Behavioral/Cognitive

Amygdala Modulation of Cerebellar Learning

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Previous studies showed that amygdala lesions or inactivation slow the acquisition rate of cerebellum-dependent eyeblink conditioning, a type of associative motor learning. The current study was designed to determine the behavioral nature of amygdala– cerebellum interactions, to identify the neural pathways underlying amygdala– cerebellum interactions, and to examine how the amygdala influences cerebellar learning mechanisms in rats. Pharmacological inactivation of the central amygdala (CeA) severely impaired acquisition and retention of eyeblink conditioning, indicating that the amygdala continues to interact with the cerebellum after conditioning is consolidated (Experiment 1). CeA inactivation also substantially reduced stimulus-evoked and learning-related neuronal activity in the cerebellar anterior interpositus nucleus during acquisition and retention of eyeblink conditioning (Experiment 2). A very small proportion of cerebellar neurons responded to the conditioned stimulus (CS) during CeA inactivation. Finally, retrograde and anterograde tracing experiments identified the basilar pontine nucleus at the confluence of outputs from CeA that may support amygdala modulation of CS input to the cerebellum (Experiment 3). Together, these results highlight a role for the CeA in the gating of CS-related input to the cerebellum during motor learning that is maintained even after the conditioned response is well learned.

Key words: amygdala; cerebellum; conditioning; interpositus; learning

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Significance Statement

The current study is the first to demonstrate that the amygdala modulates sensory-evoked and learning-related neuronal activity within the cerebellum during acquisition and retention of associative learning. The findings suggest a model of amygdala– cerebellum interactions in which the amygdala gates conditioned stimulus inputs to the cerebellum through a direct projection from the medial central nucleus to the basilar pontine nucleus. Amygdala gating of sensory input to the cerebellum may be an attention-like mechanism that facilitates cerebellar learning. In contrast to previous theories of amygdala– cerebellum interactions, the sensory gating hypothesis posits that the gating mechanism continues to be necessary for retrieval of cerebellar memory after learning is well established.

Introduction

Memory system interactions play a critical role in complex behavior (Poldrack and Packard, 2003). Interactions between emotional and motor memory systems are particularly important because memory for the emotional significance of stimuli provides essential contextual information for acquisition of motor responses. Memory for the significance of stimuli can also modify the strength and speed of a learned motor response. The early theoretical framework for this type of two-process learning, developed by Mowrer (1947) and elaborated by others (Konorski, 1967; Prokasy, 1987), has been adapted more recently to a neural two-process theory. In the neural two-process theory, the amygdala-mediated memory system facilitates the cerebellum-mediated motor memory system during associative learning (Thompson, 1987; Weisz et al., 1992; Mintz and Wang-Ninio, 2001; Lee and Kim, 2004). The main evidence supporting this theory is that lesions or inactivation of the amygdala slow the rate of acquisition of cerebellum-mediated eyeblink conditioning (Lee and Kim, 2004; Blankenship et al., 2005; Burhans and Schreurs, 2008). A crucial part of the neural two-process theory is that the amygdala forms a memory for the emotional significance of the conditioned stimulus (CS), which then facilitates the formation of a second memory in the cerebellum essential for the eyeblink conditioned response (CR).

The neural pathways for an auditory CS and the air puff or periorbital shock unconditioned stimulus (US) are well characterized for eyeblink conditioning (Freeman and Steinmetz, 2011). For an auditory CS, the transynaptic pathway that is necessary and sufficient for eyeblink conditioning includes the auditory brainstem nuclei, medial auditory thalamus, and the lateral aspect of the basilar pontine nucleus (BPN; Halverson and Freeman, 2006, 2010a; Campolattaro et al., 2007; Freeman et al., 2007;

Halverson et al., 2008, 2015). The BPN gives rise to the mossy fiber projection into the cerebellar cortex and deep nuclei (Brodal and Bjaalie, 1992; Steinmetz and Sengelaub, 1992; Shinoda et al., 2000). The base of the primary fissure in the cerebellar cortex and the anterior interpositus nucleus (AIN) are the essential sites of plasticity for eyeblink conditioning in rodents (Steinmetz and Freeman, 2014). The amygdala is thought to facilitate cerebellar learning through projections to the CS pathway, thereby increasing CS salience (Taub and Mintz, 2010; Siegel et al., 2015). However, the effects of altering amygdala activity on cerebellar function have not been examined directly.

The current study was designed to examine how the amygdala influences cerebellar learning mechanisms. We first used pharmacological inactivation of the central amygdala (CeA) during acquisition and retention of eyeblink conditioning (Experiment 1). CeA inactivation was then done while recording neuronal activity from the AIN from multiple tetrodes during acquisition and retention of eyeblink conditioning (Experiment 2). The final experiment used anterograde and retrograde axonal tracers to investigate projections from the CeA to the CS pathway (Experiment 3). The results are inconsistent with the two-process theory, indicating that the CeA gates CS input to the cerebellum during motor learning and continues to serve this function after the motor CR is well learned.

Materials and Methods

Subjects
Subjects were 71 male Long–Evans rats (250–300 g) that were ~3 months of age at the beginning of the study. Thirty-six rats were used for Experiment 1, 15 for *in vivo* neurophysiology in Experiment 2, and 20 for axonal tract tracing in Experiment 3. Rats were housed singly in Spence Laboratories of Psychology at the University of Iowa on a 12 h light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Surgery
For all experiments, rats were anesthetized with 2–3% isoflurane and administered an analgesic subcutaneously (Ketofen, 5 mg/kg). All stereotaxic coordinates are relative to bregma. For amygdala infusion and pressure injection surgeries, the coordinates used were as follows: anteroposterior, −2.4 mm; mediolateral, ±4.2 mm; dorsoventral, −7.1 mm (Paxinos and Watson, 2007). The skull was exposed, and 0.5 mm holes were drilled bilaterally over the CeA. Guide cannulae (27 gauge) were lowered to within 1 mm of the dorsal border of the CeA and were sealed with a 32-gauge stylet. Rats were then fitted with eyelid EMG electrodes and a bipolar electrode just caudal to the eye (to deliver the US), as described previously (Freeman and Nicholson, 1999).

In Experiment 2, microdrives were implanted stereotaxically over the AIN ipsilateral to the trained eye (anteroposterior, −11.1 mm; mediolateral, 2.7 mm). A small craniotomy was made with a 1.0 mm hole saw drill bit. The dura mater was punctured carefully and removed to allow for unencumbered passage of tetrode wires into the brain. The bundle/brain interface was sealed with sterile silicon (KwikSil; World Precision Instruments). The microdrive was grounded with a skull screw, and its base was secured to the rat with bone cement. Tetrode drivers were initially lowered 1.5 mm during surgery. Drivers were subsequently lowered to the target over a 3–4 d period taking note of neuronal landmarks, such as the dense layer of Purkinje cells and cerebellar white matter while descending to deep cerebellar nuclei.

In Experiment 3, we surveyed putative anatomical substrates that may account for the capacity of CeA to modulate cerebellar learning. This issue was examined in the context of anatomical circuitry relating to the pons, because these cell groups are known to serve as critical relays for descending CS-related information en route to the cerebellum and issues direct projections to the dentate and AIN (Brodal and Bjaalie, 1992; Steinmetz and Sengelaub, 1992; Tracy et al., 1998; Campolattaro and

Freeman, 2008). One group of rats received unilateral pressure injections of 10% biotinylated dextran amine (BDA) delivered in volumes of 100 nl via micropipettes (10–25 μm, inner diameter) for anterograde labeling of CeA neurons. A different group of rats received unilateral pressure injections of 100 nl of 2% Fluoro-Gold (FG; Flourochrome; Schmued and Fallon, 1986) into the BPN (anteroposterior, −7.68 mm; mediolateral, 1.20 mm; dorsoventral, −9.90 mm from dura) for the identification of candidate cell groups inclusive of, or that may be intermediary to, outputs from the CeA.

Conditioning apparatus
Conditioning chambers (12 × 11 × 10 inches) were housed within sound-attenuated boxes that were ventilated with small exhaust fans that also provided audible background noise. One wall of the chamber was fitted with speakers for the tone CS. An EMG and bipolar electrode tether passed through a commutator and were connected to a direct current amplifier and a stimulus isolator, respectively. The amplified EMG was filtered (0.5–5.0 kHz) and integrated before being digitized by a desktop computer. The stimulus isolator was also connected to the computer that specified the timing of stimulation events (CS and US). All surfaces of the conditioning chamber were wiped with 70% ethanol before the beginning of each session.

