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EXTENDED FEAR CONDITIONING REVEALS A ROLE FOR BOTH N-METHYL-D-ASPARTIC ACID AND NON-N-METHYL-D-ASPARTIC ACID RECEPTORS IN THE AMYGDALA IN THE ACQUISITION OF CONDITIONED FEAR

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Abstract

Pavlovian conditioning is a useful tool for elucidating the neural mechanisms involved with learning and memory, especially in regard to the stimuli associated with aversive events. The amygdala has been repeatedly implicated as playing a significant role in the acquisition and expression of fear. If the amygdala is critical for the acquisition of fear, then it should contribute to this processes regardless of the parameters used to induce or evaluate conditioned fear. A series of experiments using reversible inactivation techniques evaluated the role of the amygdala in the acquisition of conditioned fear when training was conducted over several days in rats. Fear-potentiated startle was used to evaluate the acquisition of conditioned fear. Pretraining infusions of *N*-methyl-D-aspartic acid (NMDA) or non-NMDA receptor antagonists alone into the amygdala interfered with the acquisition of fear early in training, but not later. Pretraining infusions of a cocktail consisting of both an NMDA and non-NMDA antagonist interfered with the acquisition of conditioned fear across all days of training. Taken together these results suggest the amygdala may potentially be critical for the acquisition of conditioned fear regardless of the parameters utilized.

Keywords

fear conditioning; rats; learning; memory; NBQX; APV

In a typical pavlovian fear-conditioning procedure, a neutral stimulus, such as a tone, is paired with an aversive unconditioned stimulus, such as shock. As a consequence of this pairing, the tone acquires the ability to elicit a variety of behaviors indicative of a central state fear (McAllister and McAllister, 1971). Because this learning is rapid, stable and present in a variety of species (including humans), pavlovian fear conditioning has become a favored model system for investigating the neural substrates of mammalian learning and memory.

Several investigators have demonstrated that the amygdala plays a critical role in pavlovian fear conditioning. For example, lesions of the amygdala prior to fear conditioning prevent the acquisition of conditioned fear (e.g. Kapp et al., 1979; Phillips and LeDoux, 1992), and lesions

after fear conditioning disrupt the expression of conditioned fear (e.g. Hitchcock and Davis, 1986; Campeau and Davis, 1995; Maren et al., 1996a; Cousens and Otto, 1998; Heldt et al., 2000). In addition, electrophysiological recordings made from the amygdala suggest that neurons in the amygdala respond to the tone and shock and undergo plastic changes with fear conditioning (Maren et al., 1991; Bordi et al., 1993; Quirk et al., 1995, 1997; McKernan and Shinnick-Gallagher, 1997). Moreover, several studies have investigated the contribution of second messenger systems in the amygdala to conditioned fear and have documented changes in gene expression in the amygdala associated with fear conditioning (Goosens et al., 2000; Lin et al., 2001; Stork et al., 2001; Malkani et al., 2004; Rattiner et al., 2004). These data, together with data showing that structures involved in processing of the tone (or light) conditioned stimulus (CS) and shock unconditioned stimulus project to the amygdala (LeDoux et al., 1990a; Romanski and LeDoux, 1992; Shi and Davis, 1998, 2001), have led many investigators to suggest that the amygdala is the site for learning and memory associated with pavlovian conditioned fear (Davis, 1998; Maren, 1999a; LeDoux, 2000). *N*-methyl-D-aspartic acid (NMDA) receptors in the amygdala have been implicated in synaptic plasticity and long-term potentiation (LTP) (Chapman et al., 1990; Clugnet and LeDoux, 1990), and evidence indicates these receptors play role in the acquisition of conditioned fear (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Fanselow et al., 1994; Maren et al., 1996b), while non-NMDA receptors appear to play a role in the expression of conditioned fear (Kim et al., 1993; Walker and Davis, 1997; Zhao and Davis, 2004).

If the amygdala is essential for the acquisition of conditioned fear, then inactivation of the amygdala prior to training should interfere with fear conditioning regardless of the experimental parameters utilized. However, there are challenges to this idea. For example, rats with lesions of the amygdala can learn to avoid a compartment where shock has been delivered (Vazdarjanova and McGaugh, 1998), and rats with significant amounts of fear conditioning prior to a lesion of the central nucleus of the amygdala can re-acquire conditioned fear (Kim and Davis, 1993). Studies by Maren (1998, 1999b) indicate the amygdala may not be necessary during overtraining procedures.

