Pretraining NMDA Receptor Blockade in the Basolateral Complex, but Not the Central Nucleus, of the Amygdala Prevents Savings of Conditional Fear

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The acquisition of conditional freezing is abolished by N-methyl-D-aspartate (NMDA) receptor antagonism in the basolateral complex of the amygdala (BLA) during fear conditioning, suggesting that memory formation is prevented. The present study examined whether there is residual memory, or “savings,” for fear conditioning in rats trained under amygdaloid NMDA receptor blockade. Rats infused with D,L-2-amino-5-phosphonovalerate (APV) into the BLA or central nucleus of the amygdala (CEA) during fear conditioning did not acquire either auditory or contextual fear conditioning. However, savings of conditional fear was exhibited by rats infused with APV into the CEA but not the BLA. These results suggest that both the BLA and CEA play a critical role in the acquisition of conditional fear but that the BLA is able to process and retain some aspects of aversive memories in the absence of the CEA.

Pavlovian fear conditioning in rats is a model system used to study the neural mechanisms underlying emotional learning and memory. In a typical experiment, naive rats are placed in a chamber (the conditioning “context”) and presented with a conditional stimulus (CS), such as a tone, followed by an aversive footshock unconditioned stimulus (US). After several pairings, the CS comes to elicit many conditional responses, including increased heart rate, blood pressure, and acoustic startle and somatomotor immobility (i.e., freezing). These conditional responses may be measured in later extinction sessions as an index of memory for previous training.

Considerable evidence points to the nuclei of the amygdala as being particularly important for fear conditioning (Davis, 1992; Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001b). Lesions of either the basolateral complex (BLA; consisting of the lateral [LA], basolateral [BL], and basomedial [BM] nuclei) or the central nucleus (CEA) produce deficits in the acquisition and expression of conditional fear (Campeau & Davis, 1995; Goosens & Maren, 2001; Y. Lee, Walker, & Davis, 1996; Maren, 1998, 1999b; Maren, Aharonov, & Fanselow, 1996; Sananes & Davis, 1992). Moreover, fear conditioning induces associative neuronal activity in both the CEA (Applegate, Frysinger, Kapp, & Gallagher, 1982; Pascoe & Kapp, 1985) and the BLA (Maren, 2000; Maren, Poremba, & Gabriel, 1991; Quirk, Armony, & LeDoux, 1997; Quirk, Repa, & LeDoux, 1995; Repa, Apergis, Desrochers, Zhou, & LeDoux, 2001).

The amygdala also appears to be a site of convergence for information about both the CS and the US. LA neurons receive input from neurons in the medial geniculate nucleus of the thalamus (MGN), thought to be the primary relay of auditory information to the amygdala (Quirk et al., 1995, 1997). Neurons in the auditory cortex, which are also capable of transmitting sensory information to the amygdala (Romanski & LeDoux, 1992), project to the same amygdaloid nuclei as do MGN neurons (LeDoux, Farb, & Romanski, 1991). In contrast, contextual cues are processed in the hippocampus (Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Phillips & LeDoux, 1992), and hippocampal afferents to the amygdala synapse primarily on neurons of BL and BM (Canteras & Swanson, 1992; Maren & Fanselow, 1995). The CEA receives direct inputs from BL and BM. It projects to the brainstem areas controlling the expression of fear responses (LeDoux, Iwata, Cicchetti, & Reis, 1988), including the ventral periaqueductal gray, which generates freezing behavior (De Oca, DeCola, Maren, & Fanselow, 1998; Kim, Rison, & Fanselow, 1993). This anatomical arrangement of connections has led to the idea that the BLA may be critical in forming the CS–US association during conditioning, whereas the CEA serves to translate this association to an appropriate motor response in brainstem structures such as the ventral periaqueductal gray (e.g., Maren & Fanselow, 1996). That is, the BLA is often considered a “learning” structure, whereas its efferents are thought to be “performance” structures.

There is an abundance of evidence indicating that the BLA makes essential contributions to the associative learning underlying Pavlovian fear conditioning (Fanselow & LeDoux, 1999; Maren, 1999a). For example, blockade of N-methyl-D-aspartate (NMDA) receptors by intra-amygdala infusion of D,L-2-amino-5-phosphonovalerate (APV; a selective NMDA receptor antagonist) has been shown to severely attenuate the acquisition (Campeau,
of the drug into nearby structures such as the caudate
savings of conditional fear could not be accounted for by diffusion
savings of both contextual and auditory fear. The effects of pre-
auditory fear. Pretraining infusion of APV into the CEA yielded
the acquisition of conditional fear. In addition, pretraining infusion
that pretraining infusion of APV into the BLA or CEA prevented
testing session but may be expressed as facilitated reacquisition
of the initial conditioning session during subsequent retraining.