Eyeblink conditioning
Figure 1A shows the onsets and offsets of the stimuli used in delay eyeblink conditioning (dEBC). The sampling window for each trial was 1000 ms, consisting of a 300 ms baseline period, 400 ms CS period, 25 ms US period, and 275 ms post-US period. The CS was an 85 dB, 2.0 kHz pure tone. The mean intertrial interval was 30 ± 10 s. Each session consisted of 10 10-trial blocks including nine paired CS–US trials and one CS-alone trial. At least 30 min before each of the first five sessions, 0.2 μl of 2.0 mM muscimol (GABA$_A$ agonist) or 0.2 μl of 0.1 M PBS (0.6 μl/h) was infused into the CeA bilaterally (Fig. 1B). Bilateral infusions were used because pilot experiments found that unilateral CeA inactivation did not impair dEBC. It is not clear why unilateral infusions were ineffective, but it might be that amygdala inputs to the pontine nuclei (ipsilateral; see Experiment 3) activate neurons that project to the contralateral PN and/or ipsilateral cerebellar cortex. Custom-built 32 gauge infusion cannulae were lowered into the guide cannula and extended 1.0 mm ventrally beyond its tip. Infusion cannulae were removed 2 min after the end of the infusion. Pre-session infusions ended starting with session 6, and training continued until reaching a criterion of two consecutive sessions of 80% CRs or greater. Rats from both groups were then given two retention sessions with infusions of PBS or muscimol in a counterbalanced order. Because ear bars were used during surgery, a general assessment of hearing was conducted before the first training session in which the experimenter produced noises such as a whistle or snap of the fingers to generate behavioral orienting responses, eyelid EMG activity, and, in Experiment 2, changes in multiunit activity.

Behavioral data collection and analysis
Integrated EMG activity for each trial was analyzed with a MATLAB-based application. CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period, 80 ms after its onset. EMG activity that exceeded the threshold during the first 80 ms of the CS period was defined as a startle response. CR amplitude was measured from the mean of the pre-CS activity. Unconditioned responses (URs) were defined as activity that crossed the threshold after the offset of the US. Any trial in which the baseline signal crossed threshold was removed from analysis. Behavioral measures were analyzed with *t* tests, repeated-measures (RM) ANOVA, and Tukey's HSD *post hoc* tests, when appropriate. The α level for all statistical tests was 0.05. All reported behavior data were from both paired and unpaired trials.

Neuronal data collection and analysis
A 32-channel tetrode microdrive was custom fabricated and consisted of eight independently moving recording tetrodes and one reference tetrode. Tetrodes were fabricated by twisting four strands of 12.7-μm-diameter nichrome wire (Kanthal) into one bundle. Heat was applied to
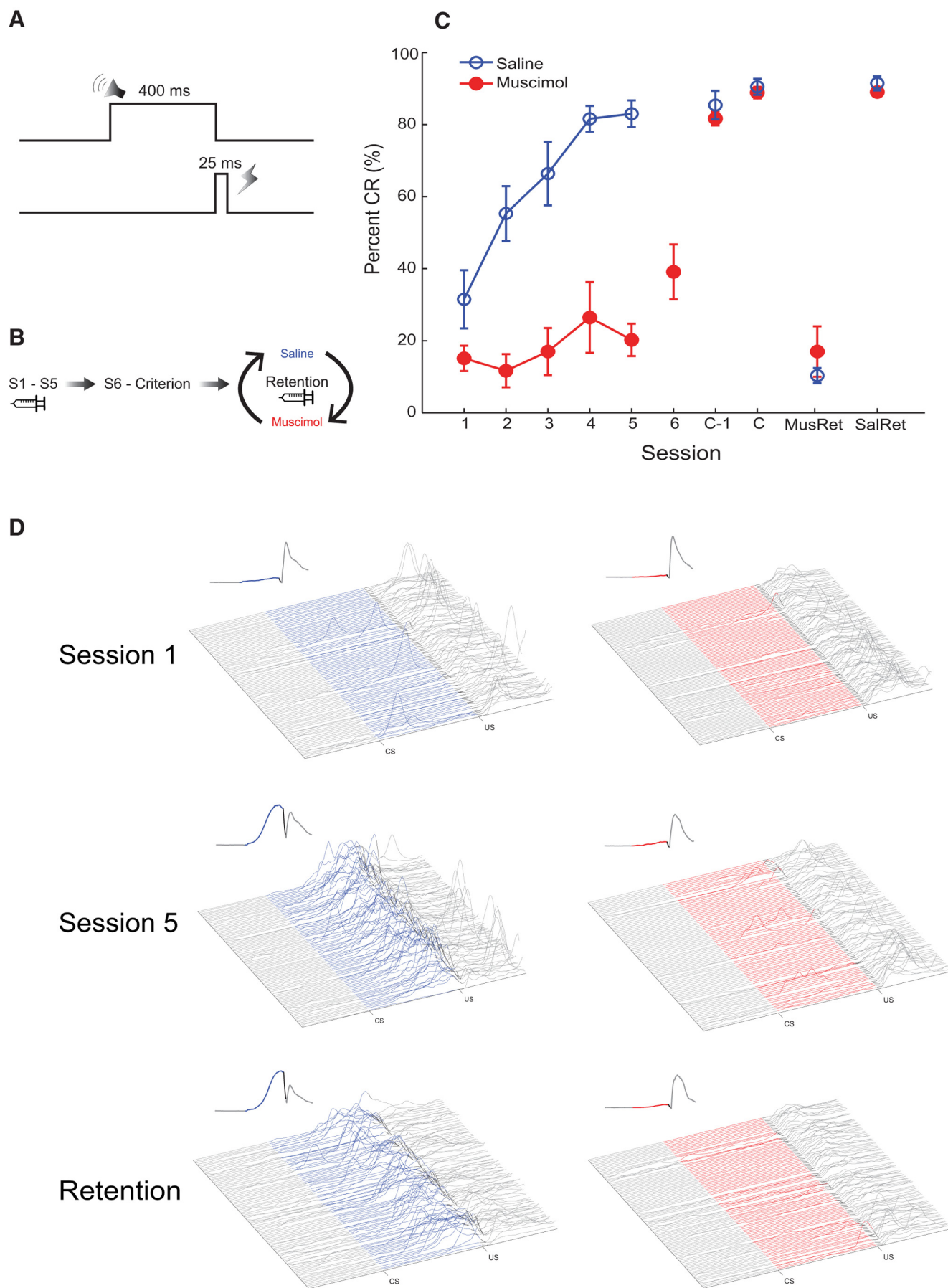
the wire bundle (tetrode) to enhance rigidity. The impedance of the tetrode tips was lowered to 250–350 kΩ by electroplating in a gold non-cyanide solution (SIFCO) at 1.0 kHz. Once training began, tetrodes were lowered to increase the quality of recording as necessary but no more than 40 μm on any single day. Rats were partially restrained by hand while tetrodes were lowered, followed by a 10–20 min waiting period before the start of a training session.

During eyeblink conditioning sessions, neuronal recordings were sampled at 32.0 kHz (Neuralynx Digital SX), with a bandpass filter set to 0.6–6.0 kHz. A 24 bit analog-to-digital converter provided ample signal resolution combined with low tetrode impedance such that signal amplification was not required. Neuronal signals were fed through a preamplifier head stage at unity gain to a motorized commutator, which allowed freely moving behavior. A waveform on one channel exceeding a peak amplitude threshold triggered data collection on all tetrode channels. Depths of tetrodes during training were only adjusted if spikes were not detected. Offline cluster cutting was initially done with Klusta-Kwik (Harris et al., 2000). Manual spike sorting was done with interactive spike sorting MATLAB-based software (MClust version 3.5).

