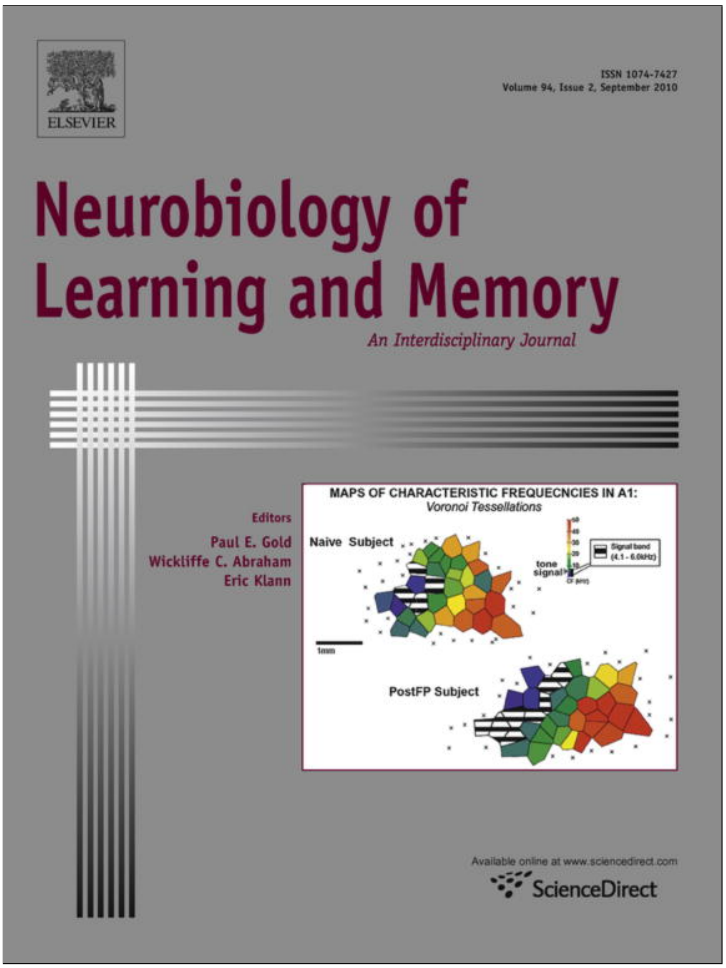

Neurobiology of Learning and Memory 94 (2010) 167–175

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Conditioned fear in adult rats is facilitated by the prior acquisition of a classically conditioned motor response

Derick H. Lindquist [a,*], Luke P. Mahoney [b], Joseph E. Steinmetz [a]

[a] The Ohio State University, Dept. of Psychology, Columbus, OH 43210, United States
[b] University of Kansas, Dept. of Pharmacology and Toxicology, Lawrence, KS 66045, United States

ARTICLE INFO

Article history:
Received 14 October 2009
Revised 30 April 2010
Accepted 10 May 2010
Available online 20 May 2010

Keywords:
Pavlovian conditioning
Two-process model
Fear conditioning
Eyeblink conditioning
Freezing
Rat

ABSTRACT

Early in eyeblink classical conditioning, amygdala-dependent fear responding is reported to facilitate acquisition of the cerebellar-dependent eyeblink conditioned response (CR), in accord with the two-process model of conditioning (Konorski, 1967). In the current study, we predicted that the conditioned fear (e.g., freezing) observed during eyeblink conditioning may become autonomous of the eyeblink CR and amenable to further associative modification. Conditioned freezing was assessed during and following Pavlovian fear conditioning in Long-Evans rats that had or had not undergone eight prior sessions of eyeblink conditioning. The amplitude and frequency of the tone conditioned stimulus (CS) was held constant across both forms of conditioning. Following fear conditioning in Experiment 1, freezing to the tone CS, but not the context, was facilitated in rats that previously experienced CS-unconditioned stimulus (US) paired eyeblink conditioning. In Experiment 2, freezing immediately following each fear conditioning trial was enhanced in rats subjected to the antecedent eyeblink conditioning, indicating a faster acquisition rate. Finally, in Experiment 3, faster acquisition was seen only in those rats fear conditioned in the same context used for the prior eyeblink conditioning. Taken together, the data indicate that the conditioned fear associated with the context and CS as a result of eyeblink conditioning can be built upon or strengthened during subsequent learning.

1. Introduction

Eyeblink classical conditioning entails the repeated pairing of a neutral conditioned stimulus (CS; e.g., light or tone) and an eyeblink-eliciting unconditioned stimulus (US; e.g., corneal air puff or periorbital shock). In well trained subjects the CS elicits a temporally precise eyeblink conditioned response (CR), with maximal eyelid closure occurring just before US onset. A relatively simple form of motor learning, the eyeblink CR nonetheless requires tens to hundreds of CS–US training trials to fully develop.

Eyeblink conditioning has been put forth as an exemplar of the two-process model of aversive conditioning (Lee & Kim, 2004; Thompson et al., 1987; Weisz, Harden, & Xiang, 1992). Initially developed to model instrumental conditioning (Mowrer, 1947; Rescorla & Solomon, 1967), two-factor or two-process accounts of Pavlovian conditioning propose that nonspecific emotional responses influence or modulate the subsequent acquisition of specific motor responses (Lennartz & Weinberger, 1992; Mintz & Wang-Ninio, 2001). The two processes emerge because the aversive US can be dually represented by its emotional and sensory attributes (Konorski, 1967; Mackintosh, 1983; Wagner & Brandon, 1989), both aspects of which can be independently associated with the CS. The training context is also thought to form connections with the emotional attributes of the US, to an even greater extent, possibly, than the CS (Konorski, 1967; Thompson et al., 1987).

The association involving a CS and the "emotional" US relies on the amygdala, and is reflected in the rapid expression of multiple fear CRs. The association involving the CS and the "sensory" US, on the other hand, critically depends on the cerebellum and leads to the development and expression of the eyeblink CR. Importantly, fear responding is proposed to precede and modulate the more slowly acquired eyeblink CR. In support, rabbits submitted to eyeblink classical conditioning display rapid autonomic changes in heart rate and blood pressure (Lavond, Lincoln, McCormick, & Thompson, 1984; Prokasy, 1972; Schneiderman, Smith, Smith, & Gormezano, 1966). Lesions of the amygdala reduce or block fear responding, including conditioned bradycardia and CS-induced reflex facilitation of the eyeblink unconditioned response (UR), and retard the rabbit eyeblink CR acquisition rate (Chachich & Powell, 1998; Weisz et al., 1992). In no case, to our knowledge, do amygdala perturbations prevent learning, indicating the amygdala contributes to but is not necessary for the development and generation of the eyeblink CR.

* Corresponding author. Address: Dept. of Psychology, The Ohio State University, Room 49, 225 Psychology Building, 1835 Neil Avenue, Columbus, OH 43210, United States. Fax: +1 614 688 4733.
E-mail address: lindquist.40@osu.edu (D.H. Lindquist).

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doi:10.1016/j.nlm.2010.05.003

In the freely moving rat, two behavioral fear CRs – conditioned freezing and ultrasonic vocalization emissions – occur at their highest rate early in training, decreasing in frequency across the latter eyeblink conditioning sessions (Britton & Astheimer, 2004; Lee & Kim, 2004). As above, amygdala lesions decelerate the eyeblink CR acquisition rate, as well as block the emission of amygdala-dependent ultrasonic vocalizations (Lee & Kim, 2004). Interestingly, freezing, though maximal during the first eyeblink conditioning session, remains elevated to the context and cue throughout training (Britton & Astheimer, 2004). That is, enhanced rates of freezing (~30%) were recorded in the last training session during the intertrial interval (ITI) between CS–US presentations and later on the same day to the tone CS in a novel context. The fact that emotional responding decreases across training suggests that conditioned fear may become autonomous of the eyeblink CR (Thompson et al., 1987), whereas the fact that fear responding does not fully extinguish suggests that it may be amenable to further associative modification.