These challenges to the idea that the amygdala is the site for learning and memory associated with pavlovian conditioned fear have highlighted the need for examining the contribution of the amygdala across a variety of fear conditioning parameters. Once again, if the amygdala is a critical site for learning and memory associated with pavlovian conditioned fear, then lesions of the amygdala should produce deficits in conditioned fear across a variety of fear conditioning situations.

In light of evidence the amygdala may play a time-limited role in conditioned fear (Breiter et al., 1996; Buchel et al., 1998; LaBar et al., 1998; Phelps et al., 2001), and the fact that most experimental fear conditioning paradigms use a relatively small number of CS-US trials over 1 or 2 days (but see Kim and Davis, 1993; Lee et al., 1996), it is critical to evaluate the role of the amygdala with a paradigm implementing more widely spaced training. Even when overtraining was examined, conditioning was conducted in a single session (Maren, 1998, 1999b). Therefore, we evaluated the contribution of the amygdala to the acquisition of conditioned fear over several days of spaced training. The purpose of the present experiments was to examine the effect of pre-training amygdala inactivation on conditioned fear trained across several sessions without overtraining, i.e. traditionally fear-potentiated startle is evaluated after two daily sessions with 10 CS+shock (CS⁺) trials for a total of 20 and the current study only used 25 CS⁺ trials. To this end, rats were given five tone (or light) and shock pairings on each of 5 days. Conditioned fear was assessed with the fear-potentiated startle procedure in which conditioned fear is defined as elevated startle amplitude in the presence versus the absence of the noise (or light) CS. Temporary inactivation of the amygdala was produced acutely by microinfusing a glutamate antagonist directly into the amygdala immediately before

fear conditioning. If the amygdala is a critical site for learning and memory associated with pavlovian conditioned fear, then infusions of glutamate antagonists into the amygdala prior to fear conditioning should produce deficits in conditioned fear regardless of the number of fear conditioning sessions.

EXPERIMENTAL PROCEDURES

Subjects

Male albino Sprague–Dawley rats (Charles River, Montreal, Quebec, Canada) weighing between 300 and 400 g were used. All rats were housed singly in standard shoebox cages. The rats were maintained on a 12-h light/dark cycle (lights on at 7 a.m.) with food and water available *ad libitum*. All studies conformed to guidelines for the ethical treatment of animals (National Institutes of Health, American Psychological Association), and were approved by the University of Vermont Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Apparatus

Conditioning and fear-potentiated startle testing were conducted in four identical stabilimeter devices. Briefly, each stabilimeter consisted of an 8×15×15-cm acrylic and wire mesh cage suspended between compression springs within an acrylic frame. Each cage was located within a custom designed 90×70×70 ventilated sound attenuating chamber. The floor of each stabilimeter consisted of four 6.0-mm diameter stainless steel bars spaced 18 mm apart, through which shock could be administered. Cage movement resulted in displacement of an accelerometer (model U321AO2; PCB Piezotronics, Depew, NY, USA) with the resulting voltage being proportional to the velocity of displacement. The analog output of the accelerometer was amplified (Finitronics, Model 483B21; Finitronics, Orange, CT, USA) and digitized on a scale of 0–10 V units by an InstruNET analog to digital converter (GW Instruments, Model 100B; Somerville, MA, USA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximum peak-to-peak voltage that occurred during the first 200 ms after onset of the startle-eliciting stimulus.

Startle responses were elicited by 50 ms white-noise bursts (5 ms rise-decay) generated by a Macintosh G3 computer sound file (0–22 kHz), amplified by a Radio Shack Amplifier (100 W; model MPA-200; Tandy, Fort Worth, TX, USA), and delivered through high frequency speakers (Radio Shack Supertweeter; Tandy) located 5 cm from the back of each cage. The CSs were a 4-s light (82 lux) produced by an 8-W fluorescent bulb (100- μ s rise time) located 10 cm behind each cage, and a 4-s white noise (80 dB) provided by a General Radio type 1390-B noise generator (Concord, MA, USA), delivered through a woofer (Kenwood; model KFC-1675, Long Beach, CA, USA) located 9 cm beside each cage. The unconditioned stimulus was a 0.5-s shock, delivered to the floor bars, and produced by a BRS/LVE shock generator (SGS-004, Laurel, MD, USA). Shock intensity was 0.6 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter, Glassbeads Inc.; Newton, CT, USA).

