than 1 same-sex littermate was assigned to a given experimental condition.

8.1.2. Apparatus, design and behavioral procedure—The apparatus and contexts used were the same as previous experiments. The behavioral protocol consisted of preexposure, training, and testing and was similar to Experiment 1 with the exception of the addition of a 1 minute postshock freezing test that occurred directly after the immediate shock on the training day. Context preexposure (PD31) consisted of multiple exposure to either Context A (*Pre* condition) or Context B (*Alt-Pre* condition). Thirty minutes prior to training on the second day (PD32), animals were given an i.p. injections of 1 ml/kg of 0 (0.9% sterile saline), 0.025, 0.05, or 0.1 mg/kg MK-801. The animals were brought back twenty-four hours later for a retention test (PD33) that consisted of a 5 minute exposure to the training context with no presentations of the unconditioned stimulus (described previously in Experiment 1). As in the previous experiment, a number of *Alt-Pre* animals were randomly assigned from each drug condition and combined to form a final group (Group *Pooled-Alt-Pre*) in order to streamline the design and analysis of this experiment.

8.1.4. Data and statistical analysis—Data analysis and statistical tests were performed using the same programs and methods as in previous experiments. Separate analyses were performed on Postshock and Retention test freezing. Postshock and retention test freezing behavior was analyzed with a repeated measures ANOVA with Drug (*0, 0.025, 0.05, 0.1, Pooled Alt Pre*) × Phase (*postshock, retention*) × Sex (*male, female*). Scores from four rats were removed from the postshock and retention freezing analysis after being identified as outliers, one from each of the following groups: MK-801-0.025, MK-801-.05, MK-801-.01, and 0 (Saline) group. Behavioral analysis was conducted on the remaining 59 animals distributed as follows: 0 (n=11), MK-801-0.025 (n=10), MK-801-0.05 (n=11), MK-801-0.1

(n=12), and Pooled-Alt-Pre (n=15). The Pooled-Alt-Pre group was derived from combining the following groups: Alt-Pre-0 (n=4), Alt-Pre-MK-801-0.025 (n=4), Alt-Pre-MK-801-0.05 (n=3), Alt-Pre-MK-801-0.1 (n=4).

8.2. Results and discussion

The results of the postshock and retention freezing tests in Experiment 5 appear in Fig. 6A and 6B, respectively. Similar to the previous experiments, ANOVA results indicated no main effect or interaction of Sex (*p*s > .25), so all of the data were collapsed across this variable and analyzed via Drug (*0, 0.025, 0.05, .1, Pooled Alt Pre*) × Phase (*postshock, retention*) mixed ANOVA. This revealed a significant main effect of Drug [$F(4,54) = 19.64$, $p < .001$] and phase [$F(1,54) = 4.26$, $p < .05$] along with a significant Drug × Phase interaction [$F(4,54) = 9.54$, $p < .001$]. A Newman-Keuls post hoc test of the Drug x Phase interaction revealed that lower doses of MK-801 (0.025mg/kg, 0.05 mg/kg) only disrupted retention test freezing but only the 0.025mg/kg group differed significantly from saline controls in a postshock freezing test (*p*s < .001), freezing significantly higher ($p<.05$). Animals receiving higher doses of MK-801 (0.1 mg/kg) did not significantly differ from non-associative pooled alternate context preexposure control animals in the both postshock and retention freezing tests (*p*s < .005).

This experiment demonstrates that only the 0.1 mg/kg MK-801 disrupts the acquisition of the context-shock association as measured by a postshock freezing test, but 0.025 or 0.05 mg/kg MK-801 is sufficient to disrupt retention test freezing. These findings suggest a possible differential involvement of NMDA receptors in the immediate acquisition versus the long-term retention of contextual fear within the CPFE paradigm.

