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Amygdala central nucleus modulation of cerebellar learning with a visual conditioned stimulus

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ABSTRACT

Previous studies found that reversible inactivation of the central amygdala (CeA) severely impairs acquisition and retention of cerebellum-dependent eye-blink conditioning (EBC) with an auditory conditioned stimulus (CS). A monosynaptic pathway between the CeA and basilar pontine nuclei (BPN) may be capable of facilitating cerebellar learning. However, given that the CeA projects to the medial auditory thalamus, a critical part of the auditory CS pathway in EBC, the CeA influence on cerebellar learning could be specific to auditory stimuli. Here we examined the generality of CeA facilitation of EBC acquisition and retention in rats using a visual CS. As in our previous studies using an auditory CS, inactivation of the CeA with muscimol severely impaired acquisition and retention of EBC with a visual CS. Extending training to 15 100-trial sessions resulted in acquisition of EBC, indicating that the CeA plays a modulatory role in cerebellar learning and is not part of the necessary neural circuitry for EBC. Tract-tracing experiments verified that axons from the CeA reach both the BPN and medial auditory thalamus (part of the necessary auditory CS pathway), but were not found in the ventral lateral geniculate (part of the necessary visual CS pathway). The neuroanatomical results suggest that the CeA most likely modulates cerebellar learning through its projection to the BPN. The findings of the current study are consistent with the hypothesis that the CeA modulates cerebellar learning by increasing CS-related sensory input to the cerebellar cortex and interpositus nucleus via the BPN. This increase in CS-related input is thought to constitute an increase in attention to the CS during EBC.

1. Introduction

The central amygdala (CeA) has been shown to modulate cerebellar learning (Blankenship, Huckfeldt, Steinmetz, & Steinmetz, 2005; Burhans & Schreurs, 2008; Farley, Radley, & Freeman, 2016; Lee & Kim, 2004; Neufeld & Mintz, 2001; Pochiro & Lindquist, 2016). Lesions or inactivation of the CeA slow the rate of eye-blink conditioning (EBC), a cerebellum-dependent Pavlovian learning task, and post-training inactivation of the CeA impairs retention of EBC (Blankenship et al., 2005; Burhans & Schreurs, 2008; Farley et al., 2016; Lee & Kim, 2004; Neufeld & Mintz, 2001; Siegel et al., 2015; Weisz, Harden, & Xiang, 1992). Inactivation of the CeA also attenuates the learning-specific ramping of deep cerebellar nucleus activity during the conditioned stimulus (CS) in EBC (Farley et al., 2016). Amygdala modulation of cerebellar learning does not require memory formation within the CeA as inhibition of protein synthesis, mRNA transcription, and NMDA receptors impaired cue and contextual fear conditioning but had no effect on eyeblink conditioning (Steinmetz, Ng, & Freeman, 2017). Rather, the CeA is thought to facilitate CS sensory input to the cerebellum via projections to the basilar pontine nucleus (BPN) (Farley et al., 2016; Pochiro & Lindquist, 2016; Siegel et al., 2015; Taub & Mintz, 2010).

All of the studies that have examined the effects of amygdala lesions or inactivation on acquisition of EBC used an auditory CS, which raises the possibility that CeA modulation of sensory input to the cerebellum is unique to auditory stimuli. This seems unlikely since CeA inactivation impairs retention of trace EBC with an LED stimulus in mice (Siegel et al., 2015). Nevertheless, the effects of CeA inactivation on acquisition of EBC need to be determined.

Previous studies demonstrated a monosynaptic projection from the CeA to the BPN (Farley et al., 2016; Mihailoff, Kosinski, Azizi, & Border, 1989; Siegel et al., 2015); however, projections to other parts of the CS input pathway to the cerebellum could play a role in the CeA modulation of cerebellar learning (Farley et al., 2016). For example, projections from the CeA to thalamic nuclei that convey sensory input to

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the BPN might be the primary route for modulation of learning by boosting CS input to the BPN, thereby facilitating CS input to the cerebellar cortex and interpositus nucleus. Indeed, there is neurophysiological evidence for feedback from the amygdala to the medial division of the medial geniculate (MGm) during discriminative avoidance and cued fear conditioning (Maren, Yap, & Goosens et al., 2001; Poremba & Gabriel, 2001). A previous study from our group found preliminary evidence for a monosynaptic projection from the CeA to the medial auditory thalamus (medial division of the medial geniculate, posterior intralaminar nucleus, suprageniculate nucleus, and neighboring nuclei) (Farley et al., 2016). The medial auditory thalamus and its projections to the BPN are necessary for EBC with an auditory CS (Campolattaro, Halverson, & Freeman, 2007; Halverson & Freeman, 2006, 2010a; Halverson, Lee, & Freeman, 2010; Halverson, Poremba, & Freeman, 2008, 2015). The ventral lateral geniculate (vLGN) and its projection to the BPN are necessary for EBC with a visual CS (Halverson & Freeman, 2010b; Halverson, Hubbard, & Freeman, 2009; Kashef, Campolattaro, & Freeman, 2014; Steinmetz, Buss, & Freeman, 2013). Thus, CeA projection to the medial auditory thalamus could modulate EBC with an auditory CS, whereas a CeA projection to the vLGN, if it exists, could modulate EBC with a visual CS.