Collectively, these experiments suggest that NMDA receptor
blockade in the BLA is sufficient to prevent associative learning
during fear conditioning. However, it has been suggested that
structures other than the amygdala may be responsible for encoding
CS–US associations during fear conditioning (Cahill, Wein-
berger, Roozendaal, & McGaugh, 1999; Weinberger, Javid, &
Lepan, 1993). For example, in the case of auditory fear conditioning,
the MGN displays associative single-unit activity during fear
learning (Edeline & Weinberger, 1992; Lennartz & Weinberger,
1992; McEchron, McCabe, Green, Llabre, & Schneiderman,
1995). Furthermore, the MGN exhibits LTP (Gerren & Wein-
berger, 1983), and fear conditioning induces synaptic plasticity in the
MGN (McEchron et al., 1996). It is possible that amygdala
afferents such as the MGN, or perhaps even cortical areas (Cahill
et al., 1999), are the critical loci of associative learning during Pavlovian fear conditioning. These structures would then passively
transmit this plasticity to the amygdala, which could subsequently
modulate conditional responding (but see Maren, Yap, & Goosens,
2001, and Poremba & Gabriel, 2001). By this view, the absence of
behavioral savings during Pavlovian fear conditioning.

General Method

Subjects

The subjects were 235 adult male Long-Evans rats (200–224 g) ob-
tained from a commercial supplier (Harlan Sprague-Dawley, Indianapolis,
IN). After arrival, the rats were individually housed in standard Plexiglas
hanging cages on a 14:10-hr light–dark cycle (lights on at 7 a.m.) and
provided free access to food and tap water. After housing, rats were
handled for 30 s per day for 5 days to acclimate them to the experimenters.
All procedures were approved by the University Committee on Use and
Care of Animals at the University of Michigan.

Surgery

One week before behavioral testing, rats were implanted with bilateral
guide cannulas (26 gauge, 15 mm; Plastics One, Roanoke, VA) aimed at
the BLA (2.3 mm posterior to bregma, 5.0 mm lateral to the midline, 6.3
mm ventral to dura), CEA (2.0 mm posterior to bregma, 4.0 mm lateral to
the midline, 5.7 mm ventral to dura), or CPu (2.0 mm posterior to
bregma, 4.0 mm lateral to the midline, 3.7 mm ventral to dura). Rats were
anesthetized with sodium pentobarbital (65 mg/kg ip) and were adminis-
tered atropine methyl nitrate (0.04 mg/kg ip) to prevent obstruction of
the airway. After mounting in a stereotaxic apparatus (Kopf Instruments,
Tujunga, CA), the scalp was incised and retracted, and lambda and bregma
were placed in the same horizontal plane. Small burr holes (1 mm diam-
eter) were drilled for placement of the guide cannulas and three small
jeweler’s screws. The guide cannulas were lowered and dental acrylic was
applied to the skull to hold the cannulas in place. After surgery, dummy
cannulas (33 gauge, 16 mm; Plastics One, Roanoke, VA) were inserted into
the guide cannulas, and the rats were returned to their home cages. The
dummy cannulas were replaced every other day during the week of
recovery.

Behavioral Apparatus

All training and testing occurred in eight identical observation chambers
(30 × 24 × 21 cm; MED Associates, Burlington, VT) located in sound-
attenuating cabinets in an isolated room. The chambers were constructed of
aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged
front door). The floor of each chamber consisted of 19 stainless steel rods
(4 mm diameter) spaced 1.5 cm apart (center to center). The rods were
wired to a shock source and solid-state grid scrambler (MED Associates,
Burlington, VT) for the delivery of footshock USs. A speaker used in
delivering acoustic stimuli was mounted to a grating on one wall of each
chamber.

Each conditioning chamber was situated on a load-cell platform that
recorded chamber displacement in response to each rat’s motor activity.
The output of each chamber’s load cell was amplified (vernier knob = 8)
at a level previously determined to optimize detection of freezing behavior
(see Maren, 1998). The load-cell amplifier output from each chamber was
digitized at 5 Hz (yielding 300 observations per minute per rat) and
acquired on-line through the use of Threshold Activity software (MED
Associates, Burlington, VT). The raw load-cell output was used to quantify
activity. Freezing was quantified by calculating the number of observations
below a freezing threshold (load-cell activity level of 5 or below; see
Maren, 1998). Freezing was scored only if a rat was immobile for 1 s or
longer; thus, freezing was scored only for five or more contiguous obser-
vations. For each session, freezing observations were converted to a
percentage of total observations.
Two experimental contexts were used. For Context A, chambers were cleaned with a 5% ammonium hydroxide solution, and stainless steel pans with a thin layer of the same solution were situated under the grid floors before rats were placed in the chambers. Illumination was provided by both the room lights and a small stimulus light (15 W) in each chamber, and background noise (65 dB, A scale) was supplied by ventilation fans in each chest. For Context B, 1% acetic acid was used to clean the chambers and placed in the pans beneath the grid floors. Illumination was provided solely by a red light (30 W) located in the room, and ventilation fans were turned off.

Drug Infusion

Pairs of rats were transported to an isolated room in plastic buckets containing a thin layer of pine shavings. Dummy cannulas were removed from each rat, and injection cannulas extending 1 mm past the end of the guide cannulas (Plastics One, Roanoke, VA) were inserted. The injection cannulas were connected to a 10 μl Hamilton syringe with polyethylene tubing (PE-20; Fisher, Pittsburgh, PA) and mounted in an infusion pump (Harvard Apparatus, South Natick, MA). Rats were infused either with vehicle (VEH; 0.1 M phosphate-buffered saline, pH 7.4) or APV (10 μg/μl; Sigma Chemical, St. Louis, MO). The infusion rate was 0.1 μl/min. During this time, rats were permitted to explore freely within the buckets; however, they were distracted when attempting to groom because grooming often dislodged the injection cannulas. After the pumps were turned off, 1 min was allowed for diffusion before the injectors were removed. Rats were immediately transported to the conditioning chambers.