Surgery

Rats were anesthetized with 80 mg/kg of ketamine/xylazine given i.p. (70 mg/kg of ketamine and 10 mg/kg of xylazine), and placed in a stereotaxic instrument. The skull was exposed and 22-gauge guide cannula (model C313G, Plastics One, Roanoke, VA, USA) were implanted bilaterally aimed at the amygdala (AP=−3.0; DV=−8.3; ML=±5.0 from bregma). Screws (part no. Y-MX-080-2FL, Small Parts, Miami Lakes, FL, USA) were anchored to the skull and the assembly was cemented in place using dental cement (part no. 300CCP, Plastic Products, Roanoke, VA, USA). Dummy cannulas (model C313DC/CAC, Plastics One) were inserted

into each cannula to prevent clogging, and these were cut to be flush with the end of the guide cannula. Rats were given buprenorphine (0.5 mg/kg) every 12 h for four times after surgery. Rats were given 7–10 days' recovery before behavioral training was initiated, and their post-operative health was monitored daily. The dummy cannulas were checked daily to ensure the cannula were not clogged.

Drug infusion

2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; Sigma, St. Louis, MO, USA), (2*R*)-amino-5-phosphonovaleric acid (APV; Sigma), cocktail (3 μ g NBQX and 2.5 μ g APV) in 0.5 μ l or vehicle (phosphate-buffered saline) was infused into the vicinity of the amygdala prior to each training session. All drugs were mixed in phosphate-buffered saline. Injections were made through 28 gauge injection cannula (Model C313I, Plastic Products) connected to Hamilton microsyringes via polyethylene tubing. For the infusions the rats were transported to the infusion room, were placed in a holding cage, their dummy cannulas were removed, and the injectors were lowered into the guide cannula. The infusions were made at a rate of 0.25 μ l/min. After the infusions were complete, the injectors were left in place for 1 min to allow for drug diffusion into the amygdala.

Behavioral procedures

The behavioral procedure consisted of the following phases: startle adaptation, fear conditioning and fear-potentiated startle.

Startle adaptation—On each of three consecutive days, rats were placed into the startle cages and given 60 startle stimuli (10 each at 85, 90, 95, 100, 105 and 110 dB) using an inter-stimulus interval (ISI) of 30 s. Based on these tests the rats were matched into groups such that each group had equivalent mean levels of baseline startle amplitude.

Fear conditioning—On each of 5 days, beginning 1 day after the final startle adaptation session, rats were placed into the startle cages and, after 5 min, were given five CS⁺ trials. The duration of both the light and noise CS was 4 s. The foot shock was delivered during the last 0.5 s of the CS. The average ISI was 9 min (range: 7–11 min). The CS⁺ was either a light or noise (counterbalanced across groups).

Fear-potentiated startle—Rats were placed in the startle cages and, after 5 min, were presented with 15 startle-eliciting noise bursts (five each at 95, 105 and 110 dB; 30 s ITI). These startle stimuli (leaders) were used to establish a stable baseline. 30 s after the final leader was presented, each animal received 15 startle-eliciting noise bursts (five each at 95, 105, 110 dB) presented alone (noise-alone trials), 15 startle-eliciting noise bursts (five at each intensity) presented 3.5 s after the onset of the CS⁺ and 15 noise bursts (five at each intensity) presented 3.5 s after the onset of the CS⁰. The CS⁰ was the alternate CS modality, and was a neutral stimulus never presented during training. The use of both the light and noise as CSs was implemented to determine if any observed effect was unique to a specific stimulus type. Presentation of the CS⁰ during testing was implemented to assess the unconditioned effects of the light and noise CS on startle, and to see if the infused drugs altered these unconditioned effects. All of the trial types were presented in a pseudo-random order (ITI=30 s). For each rat, mean startle difference scores were computed by subtracting the average response to the startle-eliciting noise bursts alone from the average response to the noise burst when it was preceded by the CS⁺ or CS⁰.

Histology

Rats were given an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and immersed in a 30% sucrose-formalin solution for at least 3 days. Coronal sections (60 μ m) were cut through the amygdala; every second slice was mounted onto a gelatin-coated slide. The sections were stained with Thionin and were examined under a light microscope for cannula placement. Rats with misplaced cannula (defined as injector placement greater than 0.5 mm from the amygdala) or lesions were excluded from statistical analysis, and the numbers listed in the procedure section indicate the number of rats after removal due to histological evaluation.

