

Central amygdala lesions inhibit pontine nuclei acoustic reactivity and retard delay eyeblink conditioning acquisition in adult rats

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Abstract In delay eyeblink conditioning (EBC) a neutral conditioned stimulus (CS; tone) is repeatedly paired with a mildly aversive unconditioned stimulus (US; periorbital electrical shock). Over training, subjects learn to produce an anticipatory eyeblink conditioned response (CR) during the CS, prior to US onset. While cerebellar synaptic plasticity is necessary for successful EBC, the amygdala is proposed to enhance eyeblink CR acquisition. In the current study, adult Long-Evans rats received bilateral sham or neurotoxic lesions of the central nucleus of the amygdala (CEA) followed by 1 or 4 EBC sessions. Fear-evoked freezing behavior, CS-mediated enhancement of the unconditioned response (UR), and eyeblink CR acquisition were all impaired in the CEA lesion rats relative to sham controls. There were also significantly fewer c-Fos immunoreactive cells in the pontine nuclei (PN)—major relays of acoustic information to the cerebellum—following the first and fourth EBC session in lesion rats. In sham rats, freezing behavior decreased from session 1 to 4, commensurate with nucleus-specific reductions in amygdala Fos⁺ cell counts. Results suggest delay EBC proceeds through three stages: in stage one the amygdala rapidly excites diffuse fear responses and PN acoustic reactivity, facilitating cerebellar synaptic plasticity and the development of eyeblink CRs in stage two, leading, in stage three, to a diminution or stabilization of conditioned fear responding.

Keywords Pavlovian conditioning · Eyeblink conditioning · Fear conditioning · Cerebellum · Amygdala

Different forms of associative learning and memory rely on distinct but distributed neural substrates and circuits (Fanselow & Poulos, 2005; Rudy, 2009; Stanton, 2000). For example, learning to fear specific stimuli or events depends on the amygdala (Duvarci & Pare, 2014; LeDoux, 2014), whereas simple forms of sensorimotor learning, such as eyeblink classical conditioning, depend on the cerebellum and brainstem (Christian & Thompson, 2003; Freeman & Steinmetz, 2011). In delay eyeblink conditioning (EBC), a light or tone conditioned stimulus (CS) is repeatedly paired with a mildly aversive corneal air puff or periorbital shock unconditioned stimulus (US). Over hundreds of training trials the CS comes to elicit an adaptive eyeblink conditioned response (CR), with maximal eyelid closure occurring just before the expected US.

The neural circuitry responsible for eyeblink CR acquisition and expression has been extensively described over the last half century (reviewed in Steinmetz & Lindquist, 2009). Briefly, the CS signal is relayed to the cerebellum by pontine nuclei (PN) mossy fibers (Aitkin & Boyd, 1978; Steinmetz & Sengelaub, 1992) while the US signal is relayed via climbing fibers from the inferior olive (Türker & Miles, 1986; Yeo, Hardiman, & Glickstein, 1986). Accordingly, the CS and US signals converge on Purkinje cells in the cerebellar cortex, HVI and the anterior lobe in particular, and interpositus nucleus (IP) projection neurons (Thompson & Steinmetz, 2009). Learning-dependent increases in IP neuronal firing activate the downstream motor nuclei responsible for eyelid closure (Berthier & Moore, 1990; McCormick & Thompson, 1984), while inhibitory output from the cerebellar cortex modulates the amplitude and timing of the eyeblink CR (Green & Steinmetz, 2005; Perrett, Ruiz, & Mauk, 1993).

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Two-stage models of aversive Pavlovian conditioning posit the rapid emergence of amygdala-dependent emotional responses in stage one, which accelerates the acquisition of more slowly acquired cerebellar-dependent sensorimotor responses in stage two (Boele, Koekkoek, & De Zeeuw, 2010; Lennartz & Weinberger, 1992; Mintz & Wang-Ninio, 2001; Rescorla & Solomon, 1967; Thompson et al., 1987; Wagner & Brandon, 1989). In eyeblink conditioned subjects, for instance, a number of fear CRs are observed during the first training session—before significant numbers of eyeblink CRs are produced—including autonomic changes in heart rate and blood pressure, elevated freezing, and 22 kHz ultrasonic emissions (Britton & Astheimer, 2004; Lee & Kim, 2004; Lindquist, Mahoney, & Steinmetz, 2010; Prokasy, 1972; Schneiderman, 1972). As predicted by the two-stage model, amygdala damage or pharmacological inactivation diminishes fear responding and retards eyeblink CR acquisition (Blankenship, Huckfeldt, Steinmetz, & Steinmetz, 2005; Chachich & Powell, 1998; Lee & Kim, 2004; Neufeld & Mintz, 2001; Weisz, Harden, & Xiang, 1992). No studies, to our knowledge, have reported that amygdala damage or inactivation prevents delay EBC, signifying the amygdala contributes to (but is not necessary for) the acquisition or expression of discrete motor responses.

The precise mechanism(s) by which the amygdala facilitates cerebellar-dependent motor learning is not known, but different functions have been attributed to individual amygdala nuclei. The emotional significance (or motivational value) of the aversive US is thought to be encoded within the basolateral nucleus (BLA), which promotes association of the CS–US signals in the lateral nucleus (LA), increasing the arousal or attention directed at the CS via activation of central nucleus (CEA) projection neurons (Blair, Sotres-Bayon, Moita, & LeDoux, 2005; Penzo, Robert, & Li, 2014; Rorick-Kehn & Steinmetz, 2005; Sah & Armentia, 2003). The CEA could potentiate CS-evoked activity in the PN, the last site outside the cerebellum where CS sensory afferents converge, through monosynaptic and/or polysynaptic projections (Berger, Alger, & Thompson, 1976; Kandler & Herbert, 1991; Mihailoff, Kosinski, Azizi, & Border, 1989). In Taub and Mintz (2010), for instance, tone-evoked PN unit reactivity was increased during CS–US paired but not unpaired trials, while CEA inactivation (via lidocaine) prevented the increase in PN unit reactivity during presentation of the tone CS.