Analyses were done with custom scripts in MATLAB and NexScript (NeuroExplorer). Statistical analyses were done in SPSS. Interpositus unit activity was time locked to stimuli and analyzed with peristimulus histograms. The baseline and CS and US periods were divided into 10 ms bins (Ng and Freeman, 2012). Neural activity in each CS period bin was normalized to the baseline activity for each unit. A RM-ANOVA was used to assess neural activity differences in bins across infusion treatments and sessions. Repeated-measure analyses for neuronal data were done with the Greenhouse–Geisser correction unless stated otherwise. In a separate analysis, the baseline and CS period were divided into 100 ms bins. Average firing rates were converted into z scores and normalized to baseline activity with a confidence interval of 95%. The proportions of responsive units between infusion treatments were compared using a χ^2 analysis.

Histological and tissue processing
Experiments 1 and 2. After the end of training, rats were anesthetized deeply with sodium pentobarbital and perfused with 0.1 M PBS and 10% formalin. Brains were extracted and postfixed overnight before being cryoprotected in 30% sucrose/Formalin for 48–72 h. After sectioning at 50 μm, coronal slices were mounted on glass slides and stained with thionin. Bilateral cannula placement to the CeA and interpositus tetrode placement was then verified with bright-field microscopy.

Experiment 3. Rats used for anatomical tracing experiments were anesthetized deeply with sodium pentobarbital and perfused with 0.1 M PBS and ice-cold 4% paraformaldehyde at a flow rate of 60 ml/min. Brains were removed, postfixed overnight in the same fixative, and cryoprotected in 20% sucrose in 0.02 M potassium phosphate buffer for 24 h at 4°C. In preparations bearing BDA tracer injections in CeA, the entire brain was sectioned using a freezing microtome, collected into five one-in-five series of 30-μm-thick coronal sections in cryoprotectant solution, and stored at −20°C until histochemical processing for visualization and analysis of axonal terminal fields. For tissue containing retrograde labeling of BPN afferents, brains were sectioned at 50 μm, mounted onto glass slides, and coverslipped using Vectashield H-1000 (Vector Laboratories).

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Figure 1. Acquisition of dEBC. **A**, Diagram of the delay conditioning paradigm, specifying the onset and offset of the stimuli. **B**, Diagram of the experimental design. Saline or muscimol infusions into the CeA occurred during sessions (S) 1–5 and at retention. Both groups received saline and muscimol retention sessions, with the order of infusions counterbalanced. **C**, Mean percentage of CRs ± SE across training sessions. Amygdala inactivation resulted in a severe impairment. Session 6 is shown for the rats that received muscimol during sessions 1–5 to demonstrate the absence of savings. C-1, Session before criterion (2 consecutive sessions > 80% CR) was reached. **C**, Session criterion was reached. MusRet, Muscimol retention; SalRet, saline retention. **D**, Waterfall plots of eyeblink topography from representative cases from sessions 1 and 5 and retention. CS period of plots are colored to represent the type of infusion for that session. Blue, Saline; red, muscimol. Top left inset on each plot is the average eyeblink across all animals for the particular session.

Histochemistry. BDA localization was performed in free-floating tissue sections using an avidin–biotin peroxidase protocol (Sawchenko et al., 1990). Sections were first incubated in 0.3% hydrogen peroxide to neutralize endogenous peroxidase activity and permeabilized by treatment in PBS containing 0.3% Triton X-100. After a 1 h incubation using Vectastain Elite reagents (Vector Laboratories), the reaction product was developed using a nickel-enhanced glucose oxidase method (Shu et al., 1988). A separate series of sections were also prepared for BDA localization using fluorescence labeling. This involved incubating sections overnight in the streptavidin–fluorophore conjugate Alexa Fluor 568 (Life Technologies) at a concentration of 1 μg/ml.

Results
Experiment 1: inactivation of the CeA during eyeblink conditioning
Eyeblink conditioning
Bilateral muscimol infusions into the CeA severely impaired acquisition of eyeblink conditioning. After the cessation of muscimol infusions, the rats showed no evidence of savings, indicating that the deficit in acquisition was caused by a learning impairment and not by impaired expression. Furthermore, the acquisition rate after cessation in muscimol infusions was no different from the saline-infused rats (3.6 ± 1.6 vs 4.0 ± 1.2 sessions). However, associative learning can also be assessed by examining the difference in CR percentage between paired and unpaired groups. Unpaired controls were not run in this experiment, but previous studies using these methods have shown unpaired levels of responding in rats that range from 0 to 12% CRs (Kleim et al., 2002; Campolattaro and Freeman, 2009). Thus, the muscimol group showed a higher percentage of CRs during Sessions 1–5 than typically seen in unpaired groups, suggesting that they developed a small amount of associative learning during Sessions 1–5.

After reaching the acquisition criterion, rats from both acquisition groups were severely impaired during retention tests when muscimol was infused into the CeA. Figure 1C shows the percentage of CRs for the initial infusion sessions, criterion sessions (R-2 and R-1), and retention sessions. An RM-ANOVA on the CR percentage data for the first five sessions identified a session × group interaction ($F_{(4,68)}$ = 4.81, p = 0.002). *Post hoc* tests revealed that significant group differences in CR percentage began in the second session of conditioning and continued for the remainder of the initial infusion sessions. An independent t test found no difference between the CR percentage during session 6 in rats that had received muscimol infusions during sessions 1–5 versus session 1 of the saline group, indicating the absence of savings in the muscimol group. Both groups of rats reached asymptotic learning before the retention sessions. Regardless of the initial infusion treatment, each group was affected equally by muscimol during retention. A paired samples t test of the CR percentage data during retention sessions (saline vs muscimol, within subjects) revealed a significant reduction in CR percentage with muscimol relative to saline ($t_{(18)}$ = 32.01, p < 0.0001).

Additional metrics of eyeblink topography were analyzed, including the percentage of startle responses, amplitude of the CR, CR onset latency, and peak latency. Example eyeblink responses from individual cases for sessions 1, 5, and retention are displayed in Figure 1D. An RM-ANOVA revealed no statistical differences in the percentage of startle responses. However, a group × session interaction was found for the CR amplitude data ($F_{(4,68)}$ = 4.01, p = 0.012). Additional analysis for the retention sessions revealed differences in CR amplitude based on infusion ($t_{(18)}$ = 6.38, p < 0.0001). There were no differences observed in CR onset latency or CR peak latency during acquisition or retention.

Amygdala inactivation did not affect any measures of the UR (amplitude, peak latency, or area).

Cannula placement
Cannula placements for Experiments 1 and 2 are shown in Figure 2. Behavioral data from 19 of the original 36 rats in Experiment 1 was analyzed. The remaining rats were removed from analysis because at least one of the cannulae missed the CeA.

Spread of inactivation
The missed cannula placements were within 1.0 mm of the CeA, and the rats with missed cannulae showed very good retention during the muscimol test, averaging 74% CRs. Moreover, two of the rats with accurate cannula placements were given an additional retention test with a lower concentration of muscimol (0.876 mM), and these rats showed a retention deficit equivalent to the deficit with the higher concentration used in the rest of the experiments (2.0 mM). Last, the infusion volume was very small (0.2 μl) and therefore could not have spread much beyond the CeA in rats with accurate cannula placements. These assessments of the spread of muscimol indicate that the inactivation effects were localized to the CeA and could not have inactivated the subcortical auditory structures necessary for dEBC (Freeman and Steinmetz, 2011).
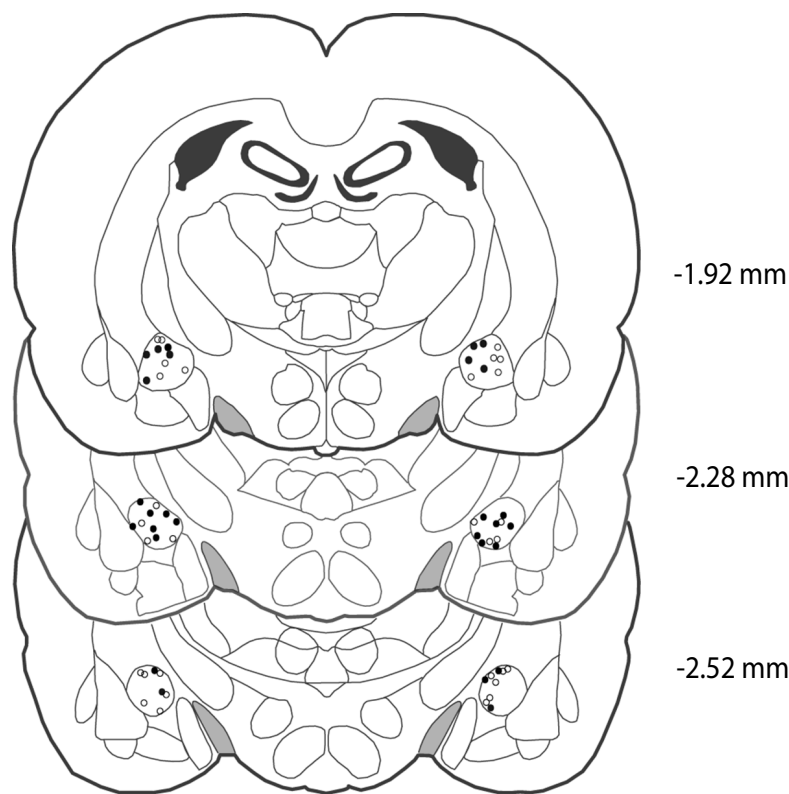

Figure 2. Bilateral cannula placements for Experiments 1 and 2. Coronal sections depicting cannula placements in the CeA for the muscimol (filled circles) and saline (open circles) groups. Measurements are relative to bregma.