To test these hypotheses, Pavlovian fear conditioning was assessed in rats that did or did not experience eight prior sessions of CS–US paired eyeblink conditioning. The amplitude and frequency of the tone CS was held constant across both forms of conditioning, while the US was switched from a periorbital shock (eyeblink conditioning) to a footshock (fear conditioning). The amygdala was posited to represent and associate the emotional attributes of both unconditioned stimuli with the tone CS and possibly the context. As such, we predicted that the synaptic plasticity in the amygdala responsible for fear conditioning (LeDoux, 2000) would benefit from the presumptive plasticity established by the prior eyeblink conditioning.

Three sets of experiments were conducted. Following fear conditioning, freezing was assessed (in counter-balanced order) to the fear conditioning context and, separately, to the tone CS in a novel context. Experiment 1 investigated whether CS–US paired eyeblink preconditioning enhanced the retrieval of conditioned fear to the context or CS, relative to rats that received fear conditioning alone. Experiment 2 examined the acquisition of fear conditioning, measuring freezing immediately following each footshock US. Experiment 3 assessed whether the putative benefits of eyeblink preconditioning were dependent on the fear conditioned to the context, the CS, or both.

2. Experiment 1

The expression of Pavlovian fear conditioning was examined as a function of prior experience. In this initial analysis, the eyeblink and fear conditioning interstimulus intervals (ISIs) were set at 350 and 6000 ms, respectively, for two reasons. First, the expression of many fear CRs is not temporally tied to the CS (e.g., freezing and ultrasonic vocalizations; Lindquist & Brown, 2004), suggesting the emotional (fearful) aspects of the excitatory CS following eyeblink conditioning can outlast the short training ISI. Second, the use of two ISIs allowed us to maintain what might be considered typical CS/US durations for each form of conditioning.

2.1. Materials and methods

2.1.1. Subjects

Thirty-five experimentally naïve, male, adult Long-Evans rats were maintained on a 12 h light/dark cycle (lights on at 0700 h) with *ad libitum* access to food and water. Surgical and behavioral procedures were conducted during the light phase. All procedures, including surgery and postoperative care, were in strict compliance with the University of Kansas animal care guidelines, and all necessary measures were taken to minimize pain and discomfort.

2.1.2. Surgical procedures

All surgical procedures were performed under aseptic conditions. Rats were anesthetized using intraperitoneal (ip) injections of an anesthetic cocktail (2.2 ml/kg), consisting of physiological saline (9.0 mg/kg), ketamine (74.0 mg/kg), xylazine (3.7 mg/kg), and acepromazine (0.74 mg/kg). Ketamine boosters were administered as required to maintain anesthesia. Each subject was surgically prepared with differential electromyographic (EMG) wires and a bipolar periocular stimulator. EMG activity was recorded in the orbicularis oculi muscle surrounding the eye by passing two ultrathin (0.003 in.) Teflon-coated stainless steel wires subdermally beneath the anterior portion of the upper eyelid. Gold-coated stainless steel wires (MS303-2B, Plastics One, Roanoke, VA) were implanted in the dorso-caudal portion of the orbicularis oculi muscle for delivery of the periorbital electrical shock US. A ground wire was connected to one of three stainless steel skull screws. The two EMG wires and ground wire all terminated in gold pins inside a six-pin plastic connector (MS 363, Plastics One). The headstage and bipolar stimulating electrodes were fixed in dental cement. The wound was salved with antibiotic ointment (Povidone), and the animals were given at least 7 days to recover before the start of training.

2.1.3. Apparatus

Rats were placed in standard operant boxes (Coulburn Instruments, Allentown, PA), contained within sound-attenuating chambers. Each operant box has two stainless steel walls, two Plexiglas walls, and a grid floor composed of 0.5 cm stainless steel bars placed approximately 1.5 cm apart. Electrode leads, attached to each subject's head, swivel freely on a 10-channel commutator connected to a counterbalanced pivoting arm, allowing subjects to move freely about in the conditioning chamber during adaptation and all subsequent eyeblink conditioning sessions. The commutator tether was disconnected from the animal during fear conditioning and context/cue testing.

2.1.4. Behavioral training and testing

Five groups of adult rats underwent various combinations of eyeblink and/or fear conditioning: paired eyeblink conditioning followed by fear conditioning (E1–EBC–FC; $n = 7$); unpaired eyeblink conditioning followed by fear conditioning (E1-Unpaired-FC; $n = 7$); context exposure in the absence of CS/US presentations followed by fear conditioning (E1-Context-FC; $n = 7$); fear conditioning only (E1–FC-only; $n = 7$); and paired eyeblink conditioning only (E1–EBC-only; $n = 7$).

Two rats were run simultaneously across all sessions. Both chambers were wiped with a water/vinegar (5:1) solution prior to placing the rat inside. Classical eyeblink conditioning entailed the presentation of a 2.8 kHz, 85 dB SPL, 450 ms tone CS, delivered from an overhead speaker, and a 100 ms train of 2.0 mA, 60 Hz, constant-current square wave electrical stimulation US. For the two groups that received paired eyeblink conditioning, the CS and US overlapped and co-terminated, resulting in a 350 ms ISI. Each of the eight training sessions was composed of 10 blocks of 10 trials: 9 CS–US paired trials and 1 CS-alone trial. The intertrial interval (ITI) was 25 ± 5 s. The Context-FC rats underwent the same conditioning protocol, but the CS and US were never presented. For unpaired eyeblink conditioning, the rats received the same number and density of CS and US presentations as in paired conditioning. Each session consisted of 100 CS and 90 US explicitly unpaired trials, with a pseudo-random ITI of 10–15 s.

Paired-FC, Unpaired-FC, Context-FC, and FC-only subjects underwent a single session of Pavlovian fear conditioning. The EBC-only rats never experienced fear conditioning. Just prior to the fear conditioning session each chamber was again wiped with the water/vinegar (5:1) solution. Fear conditioning consisted of six

pairings of the same 2.8 kHz, 85 dB tone CS (6500 ms in duration) and a co-terminating 500 ms 0.6 mA grid-shock US produced by a small-animal shock generator (Model 82400; Lafayette Instruments, Lafayette, IN) and neon grid scrambler (Model 58020; Lafayette Instruments). The ISI was 6.0 s; the ITI was 240 ± 30 s.

Table 1 documents the training/testing protocol across all three experiments and the specific contexts used, with context encompassing the sensorial characteristics of the chamber and the antecedent transport cues. Eyeblink and fear conditioning utilized Context A (Table 1). Two rats were removed from their home cage and carried to the training room in their own plastic, rectangular tub (12 in L × 9 in W × 6.5 in D) covered with a metal feeding grate. Each rat was then placed in one of two conditioning chambers (wiped with the water/vinegar solution) in the lighted training room. Contextual fear was also assessed in context A. After a 2 min baseline period, freezing was measured for 10 min and the rat was removed 2 min later. Cue testing occurred in context B (Table 1). Rats were transported in their home cage (21 in L × 12 in W × 8 in D), covered with a towel, on a metal, wheeled cart. Each rat was placed in the chamber opposite to that in which it was trained in a darkened room, save for a single, incandescent, red bulb. Both chambers were wiped with Windex®, a Plexiglas sheet was placed over the grid bars, and a 15 W incandescent bulb, hung in the top corner of the sound attenuating chamber, was turned on to illuminate the conditioning chamber from above. After a 3 min baseline period, the tone CS was continuously presented for 8 min, followed by another 3 min post-CS period before removal of the rat.