In the current study, the potentiated CEA unit activity that underlies fear CR expression (e.g., freezing) was hypothesized to increase the population of PN neurons activated in response to the tone CS, promoting enhanced CS–US synaptic plasticity in the cerebellum and the emergence of eyeblink CRs. Bilateral neurotoxic CEA lesions, or sham surgery, was performed one week prior to the start of delay EBC. All rats were submitted to one or four delay EBC sessions and then sacrificed in order to relate each subject's behavior to

posttraining neuronal activity. PN tissue was submitted to immunohistochemical (IHC) processing for c-Fos, an immediate early gene whose expression was used as an indirect marker of neuronal activity (Bullitt, 1990). If amygdala output regulates fear responding and PN acoustic reactivity, then CEA lesion rats should freeze less, have fewer Fos+ PN neurons, and demonstrate impaired EBC acquisition relative to sham controls. Furthermore, differential activation of individual amygdala nuclei across delay EBC, as suggested above, should alter the degree and persistence of neuronal activity in each nucleus. Fos+ neurons in the BLA, CEA, and LA were therefore counted and analyzed following Sessions 1 and 4 in sham rats.

Materials and method

Subjects

Experimentally naïve male Harlan Laboratories (Indianapolis, IN, USA) Long-Evans rats were individually housed and maintained in The Ohio State University Psychology vivarium. Subjects were maintained on a 12 hr light/dark cycle (lights on at 0600 hr) 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 approved by and in strict compliance with OSU institutional animal care and use committee (IACUC) animal care guidelines, and all necessary measures were taken to minimize pain and discomfort.

Surgical procedures

Based on prior EBC literature utilizing amygdala lesions or inactivation (e.g., Blankenship et al., 2005; Lee & Kim, 2004), we estimated 8 to 10 rats per group would provide sufficient power to detect significant impairments in conditioned fear and delay EBC. Approximately one week after arrival, all subjects (weighing 320–340 g) were anesthetized with respiratory isoflurane (1 %–3 %, 0.6 l/min O₂) and secured in a stereotaxic frame. The scalp was incised and two burr holes drilled in each hemisphere. Bilateral excitotoxic CEA lesions were done using the following coordinates, relative to Bregma (in mm): A/P, -2.1, M/L, ±4.3, D/V, -8.2; and A/P, -2.4, M/L, ±4.3, D/V, -8.5. A 1.0 µl Hamilton syringe, attached to a Micro4 microsyringe pump controller (model UCM4, World Precision Instruments, Sarasota, FL, USA), was lowered into each drop site. A total volume of 0.1 µl of ibotenic acid (Thermo Fisher Scientific, Geel, Belgium), dissolved in PBS vehicle (10 µg/µl), was injected in each hole at a rate of 0.1 µl/min. The syringe was left in place an additional 2 min to allow for dispersal of the drug prior to its removal. Sham lesion rats received bilateral infusions of PBS vehicle at the same rate

and volume using the same coordinates. Once injections were complete, the burr holes were sealed with bone wax.

Next, each subject was surgically prepared for EBC. Electromyogram (EMG) activity was recorded in the orbicularis oculi (OO) muscle that surrounds the left eye by passing two ultrathin (0.003-in) Teflon-coated stainless steel wires beneath the anterior portion of the upper eyelid. For delivery of the US periorbital electrical shock, gold-coated stainless steel wires were implanted in the dorso-caudal portion of the OO muscle. 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 6-pin plastic connector (M363; Plastics One Inc. Roanoke, VA). The headstage and bipolar stimulating electrodes were fixed in dental cement. The wound was salved with antibiotic ointment (Neosporin, Johnson & Johnson, New Brunswick, NJ), and rats were administered an oral dose (in water bottles) of Meloxicam (1 mg/kg; The Ohio State University Veterinary Services) for two consecutive days. All subjects were given at least 7 days to recover and handled the 2 days immediately prior to the start of training.

Conditioning apparatus

Eyeblink conditioning took place in standard operant boxes (Coulburn Instruments, Allentown, PA), contained within sound-attenuating chambers. Each operant box had 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. The interior of each chamber was illuminated by an infrared light source, and behavior was recorded with a black-and-white video camera with a wide-field lens (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ). The video signal was inputted to FreezeScan (CleverSys, Inc., Reston, VA), a software package that provides precise motion detection, quantifying the percentage of time that rats are motionless. The headstage of each rat was attached to a six channel commutator (SL6C/SB, Plastics One, Inc., Roanoke, VA, USA), allowing subjects to move freely in the conditioning chamber.

Behavioral training, testing, and analyses

Following surgery, rats were pseudo-randomly assigned to undergo one or four EBC sessions. Each chamber was sprayed with Windex prior to placing the rat inside. All rats experienced an initial 50 min stimulus-free adaptation session, followed by one session or four consecutive sessions of delay EBC, separated by ~24 hr. Each EBC session consisted of 10 blocks of 12 trials: 10 CS–US paired, 1 CS-alone, and 1 US-alone. The CS was a 450 ms, 2.8 kHz, 80 dB tone and the US was a coterminating train of 100 ms, 2.0 mA, 60 Hz, constant-current square wave periocular electrical stimulation. The

interstimulus interval (ISI), from CS onset to US onset, was 350 ms and the intertrial interval (ITI) was 25 ± 5 s.