Experiment 2: inactivation of the CeA while recording cerebellar neuronal activity
Eyeblink conditioning
Similar to Experiment 1, rats with bilateral CeA inactivation showed a severe impairment compared with controls. A group $\times$ session interaction during acquisition sessions was identified ($F_{(4,28)} = 21.2$, $p < 0.0001$), which was caused by a higher CR percentage in the control group during sessions 2–5. After criterion was reached, retention was impaired by muscimol infusions relative to the saline infusions ($t_{(8)} = 28.33$, $p < 0.0001$). No other eyeblink characteristics between these groups differed. Therefore, the behavioral results in Experiment 2 replicated the behavioral results in Experiment 1.

Cannula and tetrode placement
Single-unit analysis was done for 366 AIN neurons recorded from nine rats. Recordings of putative neurons were kept for analysis if their tetrode location was verified in the AIN (Fig. 3). Reference tetrodes were placed in cerebellar white matter ventral to the cerebellar cortex and dorsal to the interpositus nucleus. Tetrodes that were ventrally ($n = 6$) or dorsally ($n = 3$) located with respect to the AIN were removed from analysis. Six of the 15 rats were removed because of cannula misplacement or loss of the head stage.

Neuronal activity during acquisition
Firing rate, L-ratio, isolation distance, and interspike interval profiles were all considered when assessing cluster quality of a putative neuron. AIN neurons were tonically active with an average firing rate of 32.6 $\pm$ 2.7 Hz, and the average spontaneous firing rat did not differ

between groups after infusions (saline group mean, 32.53 $\pm$ 2.0 Hz; muscimol group mean, 32.95 $\pm$ 1.02 Hz). L-ratio and isolation distance are metrics of cluster separation from nearby noise spikes (Schmitzer-Torbert et al., 2005). The mean L-ratio (muscimol group, 0.82; saline group, 0.98) and mean isolation distance (muscimol group, 13.1; saline group, 15.6) did not differ between groups. Examples of single-neuron firing from both groups in Figure 4 show learning-related increases in firing with acquisition in the saline rats that is absent in muscimol rats.

The proportion of neurons with firing rates significantly higher during the CS relative to the pre-CS period was greater in the saline group than in the muscimol group during all of the infusion sessions (sessions 1–5; Fig. 5). This group difference was found for each of the 100 ms CS intervals. After training without infusions until reaching the learning criterion, the two groups did not differ in the proportion of neurons that were responsive to the CS (Fig. 5). Significant differences were found within subjects for the retention tests, in which the proportion of CS-responsive neurons was greater during the saline retention test ($p < 0.001$) relative to the muscimol retention test ($p < 0.001$).

Neuronal activity was normalized to the pre-CS baseline period to examine group differences in the magnitude of neuronal population firing during learning (sessions 1, 5, and criterion) and during retention tests (saline vs muscimol). Normalized activity during the CS differed between groups across 10 ms time bins (bin $\times$ group interaction) as early as session 1 ($F_{(12.56,1206.4)} = 3.58$, $p < 0.001$; Fig. 6A). *Post hoc* tests identified differences during the initial period of the CS (bins 3–8, $p < 0.05$). These differences reflected a neuronal response to the onset of the CS in the saline group that was absent in the muscimol group.

Neuronal activity in session 1 was then divided into 10-trial blocks to further analyze how early group differences in CS-onset
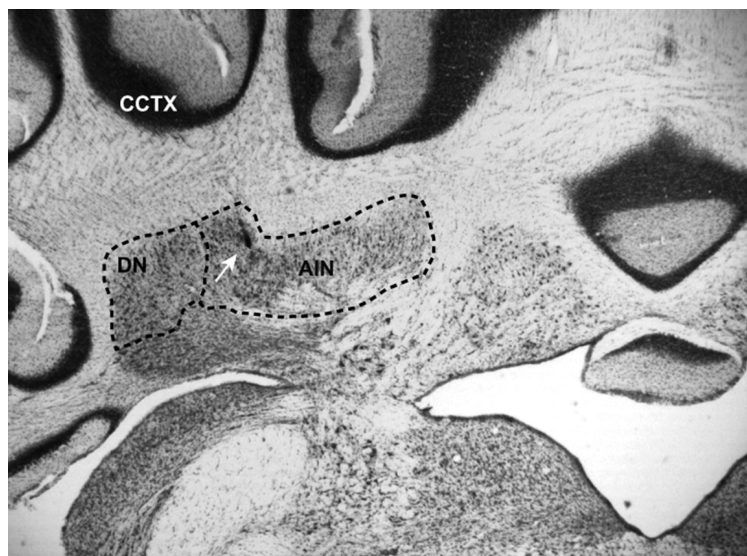
Figure 3. Thionin-stained coronal section of the cerebellum showing a tetrode marking (white arrow) in the lateral portion of the AIN. CCTX, Cerebellar cortex; DN, dentate nucleus.

responses were evident within the first session (Fig. 7). A group (saline vs muscimol) × block (1–10) × bin (1–40) analysis revealed no interaction; however, a main effect of group was found ($F_{(1,95)} = 14.1$, $p < 0.001$). Additional analysis of block 1 (trials 1–10) revealed a main effect of group ($F_{(1,96)} = 4.40$, $p = 0.04$). These subanalyses of group differences in the magnitude of neuronal firing during the CS in the first session suggest that sensory responses to the CS in the cerebellum were suppressed by inactivation of the CeA before learning started. However, the shortest latency responses to the CS (bins 1–3) during the first block did not differ between groups, indicating that the cerebellum was receiving sensory input in the inactivation group but that input was not sustained throughout the CS. The saline group then shows potentiation of the short-latency response in some of the subsequent blocks.

The magnitude of neuronal responses to the CS also differed between groups after learning was well established in the saline group on session 5 (Fig. 6B). There was a bin × group interaction ($F_{(7.5,494.7)} = 12.6$, $p < 0.001$) that was attributable to greater firing in the saline group relative to the muscimol group throughout the CS (bins 3–9, 19–20, and 23–40). The substantial group difference in neuronal firing during session 5 parallels the severe impairment in CR percentage (Fig. 1C) and amplitude.

Neuronal activity from session 1 of the saline group and session 6 of the muscimol group (Fig. 6C) was compared to examine whether there was neuronal savings in the muscimol group. Neuronal savings would be evident if neuronal activity during the CS was higher in the muscimol group during the first session without muscimol infusions (session 6) relative to the first session in the saline group. A group (saline session 1 vs muscimol session 6) × bin (1–40) RM-ANOVA revealed a bin × group interaction ($F_{(12.3,753.1)} = 3.5$, $p < 0.001$), with greater activity in the saline group during session 1 early in the CS period (bins 3–6). Thus, there was no evidence of neuronal savings in the muscimol group.

Neuronal activity from each rat's behavioral criterion session was then analyzed with a group × bin RM-ANOVA. There were no group differences in neuronal activity during the criterion session, indicating that repeated muscimol inactivation during acquisition sessions did not impair the subsequent development

of learning-related plasticity in the cerebellum in the absence of infusions (Fig. 6D).