2.1.5. EMG analysis

Throughout each eyeblink conditioning session, eyelid EMG activity was amplified (1000×) and band-pass filtered (300–1000 Hz) by a differential AC amplifier (model 1700, A-M Systems, Carlsborg, WA). The EMG signal was simultaneously digitized (500 Hz), rectified, smoothed (10 ms time constant), time shifted (10 ms, to compensate for smoothing), and stored for offline analysis using the Spike 2 waveform analysis system (CED Limited, Cambridge, England). On each trial, EMG activity from the orbicularis oculi muscle was sampled for 1500 ms, divided into three periods: (i) a 350-ms pre-CS period, prior to CS onset; (ii) a 350 ms CS–US period, between CS onset and US onset; and (iii) an 800 ms post-US period, following US onset.

The averaged EMG activity in the pre-CS period was used as a baseline for classifying behaviors and scoring trials. Trials were dropped and excluded from further analysis if EMG activity exceeded the baseline activity by five or more standard deviations during the bad trial window, which extended from 100 ms before to 15 ms after CS onset. EMG activity that exceeded the baseline activity by five or more standard deviations between 15 and 100 ms following CS onset was classified as an alpha response. A blink was scored if EMG activity exceeded the baseline activity by five or more standard deviations beginning 100 ms after CS onset. Session-wide averages were computed for blink frequencies during the ISI. The EMG data were analyzed using mixed design ANOVAs and Tukey–Kramer post-hoc tests. A significant post-hoc effect implies $p < 0.05$.

2.1.6. Freezing analysis

Freezing was defined as cessation of all movement except that required for respiration (Blanchard & Blanchard, 1969). For all groups, behavior was recorded with a black-and-white video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ), with the interior of the chamber illuminated by an infrared light source. The video signal was inputted to FreezeScan (CleverSys, Inc., Reston, VA), a video-based tool that provides precise motion detection, quantifying the percentage of time that rats are motionless. Freezing was analyzed throughout the 10 min context and 8 min cue tests. For the latter, freezing was also examined for 1 min before and 1 min after presentation of the tone CS. The freezing data were analyzed using one-way and mixed design ANOVAs and Tukey–Kramer post-hoc tests. The FreezeScan package has been used by other laboratories in the behavioral analysis of freezing (e.g., Corcoran & Quirk, 2007).

2.2. Results

2.2.1. Eyeblink conditioning

The frequency of associative (EBC–FC and EBC-only rats) and non-associative (Unpaired-FC and Context-FC rats) blinking across each of the eight sessions is illustrated in Fig. 1A. A 4 (group) × 8 (session) repeated measures ANOVA revealed a statistically significant group × session interaction, $F(21, 168) = 6.56$, $p < 0.001$. Tukey–Kramer post-hoc analysis determined that the two associative conditioning groups emitted significantly more blinks than the Unpaired-EBC–FC and Context-FC groups, with no significant difference in CR frequency between them.

2.2.2. Fear conditioning and testing

Four groups of rats—EBC–FC, Unpaired-FC, Context-FC, and FC-only—were submitted to a single Pavlovian fear conditioning session. Freezing was subsequently measured in all five groups, including the EBC-only rats, in the training context and to the CS in a novel context. Due to a corrupt video file, one Context-FC subject was dropped from the context test analysis.

A one-way (group) ANOVA revealed a significant main effect to the cue, $F(4, 30) = 14.84$, $p < 0.001$, but not the context (Fig. 1B).

Table 1
Adaptation/Training/Testing and specific contexts.

	Adaptation	EBC	Adaptation2	FC	Context test	Cue test
Group	*Tethered*		*Untethered*			
E1–EBC–FC	A	A		A	A	B
E1-Unpaired-FC	A	A		A	A	B
E1-Context-FC	A	A		A	A	B
E1–FC-only	A			A	A	B
E1–EBC-only	A	A			A	B
E2–EBC–FC	A	A		A	A	B
E2–FC-only	A			A	A	B
E3–EBC–FC–D	A	A	C	C	C	B
E3–EBC–FC–S	A	A	C	A	A	B
E3–FC-only	A			A	A	B

The various adaptation, training, and testing sessions are illustrated, with Groups for each experiment listed on the far-left column. The second row indicates whether the rat was tethered or untethered to the commutator. The letter to the right of each group signifies which of three contexts (A, B, or C) the rats were exposed to for a given session(s). Context, for the purposes of the current study, refers to the sensorial representation of the chamber into which the rat was placed, and the antecedent transport cues. See the text for details. EBC: eyeblink conditioning; FC: fear conditioning.