9. General Discussion

The current experiments investigated the involvement of NMDA-type glutamate receptors in contextual fear learning and expression across all phases of the CPFE in adolescent rats. Experiment 1 found that systemic injections of MK-801 (0.1mg/kg) given before multiple context preexposure disrupted fear conditioning, confirming our previous reports using a single context preexposure [18]. In Experiment 2, training day MK-801 injections disrupted both immediate acquisition (post-shock freezing) and 24-hour retention of contextual fear. Experiment 3 showed that MK-801 administered before a 24hr retention test did not disrupt expression of contextual fear, ruling out “performance” effects of the drug. Experiment 4 found that the consolidation of context learning was partially disrupted whereas consolidation of contextual fear was not disrupted by post-training MK-801 administration. Finally, in Experiment 5, higher doses of MK-801 (0.1 mg/kg) disrupted both postshock and retention freezing while lower doses of MK-801 (0.025 or 0.05 mg/kg) only disrupt retention test freezing. Taken together, these results indicate that the acquisition of both contextual information and contextual fear during the CPFE is NMDA receptor dependent in adolescent rats.

Experiment 1 found that systemic injections of MK-801 before multiple context preexposure disrupts the acquisition of a representation of the context, measured by retention freezing test 24 hours after immediate shock training. This confirms our previous studies using a

single context preexposure that is preceded by MK-801 administered systemically [18] or via microinfusions into DHPC [14]. This is also consistent with previous research in adult rats involving both the CPFE [17] and lesion and microinfusion studies in standard contextual fear conditioning [2, 7, 23–26]. In addition to NMDA receptor involvement on the preexposure day of the CPFE, infusing GABA agonist mucimol into the DHPC prior to any phase of the CPFE disrupts contextual fear learning and expression [16]. As a whole, our results indicate that strengthening context learning via the multiple-context-preexposure protocol does not attenuate the role of NMDA receptors in the acquisition of the context representation of the explored environment on the preexposure day of the CPFE.

The results of Experiment 2 indicate that pre-training administration of MK-801 disrupts both immediate postshock and 24hr retention freezing tests in PD31 rats. To our knowledge, this is the first study to examine the role of NMDA receptors on the training day of the CPFE in developing rats. The use of systemic injections in the present study do not speak to which brain regions are mediating this effect. In adult rats, intra-basolateral amygdala (BLA) infusions of competitive NMDA receptor antagonist D-AP5 on the training day of the CPFE disrupted subsequent 24hr retention test freezing [17]. In contrast, training-day dorsal hippocampal infusions of D-AP5 had no effect [17]. These findings suggest that NMDA receptors play a role in plasticity in the amygdala for associating the aversive shock stimulus with the retrieved context representation acquired on the previous day. Although the CPFE literature is sparse, several studies in adult rats indicate NMDA receptors in the amygdala are required for standard contextual fear conditioning [27–30]. A study by Maren et al. [27] demonstrated that intra-amygdaloid infusions of the competitive NMDA receptor antagonist AP5 disrupts both immediate acquisition and subsequent retention of contextual fear. In summary, our results indicate that NMDA receptors are involved in the acquisition of the context-shock association in the CPFE in adolescent rats. While our results do not explicitly address localization, it is very likely that NMDA receptor functioning in the basolateral amygdala mediates contextual fear learning and other brain regions such as the hippocampus or mPFC may also be needed to mediate the long-term retention of contextual fear.

The results of Experiment 5 indicate that lower doses of pre-training MK-801 (0.025 and 0.05 mg/kg) had no effect on postshock freezing but disrupted retention test freezing at similar levels as a higher dose (0.1 mg/kg). It is interesting to examine this differential dose-response effect in the context of previous intracranial-drug infusion and lesion studies concerning the involvement of DHPC and BLA NMDA receptors in standard contextual fear learning. There are several studies that show systemic, intracerebroventricular (i.c.v), or DHPC infusions of APV do not disrupt the immediate acquisition and expression of contextual fear measured by a postshock freezing test, but instead disrupt subsequent 24 hour retention test freezing [23–26, 31, 32]. Lesion studies also indicate that DHPC lesions do not disrupt post-shock freezing (contextual fear learning) but generally do impair long-term retention of contextual fear [2, 7, 28]. In contrast, comparable doses of APV infused directly into the basolateral amygdala before standard contextual fear conditioning disrupt both immediate postshock and 24hr retention test freezing [27]. Pre-training amygdala lesions also disrupt both the immediate acquisition and the long-term retention of contextual