On each trial, eyelid EMG activity from the OO muscle was sampled for 1,500 ms, divided into three periods: (i) a 350 ms pre-CS period; (ii) a 350 ms CS–US period; and (iii) an 800 ms post-US period. For each trial, the averaged EMG activity in the pre-CS period was used as a baseline for classifying blink behavior. Trials were excluded from analysis if EMG activity exceeded baseline activity by 5 or more standard deviations during the bad trial window, extending from 100 ms before and up to 20 ms after CS onset. Elevation of EMG activity within the first 100 ms after CS onset was considered an alpha (startle) response and not counted as an eyeblink CR. A conditioned blink was scored only if EMG activity exceeded baseline activity by 5 or more standard deviations beginning 100 ms after CS onset through the end of the CS–US period. CR peak amplitude data was based on CS-alone trials; UR peak amplitude was measured following US offset for US-alone and CS-US trials.

Freezing was defined as cessation of all visible body or vibrissae movements except that required for respiration (Fanselow, 1980). Freezing behavior was measured and analyzed in two ways: during the initial 60 s of each session (baseline), prior to the first CS–US trial, representing context-dependent fear behavior; and across the entire session, representing a combination of context- and CS-evoked freezing.

Histology

Subjects were sacrificed 1 hr after the first or fourth EBC session using 200 mg/kg of Euthasol (Virbac, Fort Worth, TX, USA) and immediately perfused with 0.9 % saline followed by 4 % paraformaldehyde. Brains were stored in 4 % paraformaldehyde and then transferred 2 days later to 4 % paraformaldehyde/30 % sucrose for an additional 2 to 3 days. A Leica vibrating microtome (VT1000S, Buffalo Grove, IL, USA) was used to section tissue (40 μ m) through the amygdala and the PN. Tissue sections were stored at -20 °C in a cryoprotective buffer containing ethylene glycol until processed for immunohistochemistry (Koo, Han, & Kim, 2004).

To determine the placement and extent of CEA lesions, tissue sections were stained for anti-NeuN antibody (MAB377; Merck Millipore, Billerica, MA, USA). In vertebrates, NeuN is a neuron-specific nuclear protein expressed in most neuronal cell types, making it an excellent marker for identifying neurons (Mullen, Buck, & Smith, 1992). Tissue sections from lesion rats were quenched in 0.3 % H₂O₂ / 50 % methanol and blocked in 5 % normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 hr. Sections were incubated overnight at 4 °C with 1/200 dilution of anti-NeuN antibody. The next day, sections were incubated in 1/200 dilution of biotinylated antimouse secondary antibody

(BA-2001; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 hr at room temperature and then rinsed three times in PBS. The tissue was incubated for 1 hr at room temperature with avidin-biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA), rinsed, incubated with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride (DAB substrate kit; BD Pharmingen, San Diego, CA, USA), and then rinsed in PBS to stop the reaction. Sections were mounted and allowed to dry overnight before cover-slipping. Images of CEA lesions were acquired using a Nikon 90i scope at 10X magnification and stitched using Nikon Elements software. CEA lesion reconstructions were drawn on three Paxinos and Watson (1998) plates: -1.88, -2.56, and -3.14 mm, from Bregma, and expressed in percentage form relative to the intact CEA.

Neuronal activity in the amygdala (from sham-operated rats) and the pontine nuclei (from sham and lesion rats) was quantified via immunodetection of Fos+ cells. Beginning with a pseudo-random tissue section—from Bregma, approximately -1.9 mm for the amygdala and -6.7 mm for the pontine nuclei—every sixth or fourth section, respectively, was quenched in 0.3 % H₂O₂ and blocked in 3 % normal goat serum for 1 hr. Sections were incubated for 48 hr at 4 °C with a rabbit polyclonal antibody against the c-Fos protein (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1/2000 in 3 % normal goat serum. Sections were then incubated at room temperature for 2 hr with a 1/200 dilution of biotinylated antirabbit secondary antibody (BA-1000; Vector Laboratory, Burlingame, CA, USA). Next, sections were incubated for 1 hr at room temperature with the Vectastain ABC kit, rinsed, incubated with a DAB substrate kit, then rinsed in PBS to stop the reaction. Sections were mounted on slides and, the next day, cover-slipped. For each subject, images were acquired from the PN and amygdala using a Nikon 90i scope at 10X magnification and stitched using Nikon Elements software. For each tissue section, regions of interest (ROIs) were drawn around specific nuclei in the PN or amygdala. The PN was broken into the medial pontine nuclei (MPN), which spanned the midline, and the lateral pontine nuclei (LPN), which is distributed between both hemispheres (see Fig. 3c). For the amygdala, ROIs were drawn in each hemisphere around the BLA, CEA, and LA (see Fig. 4b). All cell counts were done in a blind manner by one author and a research assistant. Total cell counts were divided by area to generate the number of Fos+ cells per unit area (mm²). Finally, the percent difference in mean Fos+ cell counts from Session 1 to 4 was calculated for each ROI: $[(\text{Session 4 mean cell counts} / \text{Session 1 mean cell counts}) - 1] * 100$.

Data analyses

Statistical analyses were performed with Windows versions of SYSTAT (v. 13) and SPSS (v. 22). Behavioral and IHC results

were analyzed using single-factor, mixed design, and repeated-measures ANOVAs, followed, when appropriate, by Tukey–Kramer post hoc testing. A significant post hoc effect implies $p < .05$.

Results

Lesion reconstructions

A total of 45 rats underwent surgery in the present study. Thirteen rats were excluded from analysis due to insufficient CEA damage (<50 % damage across three Paxinos and Watson plates in one or both hemispheres), resulting in the following sample size per group: Sham 1 session ($n = 8$), Lesion 1 session ($n = 8$), Sham 4 session ($n = 8$), and Lesion 4 session ($n = 8$). Figure 1a illustrates CEA lesions across three plates from Paxinos and Watson (1998): -1.88, -2.56, and -3.14 mm, with the smallest and largest CEA lesions shown in black and gray, respectively. Mean ($\pm SE$) CEA damage across both one and four session rats was $66 \pm 2\%$. Minor damage did occur to surrounding structures in addition to the CEA, including the BLA and LA.