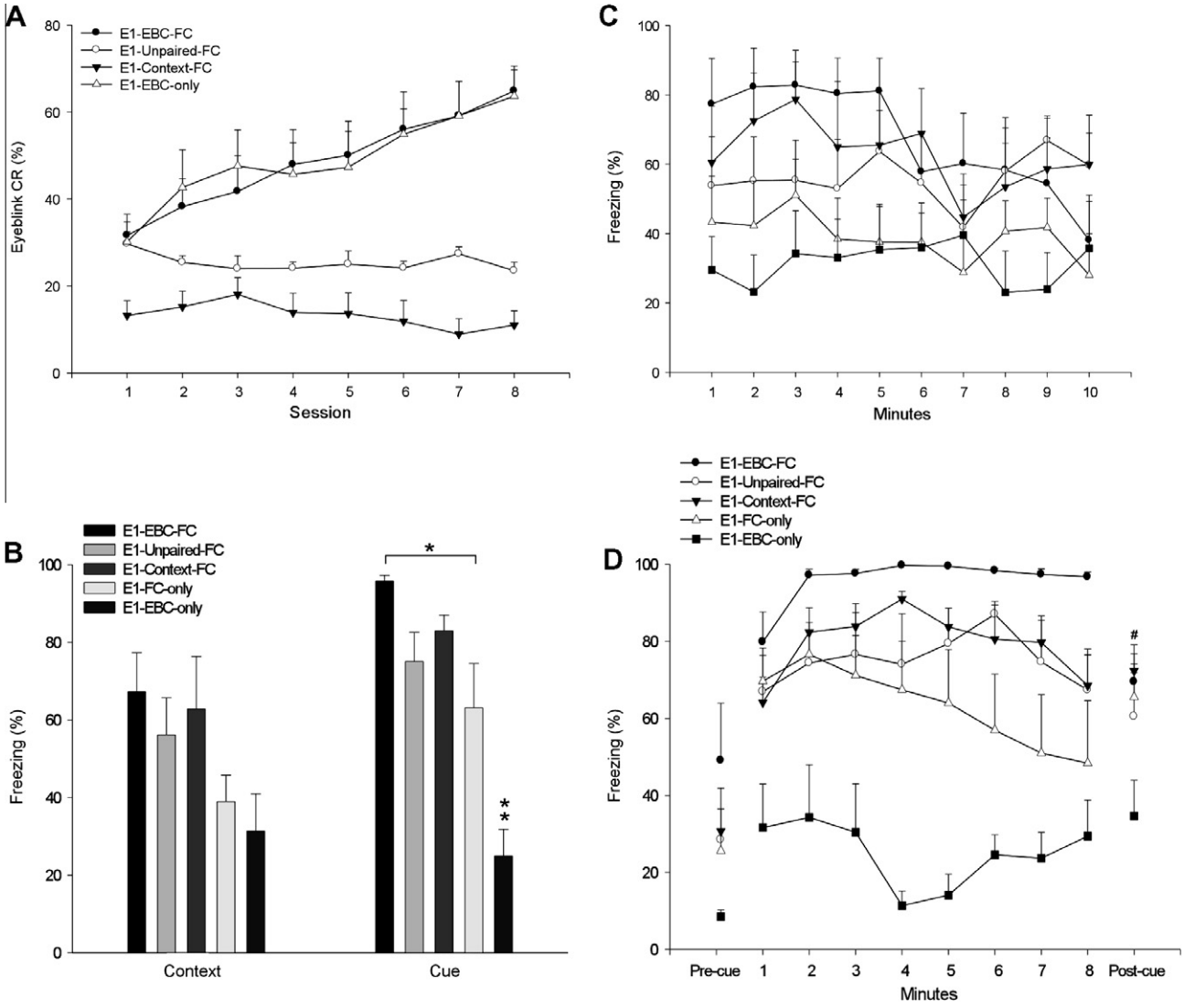

Fig. 1. Experiment 1: Eyeblink preconditioning and conditioned freezing to the context and cue, following a single session of Pavlovian fear conditioning. (A) Associative and non-associative blinking (mean ± SE) in rats that experienced: eight sessions of paired eyeblink conditioning (E1–EBC–FC and E1–EBC-only); unpaired eyeblink conditioning (E1-Unpaired-FC); or context exposure in the absence of CS/US presentations (E1-Context-FC). (B) Averaged freezing (mean ± SE), following fear conditioning, to the training chamber (context) and tone CS (cue), presented in a novel environment. Note the EBC-only rats did not undergo fear conditioning, while the FC-only rats did not experience eyeblink conditioning. Freezing to the tone CS in the EBC–FC rats was significantly enhanced relative to the FC-only rats (asterisk). Freezing to the cue in EBC-only rats was significantly lower than all other groups (double asterisk). Conditioned freezing (mean ± SE) throughout the same 10 min context test (C) and 8 min cue test (D), as well as the one min before (Pre-cue) and one min after (Post-cue) presentation of the tone CS. The Context-FC rats froze significantly more than the EBC–FC rats during the Post-cue period (pound sign).

Tukey–Kramer post-hoc analysis found significantly more freezing in the EBC–FC rats (95.8 ± 1.5%) relative to the FC-only rats (63.1 ± 11.4%), indicating CS–US paired eyeblink preconditioning increased the expression of conditioned fear to the tone CS. All groups showed significantly enhanced levels of freezing compared to the EBC-only rats.

The same pattern of results held when conditioned freezing was broken down into 1 min bins across the 10 min context test and the 8 min cue test (Fig. 1C and D). Specifically, the repeated measures ANOVA revealed a significant group × bin interaction during the cue test, $F(28, 210) = 1.87$, $p < 0.01$, but not during the context test. Post-hoc testing again found significantly higher levels of freezing in the EBC–FC rats versus the FC-only rats, and all groups froze significantly more than the EBC-only rats. One-way (group) ANOVAs computed for the 1 min pre-cue and post-cue periods found a significant effect for the latter only, $F(4, 30) = 2.84$, $p < 0.05$, with a significant difference in freezing frequency between the Context-FC and EBC-only rats (Fig. 1D).

2.2.3. Discussion

Eight sessions of paired eyeblink conditioning significantly facilitated conditioned fear to the tone CS following a single session of Pavlovian fear conditioning, even with the lengthened ISI for the latter. Neither explicitly unpaired eyeblink conditioning nor contextual exposure significantly altered cue-mediated freezing. There were no significant differences in freezing to the conditioning context among the five groups of rats.

3. Experiment 2

This experiment investigated the effects of CS–US paired eyeblink conditioning on the acquisition of Pavlovian fear conditioning, as assessed by freezing behavior immediately following each footshock US. Post-footshock freezing is considered a fear CR, tied to those cues that predict presentation of the aversive US (Fanselow, 1980). We also sought to replicate the findings of

Britton and Astheimer (2004)—that is, to observe decreased freezing across the eyeblink conditioning sessions, even as the eyeblink CR frequency increases. Finally, for this experiment the CS/US durations were held constant across both forms of conditioning. The aim was to avoid a ceiling effect (i.e., near maximal levels of freezing to the tone CS, as observed in Experiment 1) by shortening the footshock US duration from 500 to 100 ms.

3.1. Methods and materials

3.1.1. Subjects, surgery, and apparatus

Fourteen experimentally naïve, male, adult Long-Evans rats underwent the same surgical procedure described in Experiment 1. The training and testing apparatus was identical to that described above.

3.1.2. Behavioral training and testing

Two groups of rats were run: E2–EBC–FC ($n = 7$) and E2–FC-only ($n = 7$). All subjects experienced one day of adaptation to the conditioning chamber. The EBC–FC rats underwent eight sessions of paired eyeblink conditioning with a 2.8 kHz, 85 dB, 450 ms tone CS and co-terminating 100 ms, 1.5 mA (as opposed to the 2.0 mA used in Experiment 1) periorbital shock US. Fear conditioning for all subjects consisted of six trials of the same 450 ms tone CS, co-terminating with a 100 ms, 0.6 mA footshock US. The ITI for eyeblink conditioning was 25 ± 5 s; 240 ± 30 s for fear conditioning.

Freezing was measured and scored with the FreezeScan software package. As in Experiment 1, eyeblink and fear conditioning utilized context A (Table 1; see Section 2.1.4. for details). Freezing was measured throughout each of the eight eyeblink conditioning sessions. For fear conditioning, freezing to the context was assessed for the first 120 s in the chamber, prior to presentation of the first CS–US paired trial, and for 120 s following each of the six trials. Finally, freezing was recorded during the 10 min context test and before, during, and after the 8 min tone CS in context B (Table 1; see Section 2.1.4. for details).

3.2. Results

3.2.1. Eyeblink conditioning

The EBC–FC rats acquired the eyeblink CR at a rate similar to that reported in Experiment 1. An analysis of the three associative conditioning groups—E1–EBC–FC, E1–EBC-only, and E2–EBC–FC rats—revealed no statistically significant group differences in CR frequency. As seen in Fig. 2A, the eyeblink CR frequency gradually increased across training, whereas conditioned freezing was robust