fear [2, 31]. In the context of these studies, it is conceivable that our dose response findings may be due to lower doses of MK-801 not being sufficient to disrupt NMDA receptor functioning in the amygdala but are sufficient to disrupt hippocampal functioning and thus disrupt retention test freezing but not postshock freezing. As noted previously, intra-dorsal-hippocampal infusions of D-AP5 during the training day of the CPFE have no effect on retention test freezing in adult rats [17]. However, an important difference between previous studies using the CPFE and our study is the use of an immediate postshock freezing test ranging from 1–3 minutes. It is possible that this time after the shock is enough to re-engage hippocampal NMDA receptor plasticity that is otherwise not engaged in an immediate shock protocol [33]. If so, then the differential sensitivity of hippocampal NMDA receptors to dose of MK-801 might account for the impairment of retention at lower doses in the present study. In addition to possible differential involvement of the hippocampus and amygdala, it is possible that NMDA receptor antagonism disrupts mPFC plasticity occurring during the CPFE. Our lab has previously reported an increase in the immediate early-growth response gene 1 (*Egr-1*) in the mPFC in PD31 rats after context-shock association training in the CPFE [5, 6]. It is possible that there may be differential contributions of NMDA receptors in the BLA, DHPC, and mPFC in postshock and retention test freezing within the CPFE. This is a fruitful direction for further research.

Previous research in adult rats has shown that DHPC and BLA NMDA receptors are not required for the expression of contextual fear in the CPFE [17]. Experiment 3 extended this finding using adolescent animals by showing that pre-retention MK-801 injections have no effect on subsequent contextual fear expression. It is therefore unlikely that the disruption of freezing caused by MK-801 in Experiment 2A, 2B, and 5 is due to drug-induced long term hyperactivity [34, 35], or state dependency effects related to changes in drug state across training and testing phases. State dependency is unlikely because animals in the MK-801 group expressed contextual fear while under the influence of MK-801 when prior training occurred off of the drug. Previous research in our lab using comparable doses of MK-801 given systemically during a T-maze task has also shown that MK-801 effects are not due to state dependency [36]. Although it is conceivable that deficits caused by MK-801 could reflect a difference in shock sensitivity, the shock intensity used in the present study was above the threshold for this effect [35] and we observed no drug related differences in shock-elicited behavior during training. In summary, the results of Experiment 3 suggest that freezing deficits caused by MK-801 reflect learning rather than nonspecific performance effects of the drug.

The disruptive effects of MK-801 on the acquisition of the context representation (Exp. 1) and the context-shock association (Exps. 2A, 2B, 5) could possibly be accounted for by drug effects that continued after the learning experience to influence consolidation rather than learning. Experiment 4 showed that MK-801 administration prior to context preexposure or training cannot be fully accounted for by an effect on consolidation processes. This is consistent with negative effects of intra-hippocampal APV administration after preexposure or training during the CPFE in adult rats [37]. We observed no effect of post-training MK-801 and an intermediate effect of post-preexposure MK-801, depending on whether post-shock freezing or 24-hour retention was the dependent measure. It is possible that

consolidation of contextual information was only partially disrupted by NMDA receptor antagonism because of the time-course of drug action. Although the injection of MK-801 occurred immediately (<5min) after the preexposure day session, the drug may have only started to take effect about 40 minutes after the first preexposure trial so consolidation processes likely already started. Additional research examining time-course effects of NMDA receptor antagonism on consolidation is needed to fully address this possibility. Interestingly, unlike these findings in the CPFE, previous studies have highlighted a role of DHPC and BLA NMDA receptor involvement in consolidation of standard contextual fear conditioning [38–40]. It is conceivable that differences between the two behavioral paradigms (CPFE vs. standard contextual fear), age, or drug parameters could account for differences between the present findings and other findings in the literature.

In summary, our findings show that NMDA receptors are involved in the acquisition of contextual information during context preexposure and of the context-shock association during the training day of the CPFE in developing rats. The disruptive effects of MK-801 on contextual fear learning on the training day reflect effects on the acquisition rather than consolidation of fear learning whereas the effects of MK-801 on the preexposure day may reflect disruption of both acquisition and consolidation of contextual learning. MK-801 effects are unlikely to reflect performance effects such as drug-induced changes in activity or shock sensitivity. Future experiments utilizing localized intracranial drug administration are needed to address the relative contributions of mPFC, DHPC, and BLA NMDA receptors in the acquisition and retention of the CPFE in developing rats.