Neuronal activity during retention
Normalized activity was compared for the saline and muscimol retention sessions in both groups. Because the neuronal populations recorded in the two retention sessions differed within groups, separate RM-ANOVAs were done for the saline and muscimol retention sessions, with the original training condition (saline vs muscimol) as the grouping factor. During both the muscimol and saline retention sessions, neuronal firing during the CS differed between groups (muscimol, $F_{(16.3,996.8)} = 3.92$, $p < 0.001$; saline, $F_{(9.4,525.4)} = 3.82$, $p < 0.001$). *Post hoc* tests indicated that the group trained with saline during sessions 1–5 had greater neuronal firing in the early portion of the CS than the group given muscimol during sessions 1–5 in the saline (bins 3–6 and 10) and muscimol (bins 3–8) retention sessions (Fig. 6E,F). No group differences were found for the second half of the CS period, in which both groups showed robust neuronal responses during the saline retention session. These results indicate that muscimol inactivation of the CeA during initial training (sessions 1–5) resulted in weaker short-latency CS-evoked activity during retention tests (Fig. 6F), although the rats in both groups had learned to the 80% criterion.

Experiment 3: anatomical substrates of CeA modulation of cerebellar learning
Neuroanatomical tracing experiments were performed to investigate the efferent connections from CeA in relation to cerebellar eyeblink conditioning circuitry. In one group of rats ($n = 8$), pressure injections of the anterograde tracer BDA were placed into the CeA, and histochemical visualization of transported BDA was evaluated principally in the descending pathway terminating in mesencephalic and pontine regions. The size and placements of BDA were designed to balance the need for producing reliable and dense labeling of axonal and terminal elements with limiting any confounding labeling that might result from BDA diffusion to adjacent-lying structures (Fig. 8A,B). The descending trajectory of BDA-labeled elements originating in the CeA were observed to be almost exclusively ipsilateral and mostly conformed to previous descriptions of the ventral amygdalofugal pathway (Hopkins, 1975; Hopkins and Holstege, 1978; Krettek and Price, 1978; Price and Amaral, 1981; Mihailoff, 1995). These caudally projecting axons were followed from the subthalamic nucleus, between the lateral aspect of the substantia nigra par compacta and red nucleus, and further through the mesencephalic reticular formation. At the pontine level, a small contingent of labeled fibers strayed off ventrolaterally toward BPN, making an array of labeled varicosities and terminal ramifications that were most pronounced in lateral aspects (Fig. 8C). In contrast, no appreciable labeling of axonal elements was observed in the inferior olive, facial motor nucleus, and trigeminal nuclei.

In the caudal diencephalon, the trajectory of labeled fibers traversed immediately subjacent to the medial subdivision of the medial geniculate and posterior intralaminar nuclei (MGm/
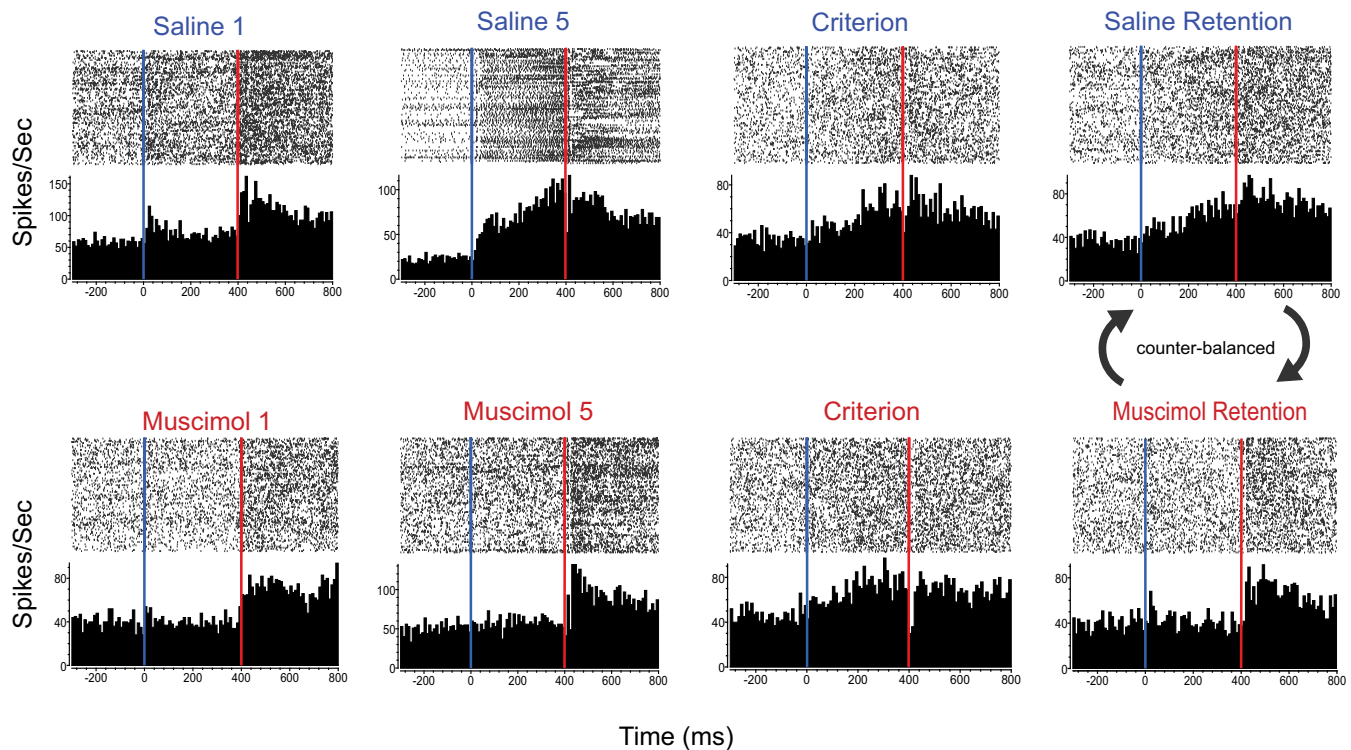
Figure 4. Representative examples of single-neuron recordings from the cerebellar AIN during eyeblink conditioning. Blue line, CS onset; red line, US onset. Increases in firing rate during the CS were absent in muscimol rats during sessions 1–5 but were acquired, along with the CR, after cessation of muscimol infusions (Criterion). CeA inactivation during retention in both groups (Muscimol Retention) abolished the learning-related activity, but learning-related activity was retained after saline infusions (Saline Retention). The order of retention sessions with saline and muscimol was counterbalanced within groups.

PIN), raising the possibility that the CeA might provide a collateral projection to the auditory thalamus. Careful examination revealed a sparse labeling of fibers with varicosities and a scattering of terminals within the MGm/PIN, but aperiodic labeling of somata were also noted, which is consistent with reports that anterograde tracing with (even with high molecular weight) dextrans is capable of producing occasional spurious retrograde labeling (for review, see Reiner et al., 2000). That efferents from the acoustic thalamus are known to densely innervate multiple amygdaloid subdivisions, including the CeA (Ottersen and Ben-Ari, 1979; LeDoux et al., 1985; cf. LeDoux et al., 1990) coupled with the lack of a clear precedent for a CeA → acoustic thalamic relay (Hopkins, 1975; Hopkins and Holstege, 1978; Krettek and Price, 1978; Price and Amaral, 1981; cf. Shammah-Lagnado et al., 1996), served as additional caveats against interpreting a CeA → auditory thalamic relay from these data.

Because BPN represents a final common pathway for relaying CS-related information to the cerebellum during eyeblink conditioning (Steinmetz et al., 1987; Halverson and Freeman, 2010a,b), a second set of tracing experiments using the retrograde tracer FG were performed to confirm that the CeA projects to the BPN ($n = 12$). These experiments were also necessitated to address concerns that spurious labeling of terminals in the BPN may have resulted from spread of anterograde tracer into the entopeduncular nucleus, which also issues dense inputs into the ventral pons (Takada et al., 1994). FG injections targeting the BPN revealed a subpopulation of retrogradely labeled neurons in the medial aspect of this CeA (Fig. 8 *D,E*), and this was observed reliably in every rat ($n = 8$) regardless of placement variability, excepting instances in which tracer placements were entirely caudal to pontine nuclei ($n = 4$). Also consistent with the foregoing anterograde tracing experiment, retrograde labeling in the CeA

was exclusively ipsilateral. These data confirm a previous report (Mihailoff et al., 1989) and provide an anatomical basis for understanding the functional gating properties of CeA in auditory eyeblink conditioning.