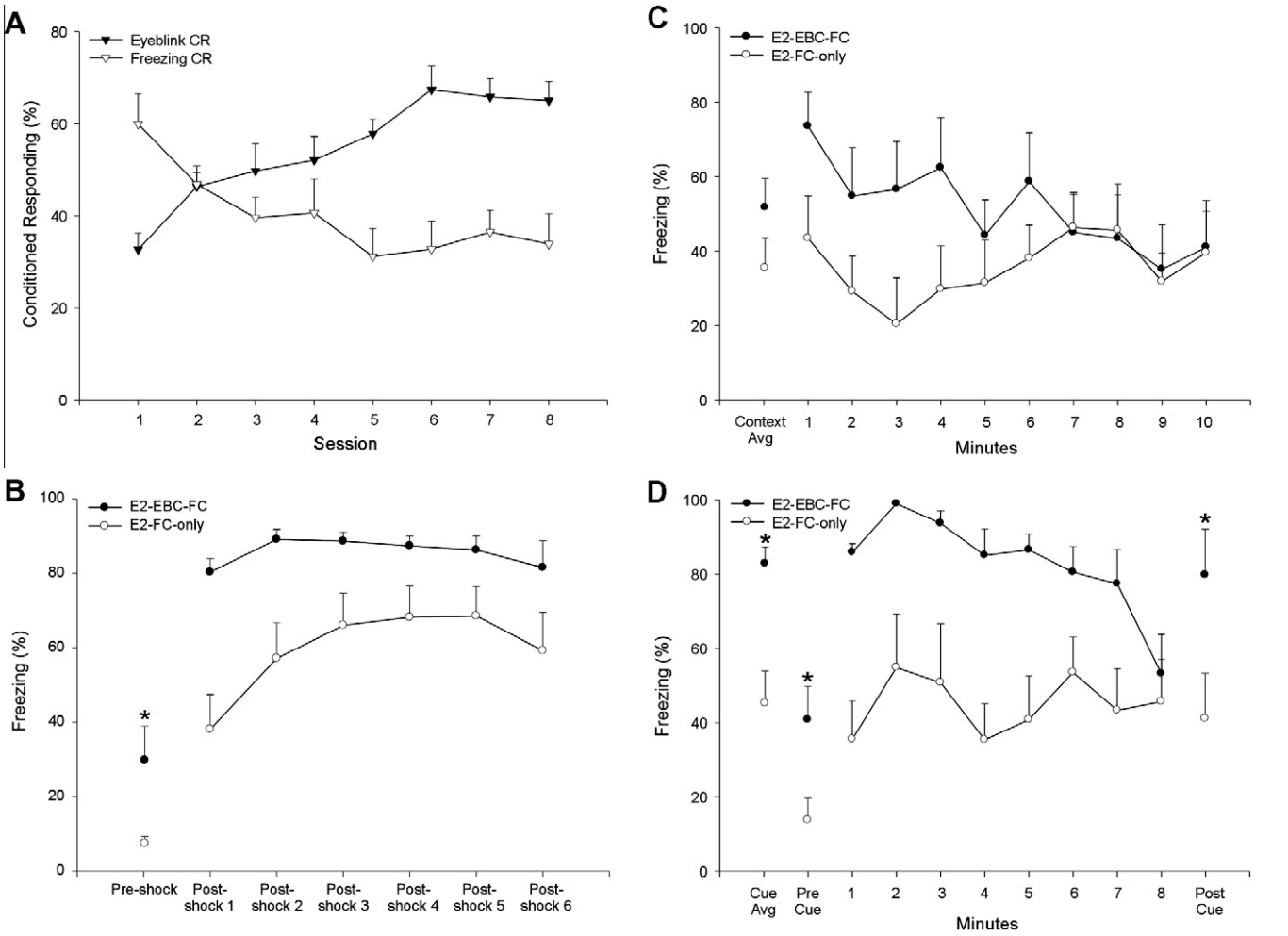

Fig. 2. Experiment 2: Conditioned freezing as a function of eyeblink conditioning, post-footshock freezing during fear conditioning, and context/cue testing. (A) Fear and eyeblink responding (mean ± SE) displayed the inverse relationship predicted by the two-process model of conditioning. (B) Fear conditioned rats that experienced prior eyeblink conditioning (E2–EBC–FC) froze significantly more than rats that did not experience eyeblink conditioning (E2–FC-only) during the initial 120 s in the conditioning chamber (Pre-shock; asterisk). Conditioned freezing (mean ± SE) throughout the 10 min context test (C) and the 8 min cue test (D), as well as the one min before (Pre-cue) and one min after (Post-cue) presentation of the tone CS. Averaged freezing was significantly higher in the EBC–FC than FC-only rats to the tone CS (Cue Avg, far left) but not the training chamber (Context Avg, far left). The EBC–FC rats froze significantly more both before (Pre-cue) and after (Post-cue) presentation of the tone CS (asterisks).

early on, declining across the later training sessions. Consistent with the two-process model of conditioning, significant decreases and increases in the fear and eyeblink CR rate, respectively, were supported by linear trend analysis: emotional responding, $F(1, 55) = 9.54$, $p < 0.001$, and motor responding, $F(1, 55) = 33.14$, $p < 0.001$.

3.2.2. Fear conditioning and testing

The EBC–FC rats displayed significantly more pre-shock freezing behavior during the initial 120 s of exposure to the conditioning chamber than the FC-only rats, $F(1, 12) = 5.74$, $p < 0.05$ (Fig. 2B, far left). The freezing rate of the EBC–FC rats was comparable to that observed during the last eyeblink conditioning session (Fig. 2A). Following each of the six fear conditioning trials, the 2 (group) × 6 (trial) repeated measures ANOVA revealed a significant group × trial interaction for freezing, $F(5, 60) = 3.94$, $p < 0.001$, with significantly higher levels in the EBC–FC rats (Fig. 2B). The increase in post-footshock freezing is taken to indicate a faster acquisition rate.

Contextual- and cue-mediated freezing was similar to that seen in Experiment 1. There was no significant group difference when conditioned freezing was averaged across the 10 min context test, nor when it was broken down into one min bins (Fig. 2C). On the other hand, the EBC–FC rats froze significantly more, averaged across the 8 min cue test, than the FC-only rats, $F(1, 12) = 14.93$, $p < 0.01$ (Fig. 2D; far left). The repeated measures ANOVA based on the test tone also revealed a significant difference, with more freezing observed in the EBC–FC rats, $F(1, 84) = 14.93$, $p < 0.01$ (Fig. 2D). Finally, freezing was analyzed during the 1 min pre- and post-cue period. Recall that cue testing occurred in a novel context. Nonetheless, the EBC–FC rats froze significantly more than the FC-only rats during the pre-cue period, $F(1, 12) = 6.32$, $p < 0.05$, and the post-cue period, $F(1, 12) = 4.96$, $p < 0.05$.

3.2.3. Discussion

Freezing was maximal during the first eyeblink conditioning session, decreasing thereafter, in accord with the two-process model of aversive conditioning. For fear conditioning, rats that underwent eyeblink preconditioning displayed faster acquisition as revealed by enhanced post-footshock freezing. The elevated level of pre-shock freezing suggests that contextual fear might underlie the facilitated rates of post-footshock freezing. And yet, this was the first session in which the rats were not connected to the commutator tether, a difference in "context" that should lead to decreased contextual fear. As in Experiment 1, the EBC–FC rats froze significantly more to the tone CS than the context, relative to the FC-only rats, while the shortened US duration resulted in decreased fear responding across the 8 min tone, compared to the continuously high freezing rates observed in Experiment 1.

4. Experiment 3

The final experiment assessed whether the facilitated fear learning observed in Experiment 2 is dependent on the context, the cue, or both. Pavlovian fear conditioning was conducted in the same or different context from that used in the prior eyeblink conditioning (see Table 1). A third group underwent fear conditioning only, as in the previous two experiments.

4.1. Methods and materials

4.1.1. Subjects, surgery, and apparatus

Twenty-one experimentally naïve, male, adult Long-Evans rats were maintained on a 12 h light/dark cycle (lights on at 0600 h) with *ad libitum* access to food and water. All procedures, including surgery and postoperative care, were in strict compliance with the Ohio State University animal care guidelines, and all necessary measures were taken to minimize pain and discomfort. The surgical procedures were the same as those described in Experiment 1, with one caveat. Animals were anesthetized with inhalant isoflurane (2%, 0.6 l/min, O_2). The training and testing apparatus was identical to that described above.

4.1.2. Behavioral training and testing

All rats experienced a single adaptation session in the absence of any stimuli. Two groups of rats then underwent eight sessions of paired eyeblink conditioning in context A, as described in Experiment 2 (see Section 3.1.2.). Following the last eyeblink conditioning session, both groups experienced a second adaptation session (with the commutator tether removed) in novel context C (see Table 1; described below). The following day, one group underwent fear conditioning in context A whereas the second group underwent fear conditioning in context C. Accordingly, the E3–EBC–FC–S rats ($n = 7$) experienced eyeblink and fear conditioning in the same chamber, whereas E3–EBC–FC–D rats ($n = 7$) were fear conditioned in a chamber different from that used in eyeblink conditioning. The third group (E3–FC-only; $n = 7$) was both adapted and fear conditioned in context A. Freezing was measured in all rats to the fear conditioning context (A or C) and the cue (in a third, novel context, B).

For context C, transport involved removing two rats from their home cage and placing each into a metal, rounded container (10 in H × 9 in D) with a flat bottom and removable cover, drilled with five air-holes. The two rats were carried to the darkened training/testing room. A 25 W incandescent bulb, hung in the top corner of each sound attenuating chamber, was turned on to illuminate the conditioning chamber from above. Inside each chamber, the droppings tray was filled with heat-dried corncob bedding material (Bed-O'Cobs, The Andersons, Maunee, OH), a cardboard box (3 in L × 3 in W × 0.5 in D) was hung from the back wall of the cage, just above the grid bars, and a piece of cardboard was secured to the bottom of one side wall and angled upwards to the top of the cage at a 10° angle. Finally, a rose-scented solid air freshener (Xcel, East–West Distributing Co., Deerfield, IL) was placed inside the sound attenuating box, just above the conditioning chamber.