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Highlights

- In adolescent rats, the context preexposure facilitation effect depends on NMDA receptor activation on either the preexposure or training day but not the testing day.
- These effects appear during fear acquisition (post-shock freezing) and 24-hour retention.
- NMDA receptor activation following preexposure or training has little or no effect on the CPFE in adolescent rats.
- Acquisition (post-shock freezing) vs. 24-hour retention show different dose-response functions. Lower doses of NMDA antagonists disrupt 24-hour retention but not post-shock freezing.

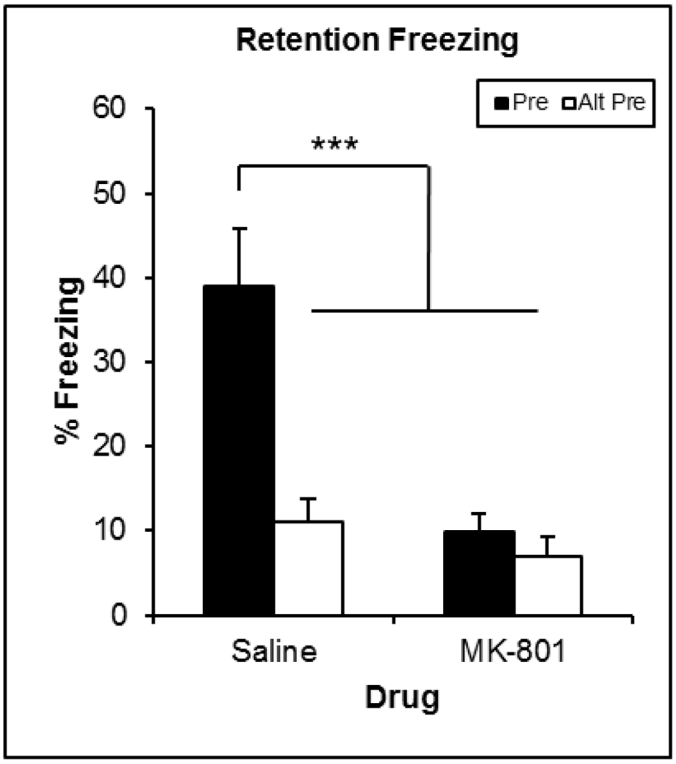

Figure 1.

Mean percent freezing (± SEM) in Experiment 1 depicted for Pre (black bars) and Alt Pre (white bars) groups across drug treatment conditions when the drug is administered prior to multiple context preexposure. The CPFE was observed in a 24 hour retention test for the Saline control group but not when MK-801 was injected prior to context preexposure (***p<.001).

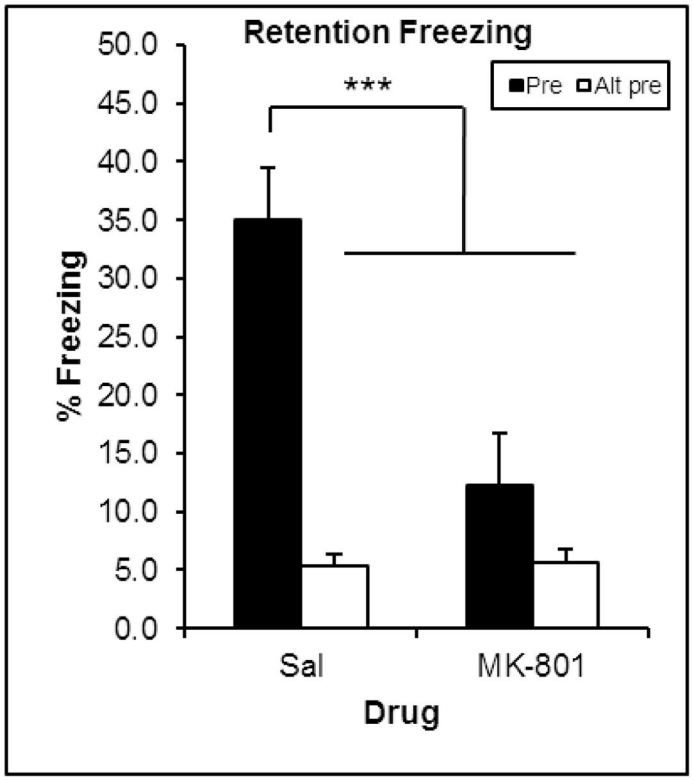

Figure 2.