The CeA projection to the BPN would be sufficient for EBC with visual, as well as auditory CSs, given that the BPN mossy fiber projection has been established as a multi-sensory input pathway in EBC (Halverson & Freeman, 2010a, 2010b; Lewis, Lo Turco, & Solomon, 1987). On the other hand, projections from the CeA to the sensory thalamic nuclei could be modality-specific and the CeA might not project to the vLGN. If acquisition of EBC with an LED CS is impaired by CeA inactivation, the absence of a CeA projection to the vLGN would suggest that the CeA modulates cerebellar learning primarily through its projection to the BPN.

In the current study, we examined whether CeA inactivation impairs acquisition or retention of EBC using a visual CS (LED). Rats were given muscimol or saline infusions into the CeA before each of 5 consecutive EBC sessions. The rats were then given further EBC sessions without infusions until reaching a criterion of 80% conditioned responses (CRs) for two consecutive days. After reaching the criterion, retention sessions were given with muscimol and saline infusions on different days (within subjects design). In our previous study with inactivation of the CeA during EBC with an auditory CS, there was very little evidence of learning after 5 days. However, other studies found that animals with CeA lesions could acquire EBC with extended training (Blankenship et al., 2005; Lee & Kim, 2004), suggesting that rats with CeA inactivation in our previous study (Farley et al., 2016) would have acquired EBC with more training. We therefore examined whether extended training with CeA inactivation would result in acquisition of EBC. Finally, anterograde and retrograde tracers were used to investigate the possibility of projections from the CeA to the medial auditory thalamus and vLGN.

2. Materials and methods

2.1. Subjects

Subjects were 60 Long Evans adult male rats. Rats were assigned to Experiment 1 (n = 13), Experiment 2 (n = 8), or Experiment 3 (n = 39). 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 University of Iowa's Institutional Animal Care and Use Committee.

2.2. Surgery

Approximately one week before training, rats were anesthetized with 4.5% isoflurane and maintained at 2% during stereotaxic surgery.

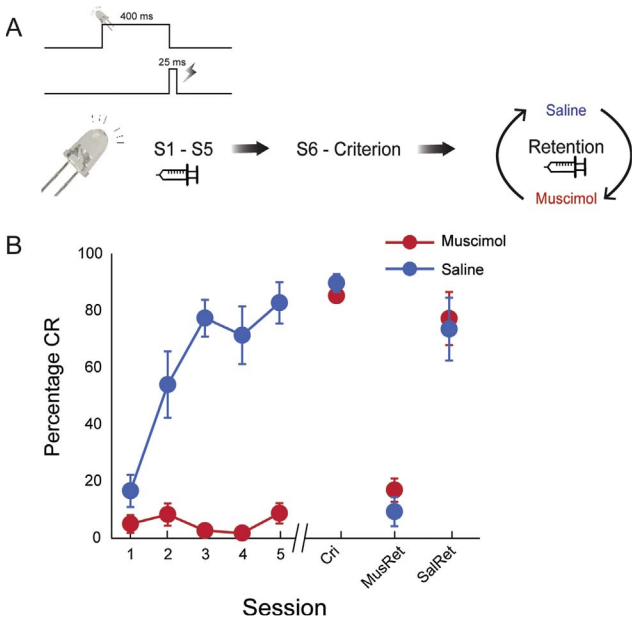

Fig. 1. (A) Timeline for Experiment 1. Rats were given delay eyeblink conditioning with an LED CS (400 ms) paired with a periorbital electrical stimulus (25 ms) for 5 training sessions (S1–S5), muscimol or saline infusions (syringe) were administered before each session. The rats were then trained to a criterion of 80% CRs for two consecutive sessions, followed by retention tests with muscimol and saline infusions on separate sessions (order of infusion type counterbalanced). (B) Percentage of CRs across training sessions in rats given muscimol (red) or saline (blue) infusions into the CeA prior to S1-S5. All rats received muscimol (MusRet) and saline (SalRet) infusions into the CeA prior to retention sessions with the LED CS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The skull was exposed and 0.7 mm diameter holes were burred over the CeA. Custom-fabricated 27-gauge guide cannulae were lowered to within 0.5 mm of the dorsal boundary of the CeA bilaterally. Guide cannulae were plugged with a stylet that extended 0.5 mm beyond the tip of the guide. Coordinates relative to bregma were AP −2.4, ML ± 4.2, and DV −7.1. A bipolar stimulating electrode (PlasticsOne # MS303/1-B/SPC) was implanted in the superficial fascia caudal to the left eye (for US delivery). Two stainless steel PFA insulated wires (AM-Systems # 791000) were implanted within the orbicularis oculi to measure differential electromyography (EMG) during eyelid closure. The EMG electrode wires were soldered to gold pins (AM-Systems # 520200) housed within a nylon connector strip affixed to the animal's head. The rats were given post-surgical analgesics for 48 h.