4.2. Results

4.2.1. Eyeblink conditioning

Eyeblink conditioning in the EBC–FC–D and EBC–FC–S rats was comparable to the CR acquisition rates found in Experiments 1 and 2. A repeated measure ANOVA found no significant difference in CR frequency between the two groups (data not shown).

4.2.2. Fear conditioning and testing

Freezing was measured during the initial 120 s of exposure to the fear conditioning chamber (Fig. 3A, far left). A one-way ANOVA (group) found a significant main effect, $F(2, 18) = 7.50$, $p < 0.01$, with higher levels of freezing in the EBC–FC–S rats relative to the other two groups. As in the prior experiment, rats exposed to the same chamber in which eyeblink conditioning occurred froze at an elevated rate. Freezing following the six CS–US fear conditioning trials was analyzed with a 3 (group) × 6 (trial) repeated measures ANOVA. Both main effects were statistically significant: group, $F(2, 90) = 4.98$, $p < 0.05$ and trial, $F(5, 90) = 2.33$, $p < 0.05$. Compared to the FC-only rats, post-hoc testing revealed significantly higher post-footshock freezing rates in the EBC–FC–S rats (Fig. 3A). The increase in post-shock freezing is taken to indicate a faster acquisition rate.

The EBC–FC–D rats showed minimal pre-shock freezing, on par with the FC-only rats. And yet they showed higher rates of

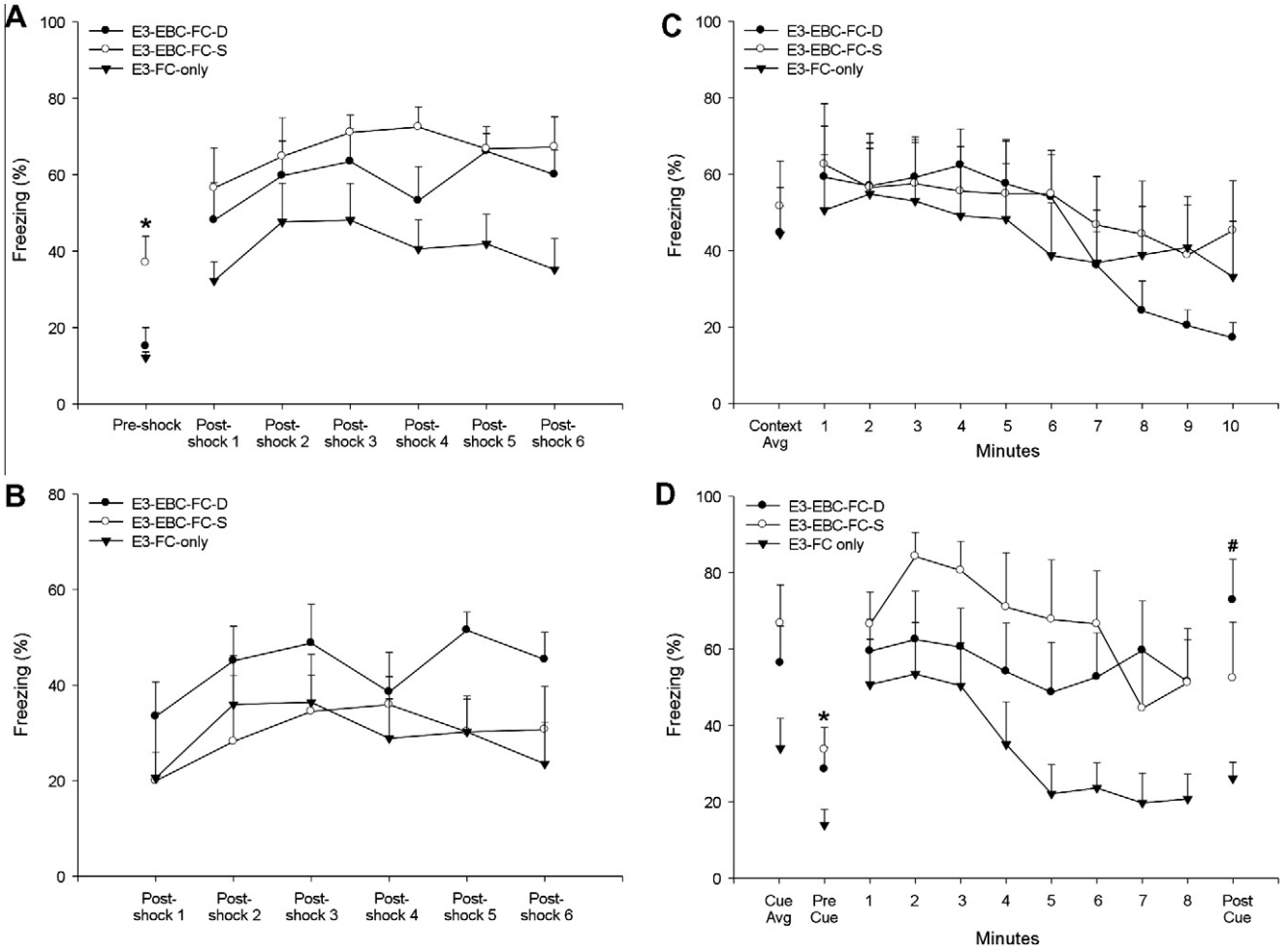

Fig. 3. Experiment 3: Post-footshock freezing during fear conditioning and cue/context testing. (A) Freezing (mean ± SE) before (Pre-shock) and immediately after six CS–US fear conditioning trials, in 120 s blocks. Rats that experienced eyeblink and fear conditioning in the same context (E3–EBC–FC–S) froze significantly more during the Pre-shock period (asterisk) than did rats fear conditioned in a different context (E3–EBC–FC–D) and rats that did not receive eyeblink preconditioning (E3–FC-only) (A). The same post-footshock freezing data is illustrated in (B), normalized to the Pre-shock freezing rate for each animal. This time, the EBC–FC–D rats showed the highest levels of post-footshock freezing. Conditioned freezing (mean ± SE) throughout the 10 min context test (C) and the 8 min cue test (D), as well as the one min before (Pre-cue) and one min after (Post-cue) presentation of the tone CS. No significant differences were observed for the context test. For the cue test, group differences in freezing approached but did not reach statistical significance ($p = 0.07$). The EBC–FC–S rats froze significantly more than the FC-only rats before (Pre-cue) presentation of the tone CS (asterisk). Following CS offset (Post-cue), the EBC–FC–D rats froze significantly more than the FC-only rats (pound sign).

post-footshock freezing. To examine this effect further, post-footshock freezing rates for each animal were normalized to their pre-shock freezing frequency. Fig. 3B shows that the highest levels of post-footshock freezing occurred in those rats fear conditioned in a context different from that in which eyeblink conditioning was conducted, albeit the repeated measures ANOVA revealed a significant effect for trial only, $F(5, 90) = 2.33$, $p < 0.05$. A one-way (group) ANOVA averaged across the six post-footshock periods did result in a statistically significant main effect, $F(2, 123) = 5.77$, $p < 0.01$, with post-hoc testing revealing a higher percent freezing for the EBC–FC–D rats (43.8 ± 2.8%) than the EBC–FC–S rats (29.9 ± 4.0%) or FC-only rats (29.3 ± 3.4%).

As reported above, there were no significant group differences when conditioned freezing was averaged or analyzed across the 10 min context test (Fig. 3C). Likewise, freezing to the 8 min tone CS was elevated in the EBC–FC–S rats (Fig. 3D), although the main group effect derived from the repeated measure ANOVA just missed statistical significance ($p = 0.07$). Freezing prior to and immediately following the test tone was analyzed with one-way (group) ANOVAs (Fig. 3D). As in Experiment 2, the EBC–FC–S rats froze significantly more prior to CS onset than the FC-only rats, $F(2, 18) = 3.80$, $p < 0.05$, as verified by post-hoc Tukey–Kramer testing. The group main effect was likewise significant for post-cue freezing, $F(2, 18) = 4.74$, $p < 0.05$, with the EBC–FC–D rats freezing more than the FC-only rats.