Procedure

In experiment 1, 32 rats were divided into six groups: APV 10 μ g-Light CS ($n=6$); APV 2.5 μ g-Light CS ($n=5$); Vehicle-Light CS ($n=5$); APV 10 μ g-Noise CS ($n=5$); APV 2.5 μ g-Noise CS ($n=5$); Vehicle-Noise CS ($n=5$). The first 3 days consisted of startle adaptation. On day 4 all groups were given a session of fear conditioning, and pretraining infusions were administered. On day 5 all groups were given a test for fear-potentiated startle without any infusions. This training-testing protocol was conducted four more times for a total of five fear conditioning sessions and five tests for fear-potentiated startle.

In experiment 2, 40 rats were divided into six groups: NBQX 3 μ g-Light CS ($n=9$); NBQX 9 μ g-Light CS ($n=4$); Vehicle-Light CS ($n=7$); NBQX 3 μ g-Noise CS ($n=8$); NBQX 9 μ g-Noise CS ($n=4$); Vehicle-Noise CS ($n=9$). The procedure for this experiment was identical to experiment 1 except that rats were given pretraining infusions of NBQX instead of APV.

In experiment 3, 22 rats were divided into four groups: APV 2.5 μ g/NBQX 3 μ g-Light CS ($n=5$); Vehicle-Light CS ($n=6$); APV 2.5 μ g/NBQX 3 μ g-Noise CS ($n=5$); Vehicle-Noise CS ($n=6$). The procedure was identical to experiments 1 and 2 except that rats were given pretraining infusions of the cocktail instead of NBQX or APV alone. In this experiment rats (previously trained with the cocktail) were also given 10 further CS⁺ trials drug free 24 h after the 5th test for fear-potentiated startle. A 6th test for fear-potentiated startle was given 24 h later without drug infusions. These further training and test sessions were conducted to evaluate if the blockade of acquisition resulted from damage to the amygdala caused by repeated infusions of the cocktail.

Data analysis

Fear-potentiated startle was calculated by subtracting the amplitude of startle to the noise burst alone from the amplitude of startle to the noise burst when it was preceded by the CS⁺ (CS⁺ difference). Fear-potentiated startle was defined as greater startle amplitude in the presence of the CS⁺ compared with startle amplitude in the presence of the noise burst alone. To evaluate the acquisition of fear-potentiated startle a priori within-subject *t*-tests were computed comparing startle to the noise burst alone with startle preceded by the CS⁺ for each group at each test. ANOVAs were computed on the CS⁺ difference scores with Test (fear-potentiated startle tests 1–5) as a within-subject variable, and Drug (vehicle, NBQX 3 μ g, NBQX 9 μ g, APV 2.5 μ g, APV 10 μ g or NBQX 3 μ g and APV 2.5 μ g) as a between-subject variable.

Experiment 1

In order to evaluate the effect of pretraining infusions of the NMDA antagonist APV over the course of fear conditioning, rats were given 1 day of training (five CS⁺ trials) followed by a test for fear-potentiated startle 24 h later. Prior to each training session, the rats were given infusions of either APV or vehicle, and testing was then conducted drug-free. This design

allowed for the assessment of APV's effects on fear conditioning at multiple time points during acquisition. Based on evidence from previous studies (Miserendino et al., 1990; Campeau et al., 1992; Kim and McGaugh, 1992; Fanselow and Kim, 1994; Maren et al., 1996b), it was predicted that inactivation of the amygdala with APV would block the acquisition of fear-potentiated startle.

To evaluate their contribution during extended fear conditioning rats were given APV 2.5 μ g/side, APV 10 μ g/side, or vehicle prior to each training session. The 2.5 μ g dose of APV effectively blocked the acquisition of fear in previous studies (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Fanselow et al., 1994; Maren et al., 1996b), and the 10 μ g dose of APV was used to insure that the spaced training procedure did not alter the minimum effective dose for APV.

RESULTS

The data from six rats were excluded from the study due to incorrect cannula location or damage in the vicinity of the amygdala. The locations of injector placements are shown in Fig. 1A and the timeline for the experiment is shown in Fig. 1B. Pretraining infusions of APV attenuated, but did not block the acquisition of conditioned fear over five tests for fear-potentiated startle (see Fig. 1C). Paired *t*-tests comparing startle to the NB alone vs. startle to the CS⁺ indicated the vehicle group exhibited fear-potentiated startle at all five tests. Pretraining infusion of 2.5 μ g APV interfered with the acquisition of fear-potentiated startle at test 1, but not tests 2–5, and pretraining infusions of 10 μ g APV interfered with acquisition at tests 1–3, but not 4 and 5. The Drug $\times$ Test ANOVA for CS⁺ difference scores indicated a main effects for Test ($F_{(4,116)}=11.347$, $P<0.001$) and Drug ($F_{(2,29)}=4.583$, $P=0.019$), but no Test $\times$ Drug interaction ($F_{(8,116)}=1.436$, $P=0.189$). There was no effect of APV on startle preceded by the CS⁰.