Histology

Histological verification of cannula placement was performed after behavioral testing. Rats were perfused across the heart with 0.9% saline followed by 10% Formalin. Brains were removed from the skull and placed in 10% Formalin for 2 days and 10% Formalin/30% sucrose until sectioning. Coronal sections (40 μm thick, taken every 120 μm) were cut on a cryostat (−18°C) and wet mounted on glass microscope slides with 95% ethanol. After drying, sections were stained with 0.25% thionin to allow visualization of cell bodies. Cannula placements were reconstructed on stereotaxic atlas templates (Swanson, 1999). Rats were excluded from statistical analyses if the cannulas were not both located in a single target structure (BLA, CEA, or CPu).

Data Analysis

Freezing was calculated as a percentage of total observations, a probability estimate amenable to analysis with parametric statistics. Probability estimates of freezing were assessed through analysis of variance (ANOVA). Post hoc comparisons in the form of Fisher’s protected least significant difference tests were performed on the freezing averages after a significant omnibus F ratio. All data shown in the figures are represented as means plus or minus standard errors of the means.

Experiment 1

The first experiment examined whether NMDA receptor blockade in the BLA or CEA during auditory fear conditioning would affect the acquisition and savings of auditory fear memories. Rats were fear conditioned after intra-amygdala infusion of either APV or VEH and later administered additional infusion-free conditioning. Savings was measured relative to a VEH-infused group that did not receive fear conditioning on the first day of training.

Method

The experiment was conducted in two phases (see Figure 1A). The first phase consisted of a single day of massed-trial auditory fear conditioning administered immediately after infusion of APV or VEH. The second phase (Test Days 1–4) consisted of 4 days of one-trial-per-day gradual reacquisition with no infusion before any session. Each test day was separated by several days of exposure to the training context to extinguish fear of this context before each CS test trial. Only when the rats had extinguished contextual fear were they administered extinction testing and retraining for auditory fear. Rats (n = 53) were assigned to one of three groups describing treatment on the day of massed training: AT+/APV, AT−/VEH, or AT+/VEH. These groups differed in the type of infusion received before massed-trial fear conditioning (APV or VEH) and the presence (“+”) or absence (“−”) of shock during this session. All training and testing were performed in the A context. One rat died postoperatively, and 7 rats were excluded from the analyses because of improper placement of guide cannulas. This yielded the following groups: AT+/APV–BLA (n = 8), AT+/VEH–BLA (n = 5), AT−/VEH–BLA (n = 4), AT+/APV–CEA (n = 16), AT−/VEH–CEA (n = 6), and AT+/VEH–CEA (n = 6). Savings in the AT+/APV groups was assessed by comparing the rate of reacquisition on Test Days 1 through 4 (indicated by freezing behavior during tone presentation on each test day) with the rate of reacquisition in rats receiving VEH infusion before the massed training (the AT−/VEH and AT+/VEH groups). The rate of reacquisition among animals in these groups during infusion-free reacquisition days was a measure of the influence (or savings) of either context exposure in the absence of shock (AT−/VEH or context-US association (AT+/VEH)).

The day before massed-trial training, rats were preexposed to the infusion procedure. Rats were transported in squads of 4 to the infusion room. On arrival, the dummy cannulas were removed, and mock injection cannulas that did not extend into the target brain structure were placed in the guide cannulas. The infusion pumps were turned on for 5 min at a rate of 0.1 μl/min to acclimate the rats to the sound of the pumps; however, the pumps were prevented from making contact with the plungers of the syringes. At the end of the 5-min period, the mock injectors were removed and the rats were transported back to their home cages.

For massed-trial training, the rats were transported in squads of 2 to the infusion room. All infusions were conducted at a rate of 0.1 μl/min. Rats with guide cannulas aimed at the BLA received a 0.5 μl infusion of APV (5 μg/side) or VEH into each BLA. Rats with guide cannulas aimed at the CEA received a 0.25 μl infusion of APV (2.5 μg/side) or VEH into each CEA. A smaller volume and dose of APV was infused into the CEA because the CEA is composed of a smaller volume of brain tissue than the BLA. One minute after the infusion was completed, the injectors were replaced with dummy cannulas, and the rats were immediately placed into the conditioning chambers.