4.2.3. Discussion

There was significantly more post-footshock freezing in the EBC–FC–S rats relative to the FC-only rats, supporting the important role played by contextual fear. Nevertheless, when normalized and averaged across the six trials, the EBC–FC–D rats showed significantly greater post-footshock freezing than the other two groups. The results suggest that post-footshock freezing may represent a combination of both contextual- and cue-mediated fear.

5. General discussion

Results from the present set of experiments indicate that fear learning and responding can benefit from the prior acquisition of a simple motor response. In Experiment 1, only those rats first submitted to CS–US paired eyeblink conditioning displayed heightened fear to the tone CS, but not the training environment, following fear conditioning (Fig. 1B). Enhanced freezing to the CS

was observed when the CS/US durations were lengthened for fear conditioning (Fig. 1D), and when the durations were held constant across both forms of conditioning (Figs. 2D and 3D). There were no significant differences in freezing to either the context or cue among the multiple fear conditioned control groups (Fig. 1B–D). The results of Experiment 2 were also consistent with the two-process model of aversive conditioning (Konorski, 1967; Wagner & Brandon, 1989), with nonspecific emotional (fear) learning preceding the emergence of a specific motor (eyelid closure) response during delay eyeblink conditioning (Fig. 2A).

Experiments 2 and 3 examined whether eyeblink preconditioning also potentiated the acquisition of learned fear, focusing on post-footshock freezing. During fear conditioning, the EBC–FC rats froze at higher frequencies than the FC-only rats (Fig. 2B), suggesting fear learning was accelerated. Interestingly, however, significant increases in post-footshock freezing were observed only when the tone-footshock pairings occurred in the same context used for the antecedent eyeblink conditioning (E3–EBC–FC–S rats; Fig. 3A). Considering the high rate of pre-shock freezing observed in the EBC–FC rats (Figs. 2B and 3A) contextual fear might be deemed principally responsible for the faster fear conditioning. When post-footshock freezing was normalized to pre-shock freezing, however, the highest levels were observed in rats fear conditioned in the novel context (E3–EBC–FC–D rats; Fig. 3B). Thus, while post-footshock freezing is recognized as a fear CR produced in consequence of the context-US association (Fanselow, 1980), the increased freezing in EBC–FC–D rats suggests that at least part of the post-US freezing observed in the current study is dependent on the contingently paired excitatory CS. Tone-mediated freezing is not entirely unexpected considering the CS, when paired with the footshock US, was no longer a neutral stimulus—owing to the preceding eyeblink conditioning. Taken together, both contextual- and cue-mediated fear appear to modulate subsequent fear conditioning, with maximal learning occurring when the excitatory CS is presented in the emotionally arousing context.

At test, the consistently elevated pre-cue freezing shown by the EBC–FC rats is more difficult to understand, as is its potential relationship to the facilitated freezing behavior observed during the 8 min tone presentation (Figs. 1D, 2D, and 3D). Typically, rats submitted to footshocks in one environment display little freezing when placed into a new environment (Blanchard & Blanchard, 1969; Bolles & Collier, 1976). This is the case with the FC-only rats, which, as expected, displayed low levels of freezing behavior during the pre-cue period (Figs. 1D, 2D, and 3D), indicating the fault does not lie with the animal's ability to disambiguate the two contexts. The increased freezing behavior among the EBC–FC rats may lie in their continued expectancy of shock, following the nine (8 eyeblink/1 fear) conditioning sessions in which they were subjected to both periorbital shocks and footshocks. If so, it is curious that elevated pre-cue freezing is mainly seen in the EBC–FC rats, even though the Unpaired-FC rats in Experiment 1 received the same number of shock presentations across all sessions. Perhaps, then, the elevated freezing seen in the EBC–FC rats reflects unease or helplessness on the part of the animal. This still would not explain why the increase in freezing was observed only prior to the tone test, in a new environment, and never during the context test in the training chamber. Notwithstanding these considerations, we propose that the increased freezing seen during the tone test in EBC–FC rats is primarily the result of the same CS being sequentially paired with two types of aversive stimuli. Specifically, we hypothesize that the rapidly encoded amygdala-dependent plasticity responsible for fear responding throughout eyeblink conditioning may, when animals are switched to fear conditioning, accelerate or strengthen fear learning owing to enhanced amygdala unit activity to the tone CS.

Within the amygdala, distinct nuclei of the basolateral complex represent and condition different aspects of the environment and training stimuli. The lateral nucleus, for instance, is considered the site of convergence between a discrete auditory CS and footshock US during auditory fear conditioning (Nader, Majidishad, Amorapanth, & LeDoux, 2001; Romanski, LeDoux, Clugnet, & Bordi, 1993). The basal nucleus, with its strong anatomical and functional interactions with the hippocampal formation (Maren & Fanselow, 1995; McDonald, 1998; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000), constitutes an important substrate for integrating hippocampus-dependent context–US associations (Kim & Fanselow, 1992). Receiving input from the basolateral complex, the central nucleus in turn modulates autonomic and behavioral fear responding via projections to the brainstem, hypothalamus, and medulla (Hopkins & Holstege, 1978; LeDoux, Iwata, Cicchetti, & Reis, 1988).

Two points lend support to our hypothesis that enhanced CS-elicited amygdala unit activity facilitates fear learning in eyeblink preconditioned rats. First, the auditory thalamus is a relay site in the propagation of acoustic information to the cerebellum during eyeblink conditioning (Halverson & Freeman, 2006; Halverson, Poremba, & Freeman, 2008). Second, the thalamo-lateral amygdala pathway is rapidly potentiated during auditory fear conditioning (Quirk, Armony, & LeDoux, 1997; Quirk, Repa, & LeDoux, 1995). If the same pathway were potentiated by tone-periorbital shock pairings early in eyeblink conditioning, and at least some amount of increased spiking to the CS persisted to the end of training, the footshock US would necessarily be associated with more excitatory CS-initiated amygdala neuronal activity. This would require, of course, that the same lateral nucleus neurons, or some portion thereof, be innervated by somatosensory inputs relaying the eye- and footshock information.

A second potential site of plasticity is the central nucleus of the amygdala. Electrophysiological recordings have detected eyeblink CR-related changes in unit activity throughout the brain, including many structures outside the eyeblink conditioning brainstem–cerebellar neural circuit (reviewed in Christian & Thompson, 2003). In the central nucleus, approximately 30% of units exhibit eyeblink CR-related excitatory activity (i.e., increased firing to the initially neutral acoustic CS) by the end of 10 conditioning sessions (Rorick-Kehn & Steinmetz, 2005). The excitatory neuronal activity develops roughly in tandem with the eyeblink CR, is greatest just before US onset, and occurs solely on trials associated with an eyeblink CR.

In a manner similar to that described above, increased central amygdala firing to the tone CS during the late stages of eyeblink conditioning could facilitate the tone-footshock association during fear conditioning. Notably, Rorick-Kehn and Steinmetz (2005) found excitatory unit activity to be greatest at the end of 10 conditioning sessions, suggesting that potentiated spiking to the CS in EBC–FC rats could feasibly occur on some number of our fear conditioning trials. That the central nucleus might play a part in the associative plasticity underlying fear learning is suggested by the inverse effect, namely, impaired fear conditioning acquisition and consolidation in rats with a chemically inhibited central nucleus (Wilensky, Schafe, Kristensen, & LeDoux, 2006). In fact, recent research indicates the central nucleus is more than a mere output conduit for the amygdala, playing an active role in some forms of associative conditioning by contributing to the learning-dependent plasticity responsible for conditioned fear (Paré, Quirk, & LeDoux, 2004; Rorick-Kehn and Steinmetz, 2005; Wilensky et al., 2006). To be sure, these examples are not exhaustive, plasticity in other amygdala nuclei or structures upstream or downstream of the amygdala could contribute to the facilitated fear learning and responding detailed in the current study.