Behavioral analyses

Freezing and eyeblink CR rates were calculated in sham and CEA lesion rats trained with one or four EBC sessions. In rats trained for one session, a single-factor (Lesion) ANOVA revealed significantly reduced session-wide freezing rates in CEA lesion rats ($30.1 \pm 5.7\%$) compared to sham rats ($49.5 \pm 4.6\%$), $F(1, 14) = 6.88$, $p < .05$ (data not shown). Figure 2a illustrates freezing behavior in the four session rats during the first 60 s of each training session (baseline) and session-wide averages. Baseline freezing, analyzed with a 2 (Lesion) \times 4 (Session) repeated measures ANOVA, yielded significant main effects for Lesion only, $F(1, 14) = 30.47$, $p < .0001$. Tukey–Kramer post hoc testing indicates the lesion rats froze significantly less than sham rats. Session-wide freezing rates, based on the same ANOVA, yielded a significant Lesion \times Session interaction only, $F(3, 42) = 5.71$, $p < .01$. Follow-up one-way (Lesion) ANOVAs indicate the CEA lesion rats froze significantly less than sham controls during the first training session only.

Delay EBC acquisition was also adversely affected by CEA lesions in the four session but not one session rats. For the latter, a single-factor (Lesion) ANOVA applied to eyeblink CR percentage was not statistically significant ($p = .67$), as expected, considering both groups had low rates of eyeblink CRs on Session 1 (data not shown). In rats trained for four sessions, the 2 (Lesion) \times 4 (Session) repeated measures ANOVA revealed significant main effects for Lesion, $F(1, 14) = 5.35$, $p < .05$, and Session, $F(3, 42) = 5.11$, $p < .01$,

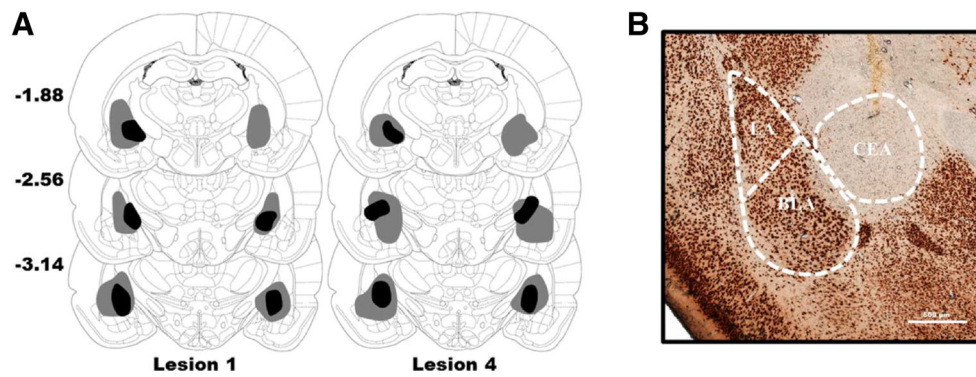


Fig. 1 Bilateral CEA ibotenic acid lesions. (a) Prior to sacrifice, both sham and lesion rats were submitted to 1 (left) or 4 (right) EBC session(s). Reconstructions are based on three atlas plates (from Bregma: -1.88, -2.56, and -3.14 mm) taken from Paxinos and Watson (1998). The largest lesions are shown in gray and the smallest lesions are shown in black. (b)

Representative unilateral photomicrograph of a CEA ibotenic acid lesion as indicated by the lack of NeuN-positive neurons. The lateral amygdala (LA), basolateral amygdala (BLA), and central amygdala (CEA) are outlined by dashed lines. Scale bar: 500 μ m

but not their interaction. Post hoc testing verified that significantly fewer eyeblink CRs were produced by lesion than sham rats (see Fig. 2b). The same ANOVA applied to CR peak amplitudes, shown in Fig. 2c, found a significant main effect for Session only, $F(3, 42) = 3.21, p < .05$. Minus a significant

interaction, follow-up one-way (Lesion) ANOVAs on each training session were Bonferroni-corrected, requiring $p < .0125$ for significance. While CR amplitude was diminished in lesion rats on Session 1, the difference from sham controls was not statistically different ($p = .035$).