Three minutes after placement in the chambers, rats in the AT+ groups received five tone (85 dB, 10 s, 2 kHz)–footshock (1 s, 1.0 mA; 70-s intershock interval) pairings. One minute after the last footshock, the rats were returned to their home cages. Rats in the AT− group received five tone (85 dB, 10 s, 2 kHz) presentations 3 min after being placed in the conditioning chambers; no footshock was delivered. All rats were returned to their home cages no more than 15 min after completion of the infusion. On all subsequent days, all groups received identical retraining and testing. Rats were transported to the conditioning room each day and placed in the conditioning chambers. For several days following each session in which shock was administered, all rats received 40 min or 60 min of context extinction testing in which no footshock occurred. Because massed-trial training and subsequent retraining and testing all occurred in the same context, this procedure was necessary to ensure that any freezing observed during the tone presentation on Test Days 1–4 was due to the tone–US association rather than the context–US association. After all rats had extinguished contextual fear, rats were given a tone extinction test and retrained (Test Days 1–4). Thus, on each test day, animals were placed in...
the conditioning chambers and, 10 min later, presented with a tone (1 min, 85 dB, 2 kHz) that was immediately followed by a footshock (1 s, 1.0 mA). This procedure was repeated (context extinction followed by tone extinction testing and retraining) until the animals exhibited asymptotic freezing levels to the tone CS. Freezing behavior was measured throughout all experimental sessions.

Results and Discussion

Histology. The injection cannula tip placements for all animals used in analyses are summarized in Figure 1B. Placements were bilaterally symmetric, and there were no differences between groups in the distribution of placements.

Behavior. Figure 2 shows that intra-amygdala infusion of APV into the BLA (Figure 2A) before massed-trial training produced severe deficits in auditory fear conditioning on Test Day 1. This was confirmed in the ANOVA by a significant main effect of group, $F(2, 14) = 8.37, p < .01$. Planned comparisons verified that rats in the AT+/APV–BLA group exhibited levels of freezing that were significantly lower than those shown by the AT+/VEH–BLA group on Test Day 1. Rats in the AT+/APV–BLA group also did not show significantly different levels of conditional freezing from rats in the AT+/VEH–BLA control group on Test Day 1. In contrast, planned comparisons revealed that rats in the AT+/VEH–BLA group exhibited significantly higher levels of freezing on Test Day 1 than rats in any other group. Thus, infusion of APV into the BLA before fear conditioning prevented the acquisition of contextual fear conditioning.

To examine the rate of reacquisition of conditional fear, we converted the percentage of freezing exhibited by each rat on each day to a percentage of the maximum level of freezing shown by that rat and calculated the mean for each group across this measure (Figure 2B). There was no difference in the maximum percentage of time spent freezing across the three groups, $F(2, 14) = 0.09, ns$, validating our use of this measure. It is clear from Figure 2B that

Figure 1. A: Experiment 1 design. Savings of auditory fear was assessed by measuring freezing to a 1-min auditory conditional stimulus on the test days. B: Schematic diagram illustrating injection sites in Experiment 1. Open symbols represent the placement of injector tips in rats infused with D,L-2-amino-5-phosphonopropionic acid (APV). Solid symbols represent the placement of injector tips in rats infused with vehicle (VEH). Numbers next to coronal sections indicate position in millimeters caudal to bregma. Coronal sections are adapted from Swanson (1999). BLA = basolateral complex of the amygdala; CEA = central nucleus of the amygdala.
rats infused with APV into the BLA before massed-trial fear conditioning reacquired conditional contextual fear at the same rate as control rats that did not receive massed-trial conditioning (AT−/VEH–BLA group). This was supported in the ANOVA by a significant main effect of group, $F(2, 14) = 5.76, p < .05$, and a significant Group $\times$ Day interaction, $F(8, 56) = 2.11, p < .05$. Planned comparisons confirmed that rats in the AT+/APV–BLA group did not exhibit different levels of freezing than rats in the AT−/VEH–BLA group on any test day. Hence, there was no evidence for savings in rats that received intra-BLA infusion of APV during massed-trial auditory fear conditioning.

As shown in Figure 3, infusion of APV into the CEA (Figure 3A) before massed-trial training also produced massive impairments in auditory fear conditioning on Test Day 1. This was confirmed in the ANOVA by a significant main effect of group, $F(2, 25) = 26.94, p < .0001$. Planned comparisons revealed that rats in the AT+/VEH–BLA group exhibited higher levels of freezing than rats in any other group, and that rats in the AT+/APV–BLA group exhibited higher levels of freezing that were not significantly different from rats in the AT−/VEH–BLA control group, on Test Day 1. Thus, infusion of APV into the CEA before fear conditioning prevented the acquisition of contextual fear conditioning.

As for BLA-infused rats, we converted the percentage of freezing exhibited by each rat on each day to a percentage of the maximum level of freezing shown by that rat and calculated the mean for each group across this measure (Figure 3B). We observed no difference in the maximum percentage of time spent freezing across the three groups, $F(2, 25) = 1.94, \text{ns}$. Although rats infused with APV into the CEA before massed-trial fear conditioning did not express conditional fear on Test Day 1, it is clear that additional training yielded savings of auditory fear conditioning in these rats (Figure 3B). Savings was manifested as a greater rate of reacquisition after the first reminder trial. An ANOVA on these data revealed a significant main effect of group, $F(2, 25) = 15.52, p < .0001$, and a significant Group $\times$ Day interaction, $F(8, 100) = 4.12, p < .001$. Planned comparisons showed that rats in the AT+/APV group exhibited significantly more freezing on Test Day 2 than rats in the AT−/VEH group. Thus, whereas infusion of APV into the CEA before massed-trial auditory fear conditioning prevented the acquisition of conditional fear, there was savings for this phase of training when the rats were administered additional footshock reminder trials.