Discussion

Inactivation of the CeA with muscimol severely impaired acquisition of dEBC (Fig. 1*C,D*). During subsequent training without infusions, the CeA inactivation group (session 6, criterion) showed an acquisition rate that was equivalent to the initial acquisition rate of the control group (sessions 1–5), which indicates a lack of savings in the CeA inactivation group. This finding demonstrates that the deficit in conditioning during the inactivation sessions was caused by impaired learning, not by impaired expression. However, it is important to note that the CeA inactivation group showed a higher percentage of CRs during acquisition than is typically seen with unpaired training (~10%), suggesting that there was a small amount of associative learning in this group during sessions 1–5. The deficit in acquisition is consistent with previous studies that showed impaired acquisition of dEBC with lesions or muscimol inactivation of the amygdala (Lee and Kim, 2004; Blankenship et al., 2005; Ng and Freeman, 2014). These findings and those of the current study indicate that the amygdala plays a critical role in acquisition, but they do not indicate that the amygdala is essential for acquisition of eyeblink conditioning. Some of the previous studies have shown that animals with amygdala lesions will eventually acquire eyeblink conditioning with additional training (Weisz et al., 1992; Blankenship et al., 2005; Burhans and Schreurs, 2008). Moreover, animals decerebrated just rostral to the red nucleus can acquire eyeblink conditioning, indicating that the forebrain is not necessary (Lovick and Zbrozyna, 1975; Norman et al., 1977; Hesslow, 1994; Kotani et al. 2002). Thus, the amygdala modulates cerebellar learning mechanisms during eye-
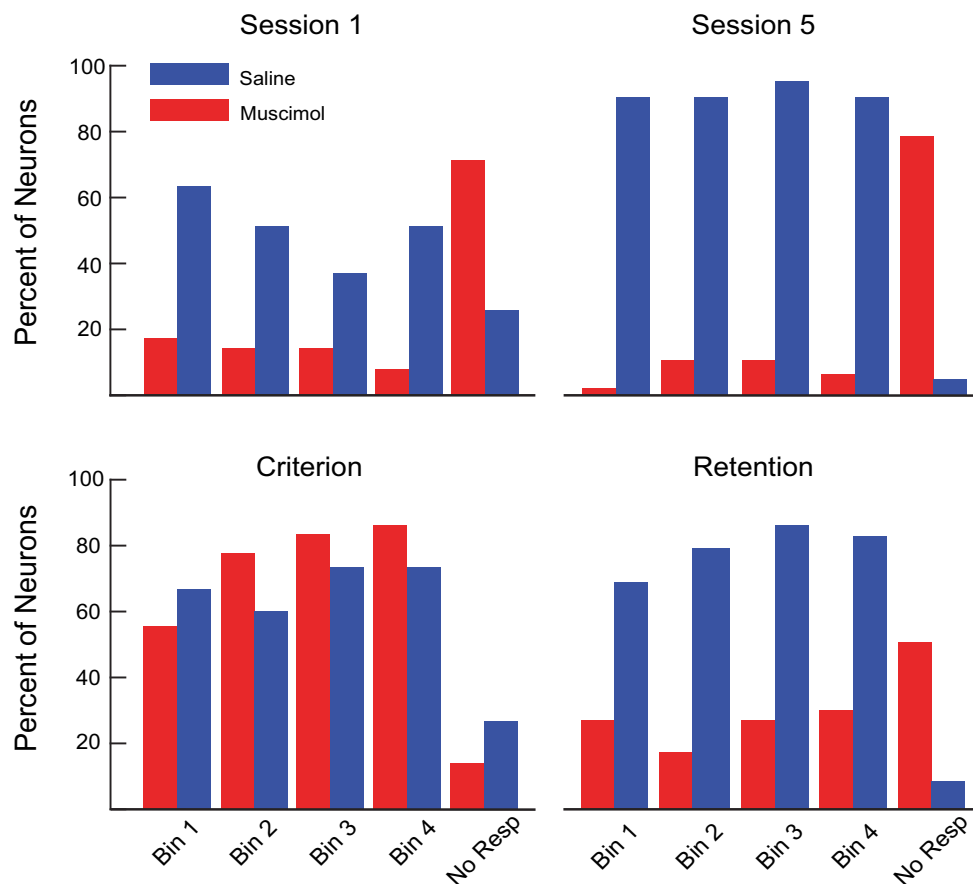
Figure 5. Percentage of AIN neurons with activity significantly above the pre-CS baseline period during the CS period (separated into 100 ms bins) during session 1, session 5, the criterion session, and the retention sessions. The majority of recorded neurons did not show a significant increase in activity during the CS period after muscimol infusions in sessions 1 and 5. By the criterion session, the majority of neurons in both groups showed an increase in activity during the CS. During the retention sessions, muscimol infusions into the CeA decreased the percentage of neurons with increased activity during the CS in both groups. No Resp, Nonresponsive neurons.

blink conditioning. The deficit in the acquisition rate of eyeblink conditioning with amygdala lesions has supported a two-process theory of eyeblink conditioning in which rapidly developing plasticity in the amygdala results in defensive response CRs early in training and facilitates subsequent plasticity in the cerebellum resulting in the motor CR (Mowrer, 1947; Konorski, 1967; Thompson, 1987; Weisz et al., 1992; Mintz and Wang-Ninio, 2001; Taub and Mintz, 2010). As cerebellar learning develops, it is thought that the role of the amygdala diminishes (Lee and Kim, 2004; Magal and Mintz, 2014). However, retention of the CR was also severely impaired in the current study by CeA inactivation, indicating that the amygdala is playing a critical role in eyeblink conditioning after the motor CR is well established (Siegel et al., 2015). Based on these behavioral results, we hypothesize that the amygdala gates auditory input to the cerebellum during conditioning, and this sensory gating function plays a critical role during learning and retrieval. The sensory gating hypothesis is supported by our analyses of neuronal activity within the cerebellar AIN. Learning-related increases in neuronal firing within the AIN were abolished by CeA inactivation during acquisition and retention of eyeblink conditioning (Fig. 6A,B,E,F). CeA inactivation had no effect on baseline firing rates in the cerebellum, indicating that the reduction in firing rate was specific to CS-elicited activity. CeA inactivation substantially reduced the number of cerebellar neurons recruited during conditioning, and those neurons that did have a CS-evoked response had lower firing rates than in controls. Not only were learning-related increases in cerebellar activity inhibited for the first five sessions, but no cerebellar neuronal

savings was noted in the inactivation group during session 6 (first session without muscimol infusions; Fig. 6C). After the cessation of CeA inactivation, there was a substantial increase in cerebellar neuronal responses to the CS in the CeA inactivation group, with no indication of a lingering deficit (Fig. 6D). After CRs and learning-related neuronal activity were acquired, both were abolished by CeA inactivation (Fig. 6E). Thus, the influence of the amygdala on cerebellar learning mechanisms appears to be as strong during retention as it is during acquisition. These findings support the sensory gating hypothesis by showing that cerebellar neuronal responses to the CS are reversibly abolished by CeA inactivation.