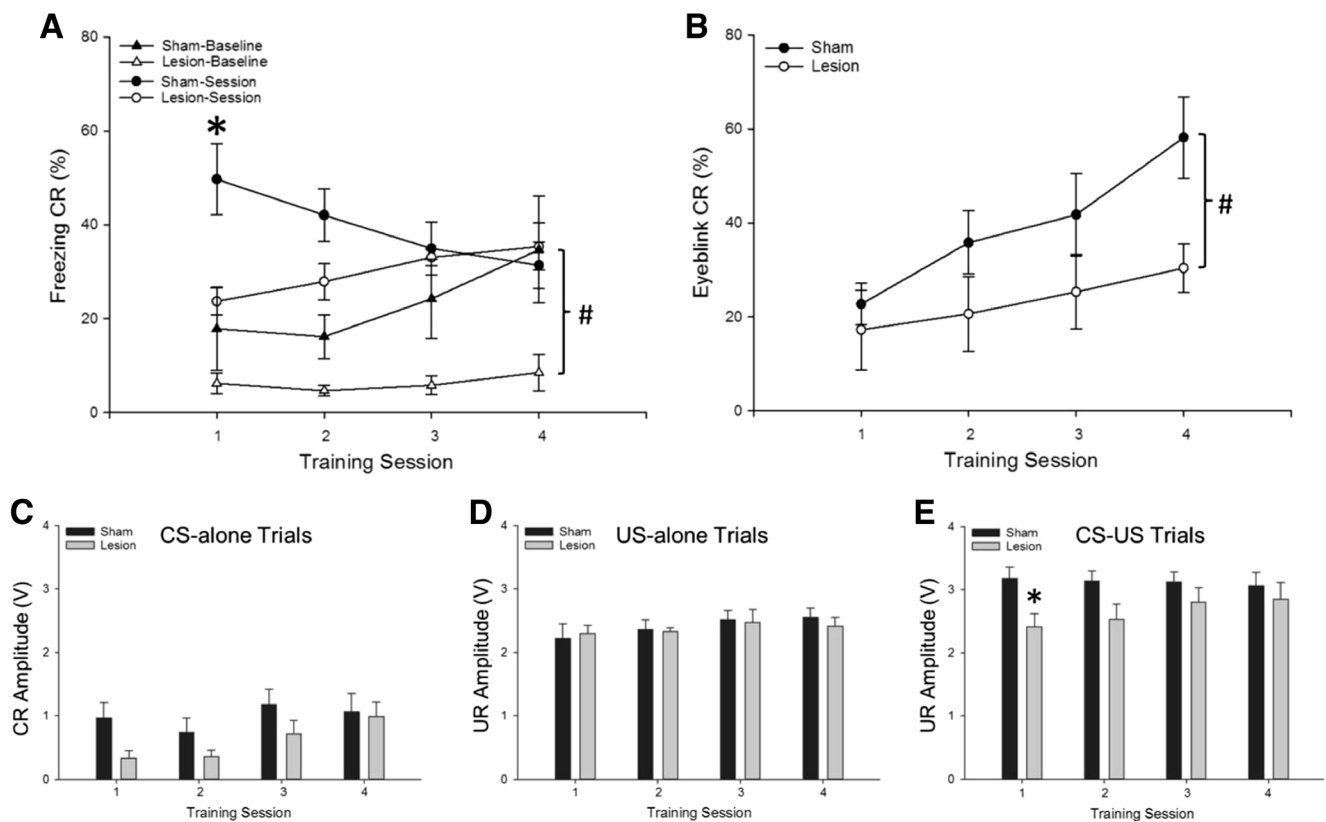


Fig. 2 Freezing behavior and eyeblink conditioned and unconditioned responding in rats trained with 4 EBC sessions. (a) Freezing (mean \pm SE) was measured during the 60 s baseline (triangles) or the entire session (circles). Relative to sham controls, results for the CEA lesion rats revealed significantly reduced baseline freezing (pound sign) and, on Session 1, significantly reduced session-wide freezing (asterisk). (b) Eyeblink CR percentages (mean \pm SE) were significantly reduced in CEA

lesion rats relative to sham controls (pound sign). (c) CR peak amplitude (mean \pm SE) was not significantly altered in CEA lesion rats. (d) UR peak amplitude (mean \pm SE), on US-alone trials, was not significantly altered in CEA lesion rats. (e) UR peak amplitude (mean \pm SE) during CS-US trials was significantly reduced, on Session 1 only, in lesion rats relative to sham controls (asterisk)

Eyeblink UR peak amplitudes were also analyzed in sham and lesion rats based on US-alone and CS–US paired trials. The former reflects UR strength while the latter measures associative UR facilitation due to the fear-enhancing effects of the tone CS following its pairing with the aversive US (Brown, Kalish, & Farber, 1951; Choi, Lindquist, & Brown, 2001). Results for US-alone trials, based on 2 (Lesion) \times 4 (session) repeated measures ANOVAs, uncovered a significant main effect for Session only, $F(3, 42) = 3.32, p < .05$, signifying CEA lesions had no deleterious effect on processing or responding to the US signal (see Fig. 2d). There was, however, a significant Lesion \times Session interaction when UR amplitude was measured following presentation of the tone CS, $F(3, 42) = 2.92, p < .05$. Follow-up one-way ANOVAs indicate associative UR facilitation was significantly reduced in lesion rats on Session 1 only (see Fig. 2e). Together, the data suggest that CEA lesions had no effect on UR production but did diminish the ability of the CS to potentiate the eyeblink UR.

Immunohistochemical analyses

PN cell counts were completed by one author and a research assistant blind to each rat's experimental group. The interrater coefficient of determination, based on a subset of ROIs, was $r^2 = 0.93$. There has been a debate in the literature regarding whether acoustic reactivity is restricted to the LPN or distributed throughout the entire PN—thus, Fos+ cells in the LPN and MPN were initially counted and analyzed independently (see Fig. 3c). ROI area encompassed by the LPN (summed across both hemispheres) and MPN was compared first, using a 2 (Lesion) \times 2 (PN region) \times 2 (Session) multifactorial ANOVA. Results revealed a significant main effect for PN region only, $F(1, 166) = 24.34, p < .0001$, with LPN area ($0.90 \pm .03$ mm) significantly smaller than MPN area ($1.10 \pm .03$ mm; data not shown). Fos+ cell counts normalized to LPN and MPN area were analyzed next using the same multifactor ANOVA. Results revealed significant main effects for PN region, $F(1, 166) = 5.87, p < .05$ and Lesion, $F(1, 166) = 42.59, p < .001$, but no interaction. Post hoc testing verified the MPN contains significantly fewer Fos+ cells than the LPN (see Fig. 3a, left) and, across both regions, the CEA lesion rats have significantly fewer Fos+ cells than sham rats (see Fig. 3a, right). Cell counts were next examined for the entire PN in subjects submitted to one or four EBC sessions. Single-factor (Lesion) ANOVAs indicate the total number of Fos+ cells was significantly reduced in CEA lesion rats for Session 1, $F(1, 40) = 12.18, p < .001$, and Session 4, $F(1, 39) = 14.34, p < .001$ (see Fig. 3b). Reduced auditory-evoked PN neuronal reactivity is proposed to contribute to the retardation in EBC acquisition seen in lesion rats (see Fig. 2b). Percent difference scores—based on between-subject differences in mean PN Fos+ cell counts—indicate cell counts were decreased from

Session 1 to 4 by just 2.4 % in sham rats and increased by 2.2 % in lesion rats.