**Experiment 2**

The second experiment examined whether NMDA receptor blockade in the BLA or CEA during contextual fear conditioning would affect the acquisition and savings of contextual fear memories (as opposed to the savings of auditory fear memories examined in Experiment 1). Rats were administered contextual fear conditioning after intra-amygdala infusion of either APV or VEH, and they later received additional training. Savings was measured by comparing the rate of reacquisition in these groups relative to a VEH-infused group that was not fear conditioned on the first day. An additional group of rats received the first conditioning session in a different context and served as a nonassociative control for exposure to the footshock US.
Method

The experiment was conducted in two phases (see Figure 4A for an illustration of the paradigm). The first phase consisted of a single day of massed-trial contextual fear conditioning that was administered immediately after infusion of APV or VEH. The second phase (Test Days 1–5) consisted of 5 days of one-trial-per-day gradual reacquisition with no infusion before any session. Rats with cannulas in the BLA, CEA, or CPu (n = 182) were assigned to one of four groups describing treatment on the day of massed training: A+/APV, A−/VEH, A+/VEH, or B+/VEH. These groups differed in terms of the type of infusion (APV or VEH) received before the massed-training session, the context in which the massed-training session occurred (Context A or B), and the presence (“+”) or absence (“−”) of shock during this session. Twenty-one rats were excluded from the analyses as a result of improper placement of guide cannulas. This yielded the following groups: A+/APV–BLA (n = 25), A+/VEH–BLA (n = 17), A−/VEH–BLA (n = 15), B+/VEH–BLA (n = 12), A+/APV–CEA (n = 28), A+/VEH–CEA (n = 23), A−/VEH–CEA (n = 21), B+/VEH–CEA (n = 15), and A+/APV–CPu (n = 5).

Savings in the A+/APV groups was assessed by comparing the rate of reacquisition during Test Days 1–5 (indicated by freezing during the first 3 min of each test day) with the rate of reacquisition in rats receiving VEH infusion before the massed training. All groups receiving VEH on the conditioning day served as controls. The rate of reacquisition among animals in these control groups during infusion-free reacquisition days was a measure of the influence of context exposure in the absence of footshock (A+/VEH), footshock exposure (B+/VEH), or context–US association (A+/VEH).

The day before massed-training trial, rats were preexposed to the infusion procedure as described for Experiment 1. On the day of massed-training trial, the rats were transported in squads of 2 to the infusion room. Rats with guide cannulas aimed at the BLA or CEA received infusions as described for Experiment 1. Rats with guide cannulas aimed at the CPu received a 0.25 μl infusion of APV at a rate of 0.1 μl/min. This yielded an infusion of 2.5 μg of APV per CPu. One minute after the infusion was completed, the injectors were replaced with dummy cannulas, and the rats were immediately placed into the conditioning chambers in either the A or B context.

Three minutes after placement in the chambers, rats in the A+ group received five unsignaled footshocks (2 s, 1.0 mA; 60-s inter-shock interval). One minute after the last footshock, the rats were returned to their home cages. Rats in the A− group were placed in the chambers for 8 min before being returned to the vivarium; no footshock was delivered. All rats were returned to their home cages no more than 15 min after completion of the infusion. This ensured that rats infused with APV would be under the influence of the drug throughout the entire massed-training session. During the following 5 days (Test Days 1–5), all groups received identical retraining and testing in Context A. Rats were transported to the conditioning room each day and placed in the conditioning chambers. Three minutes after placement in the chambers, rats received a single unsignaled shock (1 s, 0.6 mA). Rats were returned to their home cages 30 s after the shock. Pilot experiments demonstrated that naïve rats acquire conditional freezing gradually when these training parameters are used, which was essential for comparing rates of reacquisition across groups. Freezing behavior was measured throughout all experimental sessions.

Results and Discussion

Histology. The injection cannula tip placements for all animals used in analyses are summarized in Figure 4B. Placements were bilaterally symmetric, and there were no differences between groups in the distribution of placements.

Behavior. As shown in Figure 5A, it is apparent that rats infused with VEH into the BLA before massed-trial conditioning exhibited higher levels of freezing behavior after conditioning than before massed-trial conditioning. In contrast, rats that received...
Intra-amygdala infusions of APV into the BLA exhibited no conditional freezing on Test Day 1, suggesting that pretraining infusion of APV in the BLA prevented the acquisition of contextual fear conditioning. Rats that received massed-trial context conditioning in the B context (B+/VEH–BLA group) also did not exhibit conditional freezing on Test Day 1, indicating that freezing in the A+/VEH–BLA group was associative. These impressions were confirmed in the ANOVA by a significant main effect of group, $F(3, 65) = 10.69, p < .0001$. Post hoc comparisons confirmed that the levels of freezing in the B+/VEH–BLA, A+/APV–BLA, and A–/APV–BLA groups were significantly lower than those shown by rats in the A+/VEH–BLA group. Hence, APV infusion into the BLA prevented the acquisition of associative contextual freezing.