A key part of the sensory gating hypothesis is that the CeA inactivation should inhibit sensory input to the cerebellum and not just affect learning-related increases in activity. Support for the CeA affecting sensory input to the cerebellum comes from a study (Taub and Mintz, 2010) that examined the effect of CeA inactivation on multiple-unit activity in the BPN with simultaneous CeA inactivation in anesthetized rats. This study found that amygdala inactivation reduced CS-elicited activity in the BPN during conditioning. In the current study, we further examined the effects of CeA inactivation on sensory input to the cerebellum by examining cerebellar neuronal responses to the CS within the first training session, before learning developed (Fig. 7). We found that CeA inactivation caused a deficit in short-latency neuronal responses to the CS during the first session, as early as the first 10-trial block. Thus, the CeA has a substantial
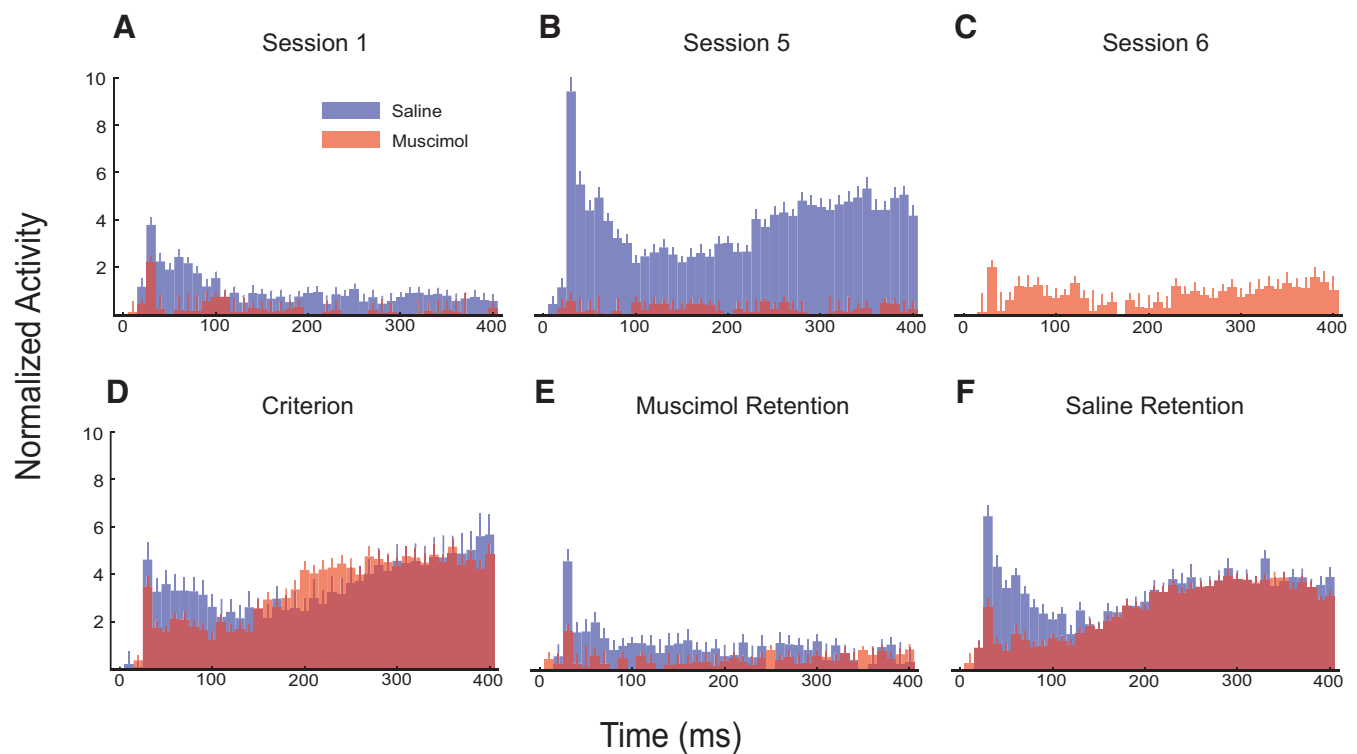
Figure 6. Mean ± SE normalized neuronal activity (spikes per seconds) in the AIN in rats given saline or muscimol infusions into the CeA. **A**, During session 1, neuronal activity was greater in the saline group than in the muscimol group during the first 10 10-ms bins. **B**, During session 5, there was a substantial increase in the magnitude of neuronal firing during the CS relative to session 1 in the saline group but not in the muscimol group. **C**, Neuronal activity from the rats that received muscimol for sessions 1–5 during session 6 did not show a substantial increase relative to session 5, indicating the absence of neuronal savings. **D**, During the criterion session, neuronal activity did not differ between the groups. **E**, During the muscimol retention sessions, neuronal activity was severely suppressed during the CS in both groups, but the saline group had greater short-latency activity than the muscimol group. **F**, During the saline retention sessions, the group difference in neuronal activity was only evident in the short-latency response to the CS. Bar opacity is reduced to show group data overlap.
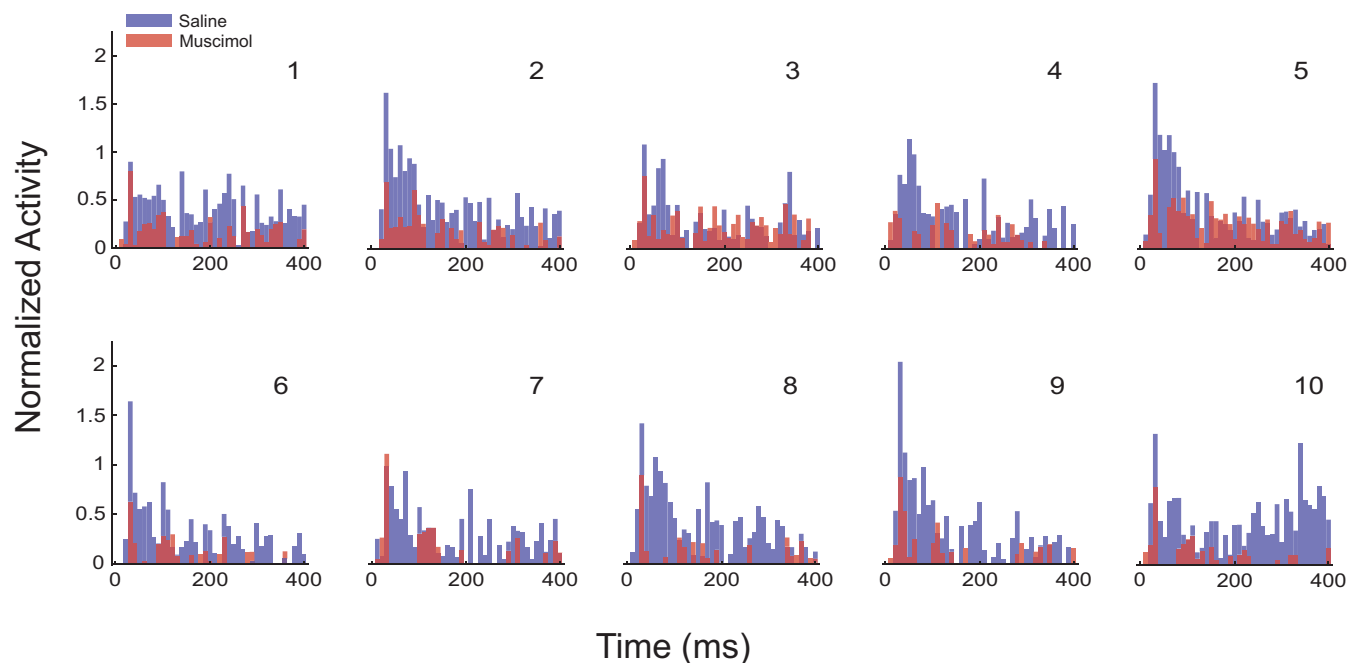