Mean percent freezing (± SEM) in Experiment 2A depicted for Pre (black bars) and Alt Pre (white bars) groups across drug treatment conditions when the drug is administered prior to immediate shock training. Administration of 0.1 mg/kg MK-801 prior to training disrupted retention test freezing 24 hours later (***p<.001).

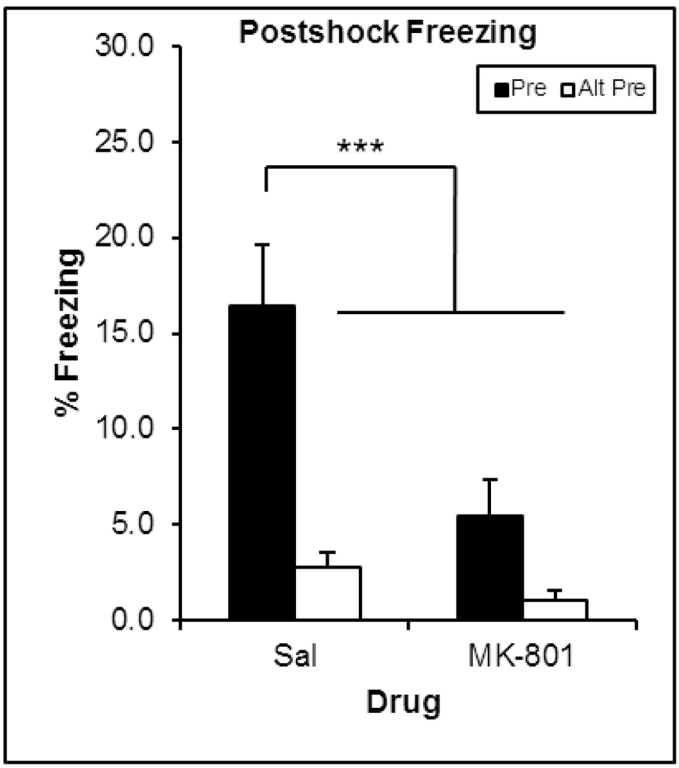

Figure 3.

Mean percent freezing (± SEM) in Experiment 2B depicted for Pre (black bars) and Alt Pre (white bars) groups across drug treatment conditions when the drug is administered prior to immediate shock training. Administration of 0.1 mg/kg MK-801 prior to training disrupted the CPFE in a 3 min postshock freezing test immediately after the shock (***p<.001).

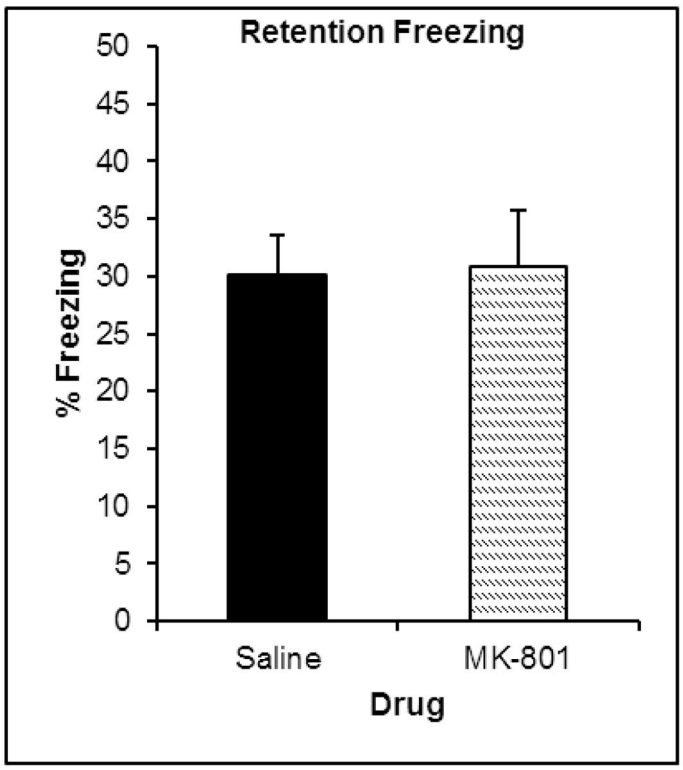

Figure 4.