Overall, the results from experiment 1 suggest antagonism of NMDA receptors alone is not sufficient to prevent the acquisition of fear-potentiated startle over several days of fear conditioning, although it was not to the same levels as vehicle infused animals (see Fig. 1C). This experiment replicated previous studies indicating antagonism of NMDA receptors in the amygdala during 1 or 2 days of fear conditioning interferes with the acquisition of conditioned fear (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Fanselow et al., 1994; Maren et al., 1996b). However, given the results from the current experiment, antagonism of NMDA receptors in the amygdala may not be sufficient to prevent the acquisition of conditioned fear over several days of fear conditioning.

Experiment 2

Since APV failed to block the acquisition of fear-potentiated startle after 5 days of training, and several studies using a variety of compounds indicate amygdala inactivation prior to training interferes with the acquisition of fear (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Fanselow et al., 1994; Helmstetter and Bellgowan, 1994; Maren et al., 1996b; Muller et al., 1997), antagonism of NMDA receptors may not be sufficient with extended training. In order to evaluate this possibility, rats underwent fear conditioning according to the same protocol used in experiment 1. Instead of APV, we used the non-NMDA receptor antagonist NBQX to inactivate the amygdala prior to training. CNQX, a less potent version of NBQX, has been used to investigate the role of the amygdala in the expression of conditioned fear (Izquierdo et al., 1993; Kim et al., 1993; Mesches et al., 1996).

Results

The data from three rats were excluded from analysis due to misplaced cannula or damage in the vicinity of the amygdala. The locations of injector placements are shown in Fig. 2A, and

the timeline for the experiment is shown in Fig. 2B. Pre-training infusions of NBQX slowed, but did not prevent, the acquisition of fear-potentiated startle (see Fig. 2C). Paired *t*-tests comparing startle to the noise burst alone with startle preceded by the CS⁺ indicated rats administered pretraining infusions of the vehicle exhibited fear-potentiated startle at tests 1–5. Pretraining infusion of 3 μ g NBQX interfered with the acquisition of fear-potentiated startle at test 1, but not tests 2–5, and pretraining infusion of 9 μ g NBQX interfered at tests 1–3 and 5, but not test 4. The Group $\times$ Test ANOVA for the CS⁺ difference score indicated main effects for Drug ($F_{(2,37)}=23.823$, $P=0.003$) and Test ($F_{(4,148)}=13.701$, $P<0.001$), but no Drug $\times$ Test interaction ($F_{(8,148)}=0.887$, $P=0.529$). There was no effect of NBQX on startle preceded by the CS⁰.

These results suggest that inactivation of the amygdala by NBQX is also not sufficient to prevent the acquisition of conditioned fear over several days of fear conditioning. Similar to the results from experiment 1, the current experiment suggests that antagonism of amygdala AMPA/kainate receptors also interferes with the acquisition of conditioned fear after 1 or 2 days of training, but not after 5 days. These results support the findings of previous experiments indicating intra-amygdala infusions of NMDA antagonists and muscimol interfere with the acquisition of conditioned fear after 1 or 2 days of fear conditioning (Miserendino et al., 1990; Campeau et al., 1992; Kim et al., 1993; Fanselow and Kim, 1994; Fanselow et al., 1994; Helmstetter and Bellgowan, 1994; Maren et al., 1996b; Muller et al., 1997).

Experiment 3

Because neither NBQX nor APV alone was sufficient to block the acquisition of conditioned fear, it is possible that in the absence of one, the other can mediate the acquisition of conditioned fear. To evaluate this possibility, two groups of rats underwent fear conditioning and testing exactly the same as for experiments 1 and 2. Prior to each fear conditioning session, one group was given bilateral intra-amygdala infusions of a cocktail (3 μ g of NBQX and 2.5 μ g of APV/side) and the other group was given bilateral intra-amygdala infusions of vehicle.