Figure 7. Mean normalized neuronal activity (spikes per seconds) in the AIN in rats given saline or muscimol infusions into the CeA during session 1 broken down into 10-trial blocks. Neuronal activity in the saline group was greater than in the muscimol group as early as Block 1, suggesting that CeA inactivation affects cerebellar responses to the auditory CS before conditioning develops. Bar opacity is reduced to show group data overlap.
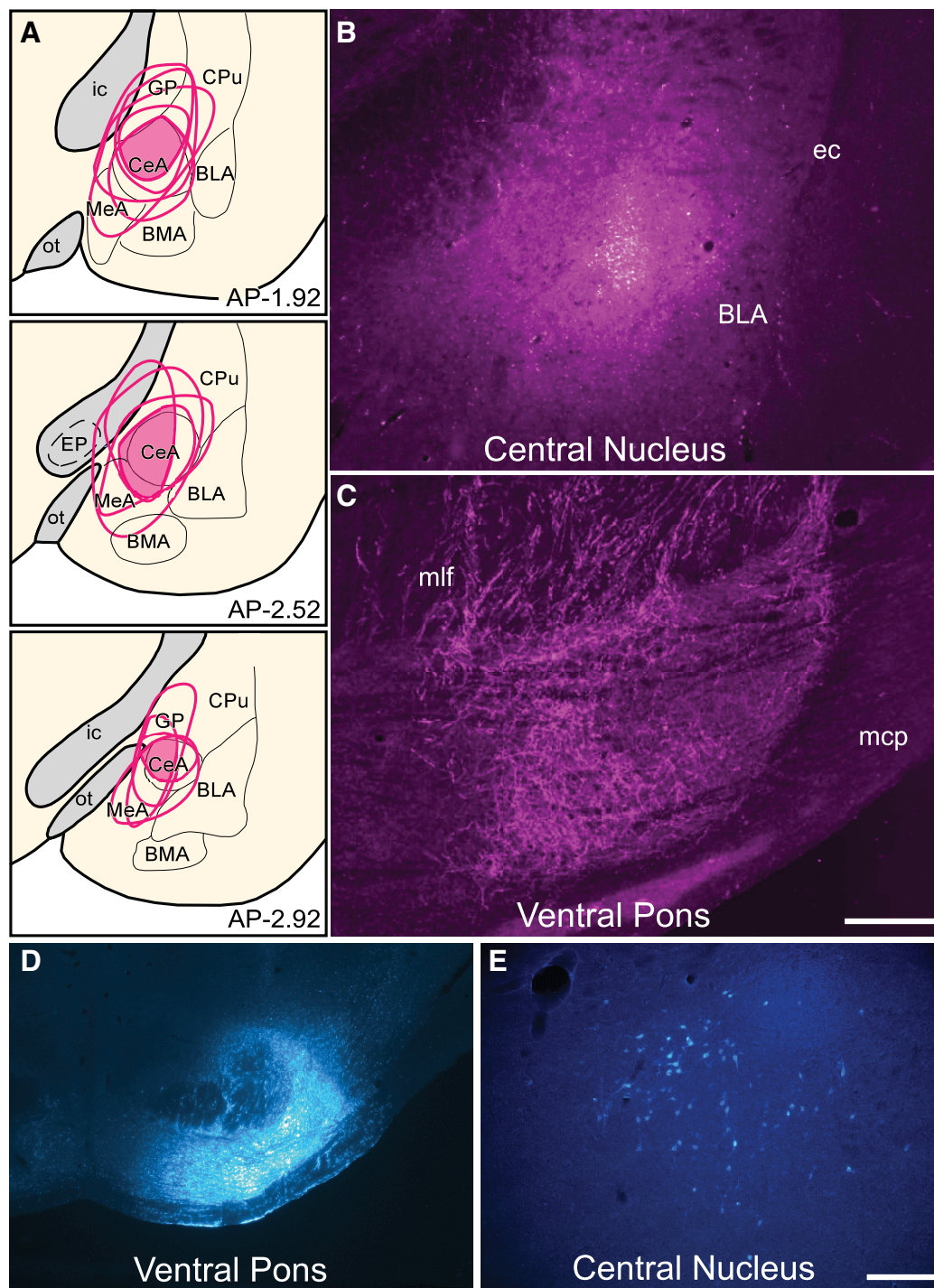
Figure 8. *A*, Reconstructions of BDA tracer injection placements in the CeA. Shaded regions indicate areas of overlap common to all tracer injections; distance in millimeters relative to bregma is indicated. AP, Anteroposterior; BLA, basolateral nucleus of the amygdala; CPu, caudate–putamen; BMA, basomedial nucleus of the amygdala; EP, entopeduncular nucleus; GP, globus pallidus; ic, internal capsule; MeA, medial nucleus of the amygdala; ot, optic tract. *B*, *C*, Epifluorescence photomicrograph illustrates a BDA tracer deposit centered within the CeA after stereotaxic injection (*B*) and a pseudocolored dark-field image photomicrograph depicting the distribution of anterogradely labeled fibers and terminals in BPN after tracer injection in the CeA (*C*). Ec, External capsule; mcp, medial cerebral peduncle; mlf, medial longitudinal fasiculus. Scale bars: *B*, 500 μm; *C*, 125 μm. *D*, *E*, Epifluorescence photomicrographs showing an FG tracer deposit centered within the lateral BPN (*D*) and retrograde labeling of BPN projecting neurons within the CeA (*E*). Scale bars: *D*, 500 μm; *E*, 125 μm.

influence on auditory CS input to the cerebellum from the start of training that persists well after conditioning is well established.

During saline retention testing, both groups showed learning-related increases in cerebellar activity that ramped up during the CS (Fig. 6*F*). However, rats trained with muscimol infusions during sessions 1–5 did not exhibit the short-latency increase in CS-elicited activity during retention seen in

rats given saline during sessions 1–5. The short-latency neuronal response to the CS is training related because it increases from session 1 to session 5 in the control group (Fig. 6*A*,*B*). Why is this short-latency neuronal response reduced in the rats given CeA inactivation during initial training, after they acquired the CR to the same levels as the controls? This effect might be related to interactions between the CeA and the au-

ditory CS pathway for eyeblink conditioning. A prominent component of the auditory CS pathway is the auditory thalamus, which includes the suprageniculate (SG), MGm, PIN, and neighboring medial auditory thalamic nuclei. These thalamic nuclei then project to the lateral BPN, which, in turn, project auditory input to the cerebellum (LeDoux et al., 1990; Shammah-Lagnado et al., 1996; Halverson and Freeman, 2010a; Fig. 8C). A study by Poremba and Gabriel (2001) found that rabbits given amygdala inactivation with muscimol during discriminative avoidance conditioning did not develop learning-related neuronal activity in the MGm, and learning-related activity in the MGm did not develop after the cessation of inactivation. Therefore, the short-latency CS-elicited activity found in the current study might be driven by plasticity in the medial auditory thalamus. If this is the case, CeA inactivation might have had the same effect as in the Poremba and Gabriel study, that is, muscimol inactivation of the CeA during initial training may have blocked plasticity in the MATN and thereby blocked the development of short-latency CS-elicited activity in the cerebellum. The mechanisms by which the amygdala transiently influences medial auditory thalamic plasticity are not known.

Axonal tract tracers were used to investigate whether the CeA could affect cerebellar learning via a direct projection to the CS pathway. (Mihailoff et al., 1989; Siegel et al., 2015). Robust anterograde labeling of axons and terminals were found in the ipsilateral BPN. Labeling was particularly strong in the lateral aspect of the BPN, which conveys auditory CS input to the cerebellum (Steinmetz et al., 1987; Campolattaro et al., 2008; Halverson and Freeman, 2010a). Recently, anterograde tracers have also revealed CeA efferents terminating in the lateral BPN in mice (Siegel et al., 2015). Retrograde neuronal labeling was also found in the CeA with tracer injections into the BPN in the current study. Thus, auditory CS information in the BPN could be gated directly by the CeA. However, it is also possible that CS input to the BPN is gated via multisynaptic pathways from the CeA to either the MGm or the periaqueductal gray (PAG). Projections from the CeA to the PAG are known to drive defense behaviors and analgesia and the PAG projects to the BPN (Paredes et al., 2000). The other multisynaptic pathway by which the CeA could influence CS input to the cerebellum is the CeA → MGm/SG → BPN pathway. In this case, the CeA would gate auditory input to the PN by modulating activity in the MGm and SG. Additional studies are needed to determine the necessary pathway(s) for auditory eyeblink conditioning. This analysis will likely necessitate optogenetic approaches to selectively manipulate different projections of the CeA.

In conclusion, the behavioral and neuronal findings presented here indicate that cerebellar learning mechanisms are attenuated with CeA inactivation. Deficits in sensory-evoked and learning-related cerebellar neuronal activity suggest a sensory gating function for amygdala–cerebellum interactions. The CeA may gate auditory input to the cerebellum through direct projections to the BPN, a putative CeA → MGm/SG → BPN pathway, or the CeA → PAG → BPN pathway. This sensory on-gating mechanism might be an attention-like mechanism (for review, see Gallagher and Holland, 1994) that increases sensory input to the cerebellum for stimuli that have acquired significance as signals for important events such as the periorbital stimulation US in the current study. In contrast to previous theories of amygdala–cerebellum interactions, the sensory gating hypothesis posits that the gating mechanism

continues to be necessary for retrieval of cerebellar memory after learning is well established.

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