Finally, the number of amygdala Fos+ cells following Sessions 1 and 4 in sham rats was quantified in three amygdala nuclei: the BLA, CEA, and LA. Single-factor (Session) ANOVAs indicate there were significant (between-subject) decreases from Session 1 to 4 in the BLA, $F(1, 124) = 4.58, p < .05$, CEA, $F(1, 124) = 13.89, p < .001$, and LA, $F(1, 124) = 10.69, p < .001$ (see Fig. 4a). The percent difference scores indicate Fos+ cell counts were decreased from Session 1 to 4 by 18.2 % in the BLA, 40.0 % in the CEA, and 35.7 % in the LA. Results confirm amygdala activation was diminished in a nucleus-specific manner across the four EBC sessions.

Discussion

Current results indicate that elevated fear responding preceded the emergence of eyeblink CRs in sham control rats, consistent with two-stage models of aversive Pavlovian conditioning (Boele et al., 2010; Lennartz & Weinberger, 1992; Mintz & Wang-Ninio, 2001; Rescorla & Solomon, 1967; Thompson et al., 1987; Wagner & Brandon, 1989). Bilateral neurotoxic CEA lesions were assessed for their effects on conditioned fear (freezing), CS-mediated UR facilitation, PN acoustic reactivity, and delay EBC acquisition. The lesions impeded fear-dependent behavior and retarded the emergence of eyeblink CRs across four EBC sessions (see Fig. 2). CEA lesions also significantly reduced the number of Fos+ PN cells, suggesting the amygdala may normally enhance CS saliency and facilitate EBC acquisition by increasing the population of tone-reactive PN neurons (see Fig. 3). Finally, bigger decreases in Fos+ cell counts in the sham rats were seen following Session 4 in LA and CEA than the BLA, compared to Session 1 (see Fig. 4). Taken together, we propose delay EBC proceeds through two well-documented stages, followed by a third putative stage involving cerebellar-mediated emotional regulation.

Two-stage models of aversive Pavlovian conditioning conceptualize sensorimotor learning to pass through two sequential stages (Neufeld & Mintz, 2001; Thompson et al., 1987; Wagner & Brandon, 1989). Broadly speaking, in response to the aversive US the amygdala is proposed to rapidly initiate a state of “conditioned arousal” early in EBC (Kapp, Wilson, Pascoe, Supple, & Whalen, 1990; Thompson et al., 1987), enhancing the salience of relevant stimuli and the rate at which eyeblink CRs emerge. Many studies have documented rapid changes in both autonomic and defensive behavior early in EBC (e.g., Lee & Kim, 2004; Prokasy, 1972), mediated by the CEA and its efferent projections to a variety of hypothalamic and brainstem targets (Applegate, Frysinger, Kapp, & Gallagher, 1982; Fox, Oler, Tromp, Fudge, & Kalin, 2015). What exactly drives the rapid increase in fear responding is

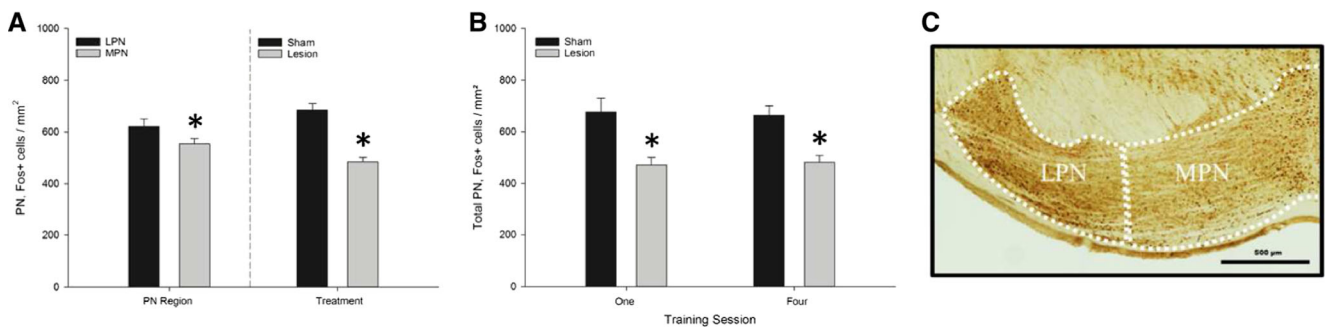


Fig. 3 Bilateral PN Fos+ cell counts in sham and lesion rats sacrificed after one or four EBC session(s). **(a)** Left side: There were significantly fewer MPN Fos+ cells (mean \pm SE) compared to counts from the LPN. Right side: Fos+ cell counts (mean \pm SE) from both LPN and MPN were significantly reduced in CEA lesion rats. **(b)** Fos+ cell counts (mean \pm SE)

from the entire PN were significantly reduced in lesion rats following session one and four. For all figures, an asterisk indicates a significant effect. **(c)** Representative photomicrograph of Fos+ cells in the LPN and MPN (outlined by dashed lines) in one hemisphere of a sham rat. Scale bar: 500 μ m

not entirely clear, however—it likely reflects a combination of unconditioned (or generalized) fear and conditioned fear specific to the tone CS and/or training context. Britton and Astheimer (2004) explored this issue by measuring CS-evoked freezing during a 12 s tone CS inserted into each EBC session or, separately, in a novel context. Conditioned freezing in the training and novel contexts was consistently higher in adult rats trained with CS–US paired trials than CS–US unpaired or US-alone trials. Further the decrease in freezing behavior seen over subsequent training sessions was dependent on the CS–US contingency—that is, rats trained with unpaired or US-alone trials demonstrated lower but more sustained freezing levels. In other words, the gradual decline in conditioned fear seen over training may be learning dependent and not the result of simple habituation. Inasmuch, we propose the initial elevation and subsequent decrease in session-wide freezing rates seen in sham rats is due, at least in part, to the associative nature of the CS–US relationship.