Results

The data from two rats were excluded due to damage in the vicinity of the amygdala. The locations of injector placements are shown in Fig. 3A, and the timeline for the experiment is shown in Fig. 3B. Pretraining infusions of a cocktail of both NBQX (3 μ g) and AP5 (2.5 μ g) interfered with the acquisition of fear-potentiated startle at all five tests (see Fig. 3C). Paired *t*-tests comparing startle to the noise burst alone with startle preceded by the CS⁺ indicated rats administered pretraining infusions of vehicle exhibited fear-potentiated startle at all five tests, and rats administered pretraining infusion of the cocktail did not show fear-potentiated startle at any of the tests. The Drug $\times$ Test ANOVA revealed a main effect for Drug ($F_{(1,20)}=6.650$, $P=0.018$), but no main effect for Test ($F_{(4,80)}=2.252$, $P=0.071$) or a Drug $\times$ Test interaction ($F_{(4,80)}=0.270$, $P=0.898$). There was no effect of the cocktail on startle preceded by the CS⁰.

The lack of fear-potentiated startle in rats given pretraining infusions of the cocktail did not result from damage to the amygdala because they were capable of acquiring fear-potentiated startle with an additional 10 trials of drug-free CS⁺ conditioning administered in one session (see Fig. 3C: FPS 6), as indicated by a significant potentiation of startle preceded by the CS⁺ compared noise burst alone startle.

Experiment 3 suggests that antagonism of both NMDA and AMPA/kainate function is capable of interfering with the acquisition of conditioned fear over several days of spaced training. Although not statistically significant, it does appear rats given infusions of the cocktail may have acquired some level of conditioned fear (see Fig. 3C). However, it appears that antagonism

of both NMDA and AMPA/kainate receptors is capable of interfering with the acquisition of conditioned fear over several days of training.

DISCUSSION

Pretraining infusions of either NBQX or APV alone blocked the acquisition of conditioned fear early in training, but not later, when fear conditioning was conducted over several days. However, pretraining infusions of a cocktail of both NBQX and APV interfered with the acquisition of conditioned fear across all 5 days of training. The current experiments indicate that without dramatically altering the number of CS⁺ conditioning trials, the role of excitatory amino acid (EAA) receptors (non-NMDA or NMDA) in the acquisition of conditioned fear becomes less clear when conditioning is conducted over several days. Fear-potentiated startle studies generally use 20 CS⁺ conditioning trials (Miserendino et al., 1990; Campeau et al., 1992), and only 25 were used in these experiments. Previous studies indicate that, when training is conducted over 1 or 2 days, antagonism of EAA receptors in the amygdala interferes with the acquisition of conditioned fear (Miserendino et al., 1990; Campeau et al., 1992; Kim et al., 1993; Fanselow and Kim, 1994; Fanselow et al., 1994; Maren et al., 1996b). These findings were replicated in the current studies, but the data presented here also indicate NMDA and non-NMDA EAA receptors individually may not be critical for the acquisition of conditioned fear conducted over several days. This suggests training over an extended period, even with a similar number of CS⁺ trials, makes the contribution of each receptor subtype less critical for the acquisition of conditioned fear.

The issue of the training paradigm implemented to establish fear conditioning is potentially a critical component for the amygdala's role in fear conditioning. Although the contribution of the amygdala to pavlovian fear conditioning has been established in a variety of studies (Sananes and Davis, 1992; Campeau and Davis, 1995; Maren et al., 1996a), these investigations of pavlovian fear conditioning often employ a conditioning paradigm requiring one or two sessions over 1 or 2 days, utilizing 1–20 conditioning trials. Even with these procedures, the amygdala appears to play an enduring role since lesions made up to 1 month after the initial conditioning are capable of eliminating fear conditioning (Lee et al., 1996; Maren et al., 1996a). A potential important factor receiving attention is the amount of training utilized to establish fear conditioning, and the belief that this may alter the role of the amygdala in fear conditioning. The questions arose from the findings that rats given overtraining can reacquire fear-potentiated startle after post-training lesions of the amygdala (Kim and Davis, 1993), and pretraining lesions did not impair acquisition of conditioned bar press suppression where extensive training is required (Killcross et al., 1997). In addition it has been shown that overtraining mitigates the effects of post-training lesions on retention of a foot shock-motivated escape task (Parent et al., 1992, 1994).