As with Experiment 1, we expressed the percentage of time spent freezing on each day relative to the maximum percentage of time spent freezing to more closely examine the rate of reacquisition of conditional fear. With this measure, it is clear that rats infused with APV in the BLA (Figure 5B) reacquired contextual fear at a rate identical to the rate of naive rats that did not receive any footshock on the initial training day (the A–/VEH–BLA group). Interestingly, rats in the A+/APV–BLA group also reacquired conditional freezing more slowly than rats that received massed-trial fear conditioning in a different context (B+/VEH–BLA group). That is, there was no evidence for savings of fear memory in rats fear conditioned after intra-BLA APV infusions. These results were supported by a significant main effect of group, $F(3, 65) = 8.28, p < .0001$, and a significant Group × Day interaction, $F(15, 325) = 4.25, p < .0001$. Also, post hoc comparisons confirmed that rats in the A+/APV–BLA group did not exhibit different freezing levels from rats in the A–/VEH–BLA group on any test day.

As shown in Figure 6A, intra-CEA infusion of APV before massed-trial contextual fear conditioning abolished the acquisition of contextual fear conditioning. This was confirmed in the ANOVA by a significant main effect of group, $F(3, 83) = 10.04,$
Post hoc comparisons revealed that rats in the A+/APV–CEA group exhibited levels of freezing on Test Day 1 that were not significantly different from rats in either the A+/VEH–CEA or B+/VEH–CEA group. However, rats in the A+/VEH–CEA group exhibited significantly higher levels of conditional freezing on Test Day 1 than rats in any other group. Hence, APV infusion into the CEA prevented the acquisition of associative contextual freezing.

In contrast to the pattern of results obtained with intra-BLA infusion of APV, rats infused with APV into the CEA before massed-trial fear conditioning (Figure 6B) showed a faster rate of reacquisition than controls that did not receive massed-trial fear.
conditioning. An ANOVA revealed a significant main effect of group, $F(3, 83) = 7.63, p = .0001$, and a significant Group $\times$ Day interaction, $F(15, 415) = 3.90, p = .0001$. The rate of reacquisition in the A+/APV–CEA group was equivalent to that of rats receiving massed-trial footshock in a different context (B+/VEH–CEA). Post hoc comparisons confirmed that the levels of conditional freezing expressed by rats in the A+/APV group were equivalent to those expressed by rats in the B+/VEH group at all time points. These data indicate that rats receiving fear conditioning after intra-CEA infusions of APV exhibit some savings of fear memory.

Importantly, the deficits in acquisition of conditional fear that we observed in rats infused with APV were due to local effects of the APV in the amygdala and not to distal effects in adjacent structures. As can be seen in Figure 7, infusion of APV into the CPu dorsal to the amygdala before massed-trial contextual fear conditioning had no effect on either acquisition or savings of conditional fear. The ANOVA revealed a significant main effect of group, $F(2, 78) = 15.96, p < .0001$, and a significant Group $\times$ Day interaction, $F(10, 390) = 7.29, p < .0001$. Post hoc comparisons revealed that rats in the A+/APV–CPu group did not differ from rats in the A+/VEH group on any test day.

In this study, rats in the A+/VEH–BLA control group exhibited relatively low levels of conditional contextual freezing on Test Day 1 (approximately 30%), especially in comparison with the levels of conditional tone-elicted freezing observed in comparable control animals in Experiment 1 (approximately 55%). It could be argued that there was insufficient associative fear after massed-trial fear conditioning to promote savings in the A+/APV–BLA group. To further examine this issue, we assessed the rate of reacquisition of conditional contextual freezing in animals exhibiting the greatest levels of conditional freezing after massed-trial training. The data from the top quartile of animals in each group (based on conditional freezing exhibited on Test Day 1) were averaged, and these averages are depicted in Figure 8. From these data, it is clear that animals in the A+/VEH–BLA group exhibited high levels of freezing after massed-trial training (roughly 60%), but animals infused with APV (A+/APV–BLA group) still exhibited no savings of conditional contextual fear. Thus, even at best, animals infused with APV in the BLA do not show savings for their original massed-trial training. These results were confirmed in the ANOVA by a near-significant main effect of group, $F(3, 13) = 3.21, p = .06$, and a significant Group $\times$ Day interaction, $F(15, 65) = 2.73, p < .01$. Post hoc comparisons showed that rats in the A+/APV–BLA group did not differ from the A–/VEH–BLA group through Test Day 4.

Thus, this experiment shows that pretraining infusion of APV in the BLA or CEA produced massive impairments in the acquisition of conditional contextual freezing. These effects were not attributable to diffusion into the overlying CPu. In addition, whereas intra-BLA infusion of APV prevented behavioral savings of context conditioning, animals receiving intra-CEA infusions of APV before training exhibited a level of savings comparable to that exhibited by rats that received footshock in a different context on the massed-trial training day (B+/VEH–CEA group).