Mean percent freezing (± SEM) in Experiment 3 depicted for animals given Saline (black bar) or 0.1 mg/kg MK-801 (striped bar) when the drug is administered prior to a 24hr retention freezing test. MK-801 did not disrupt expression of contextual fear relative to Saline controls. (p>.90).

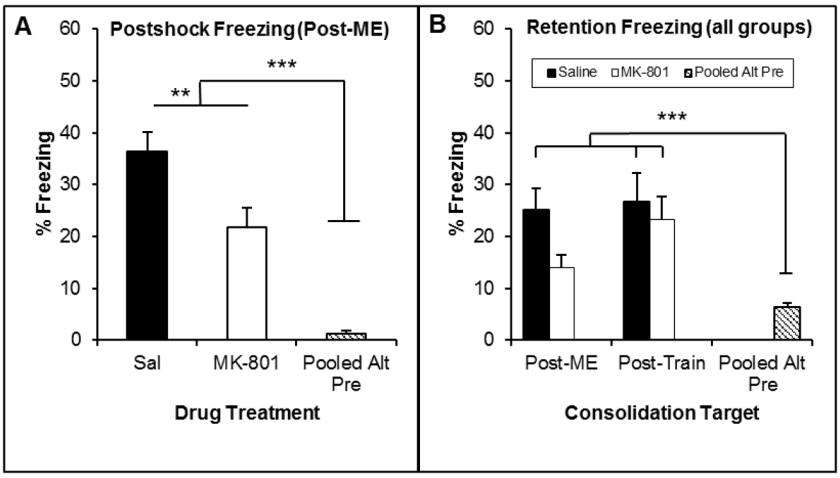

Figure 5.

Mean percent freezing (± SEM) during a 1 minute postshock and a 24 hour retention freezing test in Experiment 4 depicted for animals given Saline (black bars) or 0.1 mg/kg MK-801 (white bars) immediately after context preexposure (Panel A) or training (Panel B). The consolidation of contextual information was disrupted by post-preexposure MK-801 measured in a subsequent postshock freezing test after training (Panel A; $^{**}p<.05$) but not in a retention test 24 hours later (Panel A; $p=.06$). Post-training injections of MK-801 had no effect on retention test freezing relative to Saline and non-associative control animals (Panel B; $^{***}p<.001$). Alternate context preexposed animals were pooled across all other experimental conditions.

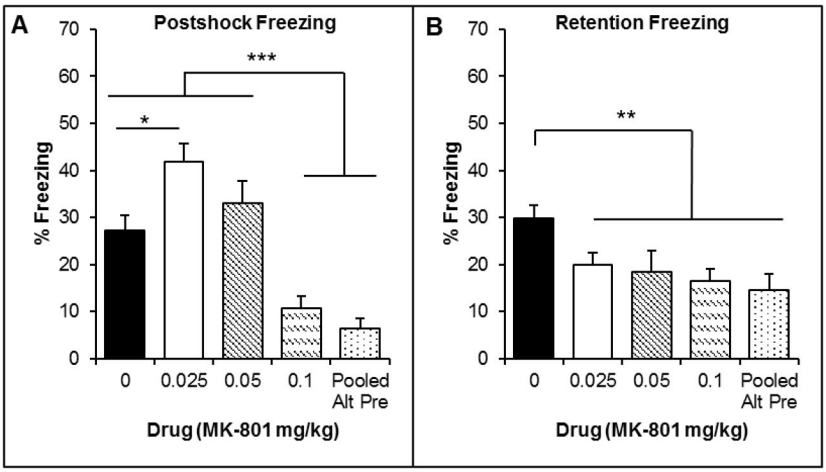

Figure 6.

Mean percent freezing (± SEM) in Experiment 5 depicted for drug conditions when the drug is administered prior to immediate shock training followed by a 1 minute postshock freezing test (Panel A) and a retention freezing test 24 hours later (Panel B). Higher doses of MK-801 (0.1 mg/kg) disrupt both postshock and retention test freezing (***p<.001) while lower doses of MK-801 (0.025 or 0.05 mg/kg) only disrupt retention freezing. (**p<.05). Alternate context preexposed animals were pooled across all drug conditions.