A few studies have directly addressed this issue. In one it was shown that moderate overtraining (25 conditioning trials) could generate low levels of conditioned freezing (Maren, 1998) in rats given pretraining lesions of the amygdala. A second study using more extensive overtraining (75 conditioning trials) found rats acquired conditioned freezing to the context, but not to a CS (Maren, 1999b). More recently, another study (Lee et al., 2005) found that discrete lesions of the basolateral or central nucleus of the amygdala impaired two measures of conditioned fear with relatively few conditioning trials. However, with increased number of trials, rats receiving discrete lesions performed identically to controls on a conditioned suppression task, and although not to the same levels as controls, they did exhibit significant conditioned freezing. Therefore it appears manipulation of the conditioning protocol with additional training may alter the contribution of the amygdala in fear conditioning.

Alternatively, as pointed out by Maren (1998) in his discussion, the issue may not be the number of trials, but rather, the distribution of the trials. A study showing complete reacquisition of fear with overtraining prior to the lesion (Kim and Davis, 1993) used distributed trials over 4 or 40 days as opposed to a single day. However, rats with complete lesions of the central nucleus and anterior BLA on a distributed schedule failed to reacquire fear-potentiated startle (Falls and Davis, 1995). Additionally, the results showing mitigation of the effect of amygdala damage with overtraining on foot shock-motivated escape used a single session for conditioning, contradicting the idea of distributed training (Parent et al., 1992, 1994).

In the end it appears that lesion extent may be the determining factor in the role of the amygdala in fear conditioning (Maren, 1998). The difference in rats being able to reacquire fear-potentiated startle appeared to be dependent on the lesion extent in the basolateral amygdala rather than the overtraining or distributed training (Kim and Davis, 1993; Falls and Davis, 1995). In a study directly examining lesion extent and overtraining, large lesions of the amygdala encompassing both the basolateral and central amygdala abolished both conditioned freezing and suppression even with overtraining (Lee et al., 2005). Although in the current study the guide cannula targeted the basolateral amygdala, it is possible with the volume infused that the infusions reached both the basolateral and central amygdala. Another explanation is that the lesion extent may be reflected by the type of compound infused. Infusion of only the NMDA receptor antagonist APV or the AMPA receptor antagonist NBQX might be classified as a less extensive lesion compared with the combined infusion of both compounds. This might explain the failure to block the acquisition of conditioned fear across all tests when evaluated individually, and the blockade across all tests when infused together.

LTP has been a leading model for evaluating learning-related plasticity involved with associative fear conditioning in the amygdala. EAA receptors in the amygdala have been critically linked to fear conditioning. Studies have shown that pre-training infusions of NMDA receptor antagonists interfere with the acquisition of fear conditioning as measured by fear-potentiated startle (Miserendino et al., 1990; Campeau et al., 1992), freezing (Fanselow and Kim, 1994; Maren et al., 1996b), and inhibitory avoidance (Kim and McGaugh, 1992). NMDA receptors have been implicated in the synaptic alterations characterizing LTP, and several studies suggest LTP can be induced in the amygdala by electrical stimulation (Clugnet and LeDoux, 1990; Maren and Fanselow, 1995) and fear conditioning (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Research also indicates LTP is prevented by NMDA receptor antagonists in these pathways (Maren and Fanselow, 1995). These studies provide evidence suggesting that NMDA receptors in the amygdala, through their involvement in LTP, may be critically involved in fear conditioning.

Similar to the hippocampus (Grover and Teyler, 1990), there are forms of LTP in the amygdala that do not require NMDA receptor activation (Chapman and Bellavance, 1992; Weisskopf et al., 1999), but do require the activation of voltage-gated calcium channels (VGCCs) (Weisskopf et al., 1999), and recent work indicates that VGCCs do contribute to the acquisition of conditioned fear (Bauer et al., 2002). Research also indicates AMPA receptors contribute to conditioned fear in the amygdala. Infusions of AMPA receptor antagonists into the amygdala interfere with the expression of conditioned fear (Kim et al., 1993; Walker and Davis, 1997). Pretraining infusion of an AMPA receptor agonist in the amygdala enhances the acquisition of conditioned freezing (Rogan et al., 1997). Other work suggests cholinergic (Rudy, 1996; Anagnostaras et al., 1999) and dopaminergic (Guarraci et al., 1999; Nader and LeDoux, 1999) neurotransmission in the amygdala contributes to the acquisition and expression of conditioned fear. Therefore, it appears likely there are a number of systems within the amygdala contributing to synaptic plasticity, and the various interactions between them. Certainly the current results further support the idea that LTP associated with fear conditioning in the amygdala can occur through both NMDA and non-NMDA dependent mechanisms (Bauer et

al., 2002), because inactivation of only one subtype was not capable of preventing the acquisition of conditioned fear.