**General Discussion**

The present results suggest that NMDA receptor activation in both the BLA and CEA is essential for the acquisition of Pavlovian fear conditioning. These experiments reveal that infusion of APV into the BLA or CEA before fear conditioning prevents the acquisition of auditory and contextual conditional fear. These experiments also reveal behavioral savings of conditional contextual and auditory fear, as measured by the rate of reacquisition during subsequent drug-free retraining, in rats infused with APV into the CEA, but not BLA, before fear conditioning. This confirms and extends the results of numerous experiments indicating that APV infusion into the BLA blocks the acquisition of conditional freeze-
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by VEH-infused rats that received footshock exposure in a differ-
shocked control rats, and no difference (i.e., no savings) was
large neurotoxic BLA lesions with the freezing exhibited by un-
tensive comparison of the freezing behavior exhibited by rats with
fear (Maren et al., 2001). Moreover, although one report has
suggested that rats with large neurotoxic lesions of the BLA
fear in rats with posttraining BLA lesions (Maren, 1998, 1999b,
2001a). We have also shown that pretraining inactivation of the
BLA with the gamma-aminobutyric-acid (GABA\textsubscript{A}) receptor ago-
muscomol prevents behavioral savings of conditional auditory
fear (Maren et al., 2001). Moreover, although one report has
suggested that rats with large neurotoxic lesions of the BLA
exhibit savings of contextual fear (Cahill, Vazdarjanova, & Set-
low, 2000), the behavior of these rats was not compared with
the behavior of unshocked controls. Our laboratory has done an ex-
tensive comparison of the freezing behavior exhibited by rats with
large neurotoxic BLA lesions with the freezing exhibited by un-
shocked control rats, and no difference (i.e., no savings) was
observed in the lesioned rats across any measure (Maren, 2001a).

In Experiment 2, we observed that the level of savings of rats in the
A+/APV–CEA group was comparable to the savings exhibited by
VEH-infused rats that received footshock exposure in a differ-
ent context on the massed-trial training day (B+/VEH–CEA). The savings exhibited by rats in the B+/VEH–CEA group may reflect
associative generalization between the training and testing con-
texts. Alternatively, these savings may be attributable to a nonas-
ociative stress-related facilitation of learning similar to the facil-
itation of the acquisition of eyeblink conditioning by tailshock stress (Shors & Mathew, 1998). Likewise, the savings observed
after intra-CEA infusion of APV may be either associative or
nonassociative. With regard to the former, a small portion of the
CEA may have remained functional during massed-trial training.
Functional CEA neurons may have acquired a subthreshold associ-
itive memory trace that produced behavioral savings with addi-
tional training. It is also possible that nonassociative effects of
footshock stress during massed-trial training enhanced the sub-
sequent acquisition of conditional fear with further training in a
different context. The latter explanation is particularly appealing,
in that nonassociative facilitation of learning by shock stress is
mediated by the BLA and not the CEA (Shors & Mathew, 1998).

Consistent with these findings, we have observed facilitated learning (i.e., savings) of conditional fear after infusion of APV in
the CEA but not the BLA. Thus, our data suggest that the BLA
may be capable of processing some aspects of the footshock US in
the absence of a functional CEA but that this spared memory is not
apparent without additional fear conditioning. In this case, our data
further indicate that NMDA receptor blockade in the BLA pre-
vents both associative and nonassociative processing during fear
conditioning. In contrast, NMDA receptor blockade in the CEA
interfered only with associative processing.

The findings reported here agree with a number of studies highlighting the importance of the amygdala in acquisition of
Pavlovian conditional fear. For example, two recent studies have
shown that much of the plasticity observed in the MGN after fear
conditioning is amygdala dependent (Maren et al., 2001; Poe-
rema & Gabriel, 2001). Thus, in the absence of the amygdala, at least
one amygdala afferent is unable to acquire long-term associative
plasticity. Our data suggest that, in the absence of functional
amygdaloid NMDA receptors, other amygdala afferents may be
similarly impaired in the acquisition of long-term plasticity, or the
plasticity acquired is simply insufficient to drive conditional
responding.

A recent model of the cellular mechanisms in the amygdala that
underlie associative fear learning (Blair, Schafe, Bauer, Rodrigues,
& LeDoux, 2001) suggests that NMDA receptors play a critical
role in initiating long-term synaptic plasticity in amygdala neu-
rons. By this model, ionic currents flowing through non-NMDA
receptors yield back-propagating action potentials that activate
dendritic voltage-gated calcium channels (VGCCs). VGCCs con-
vert the short-term plasticity enabled by the NMDA receptor to a
more enduring form of long-term synaptic plasticity (i.e., long-
term potentiation). This model predicts that NMDA receptor an-
tagonism in the BLA during fear conditioning would prevent both
short- and long-term synaptic plasticity. Our results support this
model and suggest that NMDA receptor-dependent synaptic plas-
ticity is critical for establishing long-term fear memories. Of
course, NMDA receptor antagonists may have impaired memory
by reducing the excitability of amygdaloid neurons, independent of
any effects on synaptic plasticity (Fendt, 2001; H. J. Lee et al.,