In line with previous research (Blankenship et al., 2005; Lee & Kim, 2004; Weisz et al., 1992) CEA lesions significantly retarded the emergence of eyeblink CRs (see Fig. 2b). Moreover, once a conditioned blink was generated, there was a trend toward smaller CR peak amplitudes in the lesion rats (Fig. 2c). No experimental group differences were seen following US-alone trials, however, suggesting US processing and UR production is unimpeded by CEA lesions (see Fig. 2d). As for conditioned fear, session-wide freezing rates were significantly reduced, relative to sham controls, during the first training session only (see Fig. 2a). The CEA is also capable of facilitating a variety of URs or reflexes (e.g., whole body acoustic startle) when elicited in the presence of a fear-eliciting CS (Choi et al., 2001; Hitchcock & Davis, 1991). In eyeblink conditioned rabbits, the UR was reportedly enhanced within the first 5 to 12 trials, with larger amplitudes on CS–US paired than US-alone trials (Weisz & McInerney, 1990). As illustrated in Fig. 2e, CEA lesion rats produced little

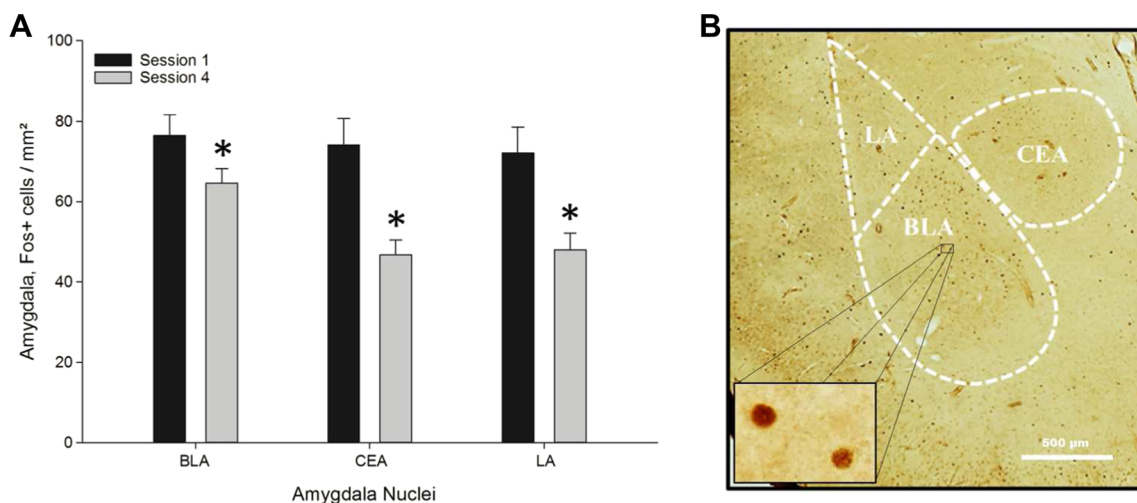


Fig. 4 Bilateral amygdala Fos+ cell counts in sham lesion rats sacrificed after one or four EBC session(s). **(a)** Fos+ cell counts (mean \pm SE) were significantly decreased from Session 1 to 4 in the basolateral amygdala (BLA, left), the central amygdala (CEA; middle), and the lateral

amygdala (LA; right), as indicated by asterisks. **(b)** Representative unilateral photomicrograph of Fos+ cells in the LA, BLA, and CEA (outlined by dashed lines) of a sham rat. Inset shows Fos+ BLA cells at high (60X) magnification. Scale bar: 500 μ m

associative UR facilitation on the first training session. The increase in UR amplitude provided by the CS (i.e., relative to US-alone trials) was negligible (5.0 %) compared to the increase seen in sham rats (42.7 %). This result runs counter to two prior EBC studies, which found no reliable experimental group differences in UR amplitude (based on CS–US trials), when the CEA was pharmacologically inactivated in young rats or, in adult rats, the whole amygdala was damaged (Lee & Kim, 2004; Ng & Freeman, 2014). Nevertheless, current data indicate that the loss of potentiated freezing and reflexive responding observed in lesion rats was, in both cases, most pronounced on the first training session.

The acoustic CS signal is relayed through the medial thalamic nuclei, the cochlear nucleus, the nucleus of the lateral lemniscus, and finally to the PN, the last precerebellar nucleus that conveys acoustic signals to the cerebellum (Freeman & Steinmetz, 2011). Auditory cues were previously reported to be selectively relayed to the cerebellum through the LPN (Bao, Chen, & Thompson, 2000; Steinmetz et al., 1987), while other research found auditory-evoked responses throughout both the LPN and MPN (Cartford, Gohl, Singson, & Lavond, 1997). Based on multi-unit recordings, Taub and Mintz (2010) argued that amygdala-dependent CRs enhance tone-mediated reactivity in PN neurons along most of its medial-lateral axis. As seen in Fig. 3, CEA lesion rats likewise had a significant reduction in Fos⁺ neurons in both the LPN and MPN. Interestingly, the mean area of the LPN was significantly smaller than the MPN, yet it also had significantly more Fos⁺ cells, suggesting the LPN may contain a higher concentration of acoustic-reactive neurons than the MPN.