Looking at the results from experiment 3, it could be argued that the acquisition of conditioned fear was not completely prevented. Although not statistically significant, rats given pretraining infusions of the cocktail appeared to acquire some level of conditioned fear (see Fig. 3C). One possible explanation is that another neural structure in the brain, albeit not as good as the amygdala, participates in fear conditioning. The medial geniculate nucleus (MGN) transmits auditory information to the amygdala (LeDoux et al., 1990b), and has been proposed as a critical site of neuronal plasticity in the case of fear conditioning to auditory stimuli (Weinberger, 1998). In fact, it has been shown that the MGN undergoes associative neuronal plasticity in fear conditioning paradigms (Gabriel et al., 1975; Supple and Kapp, 1989; Edeline and Weinberger, 1992). It has also been shown that synaptic plasticity (McEchron et al., 1996) and LTP (Gerren and Weinberger, 1983) occur within the MGN. Furthermore, electrophysiological studies indicate that the latencies of conditioning-related increases in firing of neurons in the basolateral complex of the amygdala are consistent with projections from the thalamus (Quirk et al., 1995, 1997; Maren, 2000). Because the MGN contains most of the neuronal properties considered necessary for the acquisition of conditioned fear, it seems plausible that the MGN encodes incoming stimulus information before sending it to the amygdala.

Given the results of the current study it appears that, even with a distributed training paradigm, the amygdala may play a key role in the acquisition of conditioned fear, although the current results did not use extensive overtraining. It would be interesting to see whether using the same distributed training utilized in the current study, but increasing the number of conditioning trials each day, would alter the contribution of the amygdala in fear conditioning. It also would be of interest to evaluate the effects of combined higher doses of NBQX and APV to see if even the low, non-statistically significant, levels of conditioned fear were completely eliminated. Based on the results from previous studies, in addition to the current findings, it seems apparent a number of variables must be considered when assessing the role of the amygdala in fear conditioning. There appears to be a complex interplay between a variety of variables such as the number of training trials, the distribution of the training trials, the receptor subtypes within the amygdala, the subnuclei of the amygdala and the particular paradigm being utilized to evaluate conditioned fear. All of these components may contribute differentially to the acquisition, expression and storage of conditioned fear.

Abbreviations

CS, conditioned stimulus; CS⁺, CS+shock; CS⁰, neutral stimulus never presented during training; EAA, excitatory amino acid; ISI, inter-stimulus interval; LTP, long-term potentiation; MGN, medial geniculate nucleus; NMDA, *N*-methyl-D-aspartic acid; VGCC, voltage-gated calcium channel.

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Fig. 1.

Acquisition of conditioned fear when fear conditioning and fear-potentiated startle testing was conducted on alternate days. (A) Composite of injector locations for experiment 1. (B) Timeline for experiment 1. (C) Mean difference scores for startle to the CS⁺ and CS⁰ during drug-free tests for fear-potentiated startle in rats given pretraining infusions of Vehicle, APV 2.5 μ g or APV 10 μ g. * $P < 0.05$, and # $P < 0.01$, for comparison of startle to the noise burst alone with startle to the noise burst preceded by the CS⁺.

Fig. 2.

Acquisition of conditioned fear when fear conditioning and fear-potentiated startle testing was conducted on alternate days. (A) Composite of injector locations for experiment 2. (B) Timeline for experiment 2. (C) Mean difference scores for startle to the CS⁺ and CS⁰ during drug-free tests for fear-potentiated startle in rats given pretraining infusions of Vehicle, NBQX 3 μ g or NBQX 9 μ g. * $P < 0.05$, and # $P < 0.01$, for comparison of startle to the noise burst alone with startle to the noise burst preceded by the CS⁺.

Fig. 3.

Acquisition of conditioned fear when fear conditioning and fear-potentiated startle testing was conducted on alternate days. (A) Composite of injector locations for experiment 3. (B) Timeline for experiment 3. (C) Mean difference scores for startle to the CS⁺ and CS⁻ during drug-free tests for fear-potentiated startle in rats given pretraining infusions of Vehicle or cocktail (2.5 μ g APV and 3 μ g NBQX). * $P < 0.05$, and # $P < 0.01$, for comparison of startle to the noise burst alone with startle to the noise burst preceded by the CS⁺.