Our data are not consistent with the suggestion that physiolog-
ical plasticity observed in the BLA and CEA during aversive
associative learning (e.g., Maren, 2000; Quirk et al., 1995, 1997)
is passively mirrored from afferent structures (Weinberger et al.,
1993). Similarly, our data are not consistent with claims that the
amygdala serves only to modulate Pavlovian associative memories
generated and stored elsewhere (McGaugh, Introuti-Collison, Ca-
hill, Kim, & Liang, 1992). In both of these cases, extra-amygdaloid
structures would be expected to mediate associative learning dur-
fear conditioning under NMDA receptor blockade of the
amygdala. The deficits in conditional fear observed in animals
trained under amygdaloid NMDA receptor antagonism could then
be attributed to either a retrieval failure during extinction testing or
the failure of the original training to generate a strong memory
trace. However, when additional training is administered, one
would predict that rats trained under NMDA receptor blockade of
the BLA or CEA should reacquire conditional fear at a rate faster
than that of nonshocked controls, demonstrating savings of con-
ditional fear. This pattern of results has been observed in the
eyeblink-conditioning paradigm, for example (Clark & Lavond,
1993; Krupa, Thompson, & Thompson, 1993). In this case, inac-
tivation of the cerebellum, the locus of CS–US association, pre-
vented acquisition of eyelink conditioning and resulted in no
savings for the original training. In contrast, inactivation of the red
nucleus, a performance structure that translates the CS–US asso-
ciation into the eyelink conditional response, resulted in substan-
tial savings for the original training. The results presented here
indicate that there is no savings of contextual fear or auditory fear after training under NMDA receptor blockade in the BLA. This suggests that the BLA is a critical locus of plasticity during Pavlovian fear conditioning.

Our results are also inconsistent with one study suggesting that infusion of APV in the CEA has no effect on acquisition of Pavlovian fear conditioning (Fanselow & Kim, 1994). The reason for this discrepancy is unclear; however, a more anterior portion of the CEA was targeted in the present experiments. This may have resulted in inactivation of a larger portion of the medial division of the CEA (CEAm) by APV infusion than in the study by Fanselow and colleagues. Because the CEAm is the portion of the central nucleus that projects most heavily to the brain areas responsible for driving conditional responding (LeDoux et al., 1988; Veening, Swanson, & Sawchenko, 1984), the coordinates used in the present experiments may have inactivated the portion of the CEA that plays the most critical role in fear conditioning.

Several studies have reported that lesions of the CEA affect the acquisition of Pavlovian appetitive tasks (Gallagher, Graham, & Holland, 1990; Parkinson, Robbins, & Everitt, 2000). Thus, it is clear that the CEA plays a critical role in associative learning in at least some Pavlovian paradigms. In spite of the role the CEA plays in the acquisition of other Pavlovian conditional responses, the nature of the role of the CEA in Pavlovian fear conditioning has remained elusive. It is often assumed that the largely unidirectional transmission of information that leads to a rather strict segregation of learning and performance structures in the circuitry underlying eyeblink conditioning (Clark & Lavond, 1993; Krupa et al., 1993; Medina, Repa, Mauk, & LeDoux, 2002; Steinmetz, 2000) exists as well in the circuitry supporting fear conditioning (LeDoux, 2000; Maren, 2001b). Also, the CEA is ideally situated to be a performance structure because it directly projects to the brainstem nuclei that control the conditional responses evoked by fearful stimuli (LeDoux et al., 1988). Yet, it has since been shown that there is extensive reciprocal information flow between amygdaloid nuclei (Pitkänen, Savander, & LeDoux, 1997). Thus, a strict segregation of learning and performance may not be applicable to fear conditioning circuitry. Indeed, because the CEA itself is a site of CS–US convergence, it seems likely that plasticity in the CEA is involved in the acquisition of fear conditioning (see Pascoe, Supple, & Kapp, 1991).

In support of this, there are recent reports that manipulations of CEA block the acquisition of conditional freezing. Specifically, acquisition of Pavlovian fear conditioning can be blocked by pretraining inactivation of CEA with muscimol, and this effect can be observed with smaller doses of muscimol than those required for intra-BLA infusions of muscimol (Wilensky, Schafe, & LeDoux, 2000). Similar to the data reported here, this report suggests that muscimol is interfering with the acquisition of conditional fear through local mechanisms in the CEA, rather than by diffusing to the proximal BLA. Furthermore, it has also been shown that posttraining infusion of the protein synthesis inhibitor anisomycin in the CEA blocks the formation of long-term fear memories (Wilensky, Schafe, & LeDoux, 2001), paralleling the results obtained with intra-BLA infusion of anisomycin (Schafe & LeDoux, 2000).

Our observation that NMDA receptor activation in the CEA appears to be critical for the acquisition of conditional fear suggests that the CEA could be a locus of CS–US association underlying the acquisition of conditional freezing (Pascoe & Kapp, 1985). Whereas this is consistent with reports showing associative plasticity in CEA after fear learning, we have recently shown that intra-amygdala infusion of a protein kinase A and C inhibitor blocks the acquisition of conditional fear when infused into the BLA but not the CEA (Goosens et al., 2000). This suggests that if the BLA and CEA both play roles in the long-term storage of the associative plasticity underlying fear conditioning, they must do so by different molecular mechanisms. Alternatively, the CEA might have NMDA-receptor-dependent projections that tonically modulate activity in the BLA. Because APV has been shown to block synaptic transmission in the BLA (Maren & Fanselow, 1995), intra-CEA administration of APV might also inactivate CEA neurons and indirectly disrupt synaptic transmission in the BLA. In this case, the disruption of Pavlovian fear conditioning observed after intra-CEA infusion of APV would occur through indirect mechanisms. Further research will be aimed at distinguishing between these hypotheses and clarifying the role of the CEA in Pavlovian fear conditioning.

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Received July 22, 2002
Revision received March 5, 2003
Accepted March 7, 2003

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