To conclude, we suggest that increased CS-mediated amygdala unit firing in eyeblink preconditioned rats facilitates its association

with the footshock US, leading to faster learning and enhanced freezing to the tone at test. The fact that learning is most robust when fear conditioning and the preceding eyeblink conditioning utilize the same training context and transport cues attests to the influence of a feared environment. Of course, such adaptive behavior ought not to be unexpected in organisms that must continually reevaluate previous learning in the light of current experience.

Acknowledgments

Funding provided by NIAAA grant (J.E.S.) AA011945 and NCRR grant (L.P.M.) P20 RR016475. The authors thank Benjamin Hornung, Eric Iheme and Sheila Tsau for assistance running rats.

References

Blanchard, R. J., & Blanchard, D. C. (1969). Crouching as an index of fear. *Journal of Comparative and Physiological Psychology, 67*(3), 370–375.

Bolles, R. C., & Collier, A. C. (1976). The effect of predictive cues on freezing in rats. *Animal Learning & Behavior, 4*, 6–8.

Britton, G. B., & Astheimer, L. B. (2004). Fear develops to the conditioned stimulus and to the context during classical eyeblink conditioning in rats. *Integrative Physiological & Behavioral Science, 39*(4), 295–306.

Chachich, M., & Powell, D. A. (1998). Both medial prefrontal and amygdala central nucleus lesions abolish heart rate classical conditioning, but only prefrontal lesions impair reversal of eyeblink differential conditioning. *Neuroscience Letters, 257*, 151–154.

Christian, K. M., & Thompson, R. F. (2003). Neural substrates of eyeblink conditioning: Acquisition and retention. *Learning and Memory, 11*, 427–455.

Corcoran, K. A., & Quirk, G. J. (2007). Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. *The Journal of Neuroscience, 27*(4), 840–844.

Fanselow, M. S. (1980). Conditional and unconditional components of post-shock freezing. *Pavlovian Journal of Biological Science, 15*, 177–182.

Halverson, H. E., & Freeman, J. H. (2006). Medial auditory thalamic nuclei are necessary for eyeblink conditioning. *Behavioral Neuroscience, 120*(4), 880–887.

Halverson, H. E., Poremba, A., & Freeman, J. H. (2008). Medial auditory thalamus inactivation prevents acquisition and retention of eyeblink conditioning. *Learning & Memory, 15*(7), 532–538.

Hopkins, D. A., & Holstege, G. (1978). Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. *Experimental Brain Research, 32*, 529–547.

Kim, J. J., & Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science, 256*, 675–677.

Konorski, J. (1967). Integrative activity of the brain. Chicago: University of Chicago.

Lavond, D. G., Lincoln, J. S., McCormick, D. A., & Thompson, R. F. (1984). Effect of bilateral lesions of the dentate and interpositus cerebellar nuclei on conditioning of heart-rate and nictitating membrane/eyelid responses in the rabbit. *Brain Research, 305*, 323–330.

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience, 23*, 155–184.

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *The Journal of Neuroscience, 8*(7), 2517–2529.

Lee, T., & Kim, J. J. (2004). Differential effects of cerebellar, amygdalar, and hippocampal lesions on classical eyeblink conditioning in rats. *The Journal of Neuroscience, 24*(13), 3242–3250.

Lennartz, R. C., & Weinberger, N. M. (1992). Analysis of response systems in Pavlovian conditioning reveals rapidly versus slowly acquired conditioned responses: Support for two factors, implications for behavior and neurobiology. *Psychobiology, 20*(2), 93–119.

Lindquist, D. H., & Brown, T. H. (2004). Amygdalar NMDA receptors control the expression of associative reflex facilitation and three other conditional responses. *Behavioral Neuroscience, 118*(1), 36–52.

Mackintosh, N. J. (1983). *Conditioning and associative learning*. New York: Oxford University Press.

Maren, S., & Fanselow, M. S. (1995). Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. *The Journal of Neuroscience, 15*, 7548–7564.

McDonald, A. J. (1998). Cortical pathways to the mammalian amygdala. *Progress in Neurobiology, 55*, 257–332.

Mintz, M., & Wang-Ninio, Y. (2001). Two-stage theory of conditioning: Involvement of the cerebellum and the amygdala. *Brain Research, 897*, 150–156.

Mowrer, O. H. (1947). On the dual nature of learning – A reinterpretation of "conditioning" and "problem-solving". *Harvard Educational Review, 17*, 102–148.

Nader, K., Majidishad, P., Amorapanth, P., & LeDoux, J. E. (2001). Damage to the lateral, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning. *Learning & Memory, 8*, 156–163.

Paré, D., Quirk, G. J., & LeDoux, J. E. (2004). New vistas on amygdala networks in conditioned fear. *Journal of Neurophysiology, 92*, 1–9.

Pitkanen, A., Pikkarainen, M., Nurminen, N., & Ylinen, A. (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. *Annals of the New York Academy of Sciences, 911*, 369–391.

Prokasy, W. F. (1972). Developments with the two-phase model applied to human eyelid conditioning. In A. H. Black & W. F. Prokasy (Eds.), *Classical conditioning II: Current research and theory* (pp. 199–247). New York: Appleton-Century-Crofts.

Quirk, G. J., Armony, J. L., & LeDoux, J. E. (1997). Fear conditioning enhances different temporal components of tone-evoked spike trains in auditory cortex and lateral amygdala. *Neuron, 19*, 613–624.

Quirk, G. J., Repa, C., & LeDoux, J. E. (1995). Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: Parallel recordings in the freely behaving rat. *Neuron, 15*(5), 1029–1039.

Rescorla, R. A., & Solomon, R. L. (1967). Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychological Review, 74*(3), 151–182.

Romanski, L. M., LeDoux, J. E., Clugnet, M. C., & Bordi, F. (1993). Somatosensory and auditory convergence in the lateral nucleus of the amygdala. *Behavioral Neuroscience, 107*, 444–450.

Rorick-Kehn, L. M., & Steinmetz, J. E. (2005). Amygdalar unit activity during three learning tasks: Eyeblink classical conditioning, Pavlovian fear conditioning, and signaled avoidance conditioning. *Behavioral Neuroscience, 119*(5), 1254–1276.

Schneiderman, N., Smith, M. C., Smith, A. C., & Gormezano, I. (1966). Heart rate classical conditioning in rabbits. *Psychological Science, 6*, 241–242.

Thompson, R. F., Donegan, N. H., Clark, G. A., Lavond, D. G., Lincoln, J. S., Madden, J., et al. (1987). Neuronal substrates of discrete, defensive conditioned reflexes, conditioned fear states, and their interactions in the rabbit. In I. Gormezano, W. F. Prokasy, & R. F. Thompson (Eds.), *Classical conditioning* (3rd ed., pp. 371–399). Hillsdale, NJ: Erlbaum.

Wagner, A. R., & Brandon, S. E. (1989). Evolution of a structured connectionist model of Pavlovian conditioning (AESOP). In S. B. Klein & R. R. Mowrer (Eds.), *Contemporary learning theories: Pavlovian conditioning and the status of traditional learning theory* (pp. 149–190). Hillsdale, NJ: Erlbaum.

Weisz, D. J., Harden, D. G., & Xiang, Z. (1992). Effects of amygdala lesions on reflex facilitation and conditioned response acquisition during nictitating membrane response conditioning in rabbit. *Behavioral Neuroscience, 106*(2), 262–273.

Wilensky, A. E., Schafe, G. E., Kristensen, M. P., & LeDoux, J. E. (2006). Rethinking the fear circuit: The central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. *The Journal of Neuroscience, 26*(48), 12387–12396.