The PN is thought to be innervated and diffusely influenced by the CEA through both monosynaptic and polysynaptic projections. As an example, during fear conditioning the CEA could stimulate acetylcholine release from the nucleus basalis, biasing the firing of auditory cortex neurons—which innervate the entire PN (Perales, Winer, & Prieto, 2006; Wiesendanger & Wiesendanger, 1982)—toward the frequency of the tone CS (Weinberger, 1998, 2004). CEA lesions reduced the number of PN Fos⁺ cells and decelerated EBC acquisition, as predicted. How PN neuronal kinetics were altered by CEA lesions is not known—that is, the reduction in PN c-Fos immunostaining could be due to diminished tone-evoked reactivity or could reflect a more low-level effect such as a reduction in basal neuronal activity. Regardless of the precise mechanism, however, CEA lesions are proposed to diminish the neuronal saliency of the CS signal, impeding cerebellar CS–US associative plasticity.

We hypothesized PN acoustic reactivity was amplified via CEA projection neurons, concomitant with autonomic and behavioral fear CR expression. From Session 1 to 4 in sham rats, the number of Fos⁺ cells in the CEA fell by 40.0 % while PN cell counts decreased by only 2.4 %, a result that is hard to

reconcile if the CEA is responsible for increasing the population of tone reactive cells. IP-dependent changes in spiking activity, tied to eyeblink CR production, have been recorded in numerous loci throughout the brain, including for instance, hippocampal CA1 neurons (reviewed in Lavond & Cartford, 2000). The proportion of PN neurons that discharge each time an eyeblink CR is generated, modeling its amplitude and time course, also increases over training (Bao et al., 2000). While speculative, perhaps CEA amplification of tone-evoked PN reactivity is diminished over training, but the loss is supplemented each time a conditioned blink is emitted by IP-generated excitatory feedback.

Rats exhibit conditioned fear to the training context early in EBC in that they show elevated freezing before and after CS–US presentations (Britton & Astheimer, 2004; Ng & Freeman, 2014). We propose that the context-US memory grows in strength over training, such that subsequent exposures to the context—which includes both the physical chamber and the handling required to attach each subject's headstage—leads to faster retrieval and higher baseline freezing rates (see Fig. 2a). Context-dependent fear is known to influence the emission of eyeblink CRs—for example, switching eyeblink conditioned rats or rabbits to a novel context leads to a decrease in the number of emitted eyeblink CRs (Lindquist, 2013; Penick & Solomon, 1991). The fact that baseline freezing levels increased over training sessions in sham and lesion rats suggests the amygdala may facilitate the context-US association at a slower rate than the CS–US association. Indeed, the hippocampus has been proposed to process contextual information in competition with the cerebellar CS–US association (Lee & Kim, 2004), such that hippocampal lesions actually facilitate eyeblink CR acquisition during delay EBC (Christiansen and Schmajuk 1992; Port, Mikhail, & Patterson, 1985; Schmaltz & Theios, 1972).

Anatomically, the amygdala and hippocampus share extensive connections (Pitkanen, 2000; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000). All major amygdala nuclei receive input from the hippocampus, though electrophysiological recordings suggest the BLA innervates and influences hippocampal neuronal activity to a greater degree than LA or CEA (Blankenship et al., 2005; Ikegaya, Saito, & Abe, 1994; Racine, Milgram, & Hafner, 1983). In rats sacrificed following Session 1 and 4, the BLA showed the smallest decrease in Fos⁺ cell counts (18.2 %), about half that seen for the CEA (40.0 %) and LA (35.7 %). Considering the BLA is thought to acquire and store information about the emotionally arousing properties of specific contexts (Huff & Rudy, 2004; Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014), the BLA could conceivably interact with the hippocampus throughout EBC, modulating and/or storing the context-US memory (Akirav & Richter-Levin, 1999; Kochli, Thompson, Fricke, Postle, & Quinn, 2015). Inconsistent with this idea, however, is the impaired baseline freezing seen in

CEA lesion rats (whose BLA was mostly intact) across training, relative to the sham controls (see Fig. 2a).

Altogether, results from the current study suggest the amygdala and its diffuse CEA output projections are capable of facilitating cerebellar sensorimotor learning via the enhancement of CS-mediated brainstem activity. Intriguingly, a recent study found that experimental stimulation of IP projection neurons (mimicking an eyeblink CR) can inhibit CEA neuronal responding to an aversive periorbital US, possibly as a means to regulate fear responding as conditioned eyeblinks grow in number (Magal & Mintz, 2014). It raises the interesting possibility that the robust reduction in Fos+ cells seen in the CEA (and possibly LA) from Session 1 to 4—and the concomitant decrease in conditioned freezing—is the result of learning-dependent negative feedback from the cerebellum, representing a third stage of aversive Pavlovian conditioning. As first proposed by Mintz and colleagues (Erez, Gordon, Sever, Sadeh, & Mintz, 2004; Mintz & Wang-Ninio, 2001), we suggest the two stage model should be reconceptualized as a three-stage model: rapid emotional responding in stage one, which enhances discrete motor learning in stage two, which diminishes emotional reactivity in stage three. Dynamic and reciprocal amygdalar-cerebellar modulation of this type would provide the immediate benefits of conditioned fear or arousal, while also ensuring the heightened emotional state is ultimately stabilized and returned to baseline levels. Absent such a mechanism, following cerebellar damage for instance (Lavond, Lincoln, McCormick, & Thompson, 1984), chronic increases in amygdala activation and emotional arousal could prime or even provoke various affective/anxiety disorders (Kim et al., 2011; Magal & Mintz, 2014).

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