2.3. Conditioning apparatus

Conditioning chambers (12″ L × 11″ W × 10″ H) 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 DC amplifier and a stimulus isolator, respectively. The amplified (×2000) 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 which specified the timing of stimulation events (CS and US). All surfaces of the conditioning chamber were wiped with 70% ethanol prior to the beginning of each session.

2.4. Infusions

At least 30 min prior to each of the first 5 sessions, and for retention tests, 0.2 μL of 2.0 mM muscimol ($GABA_A$-R agonist) or 0.2 μL of 0.1 M phosphate buffered saline (PBS; 6 μL/h) was infused into the CeA

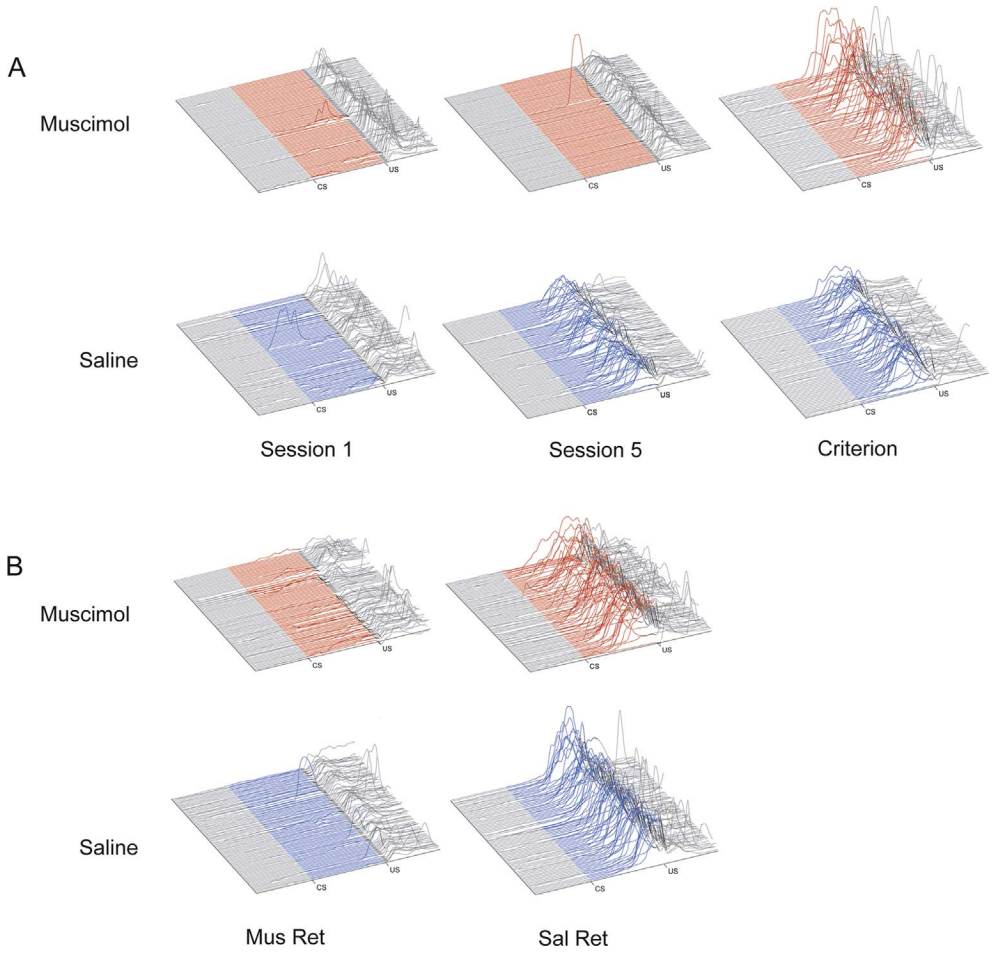

Fig. 2. Representative waterfall plots of integrated eyelid EMG signal across trials (front to back). (A) EMG responses of a rat trained with muscimol or saline infusions into the CeA during Sessions 1–5 and then trained to a criterion. (B) Retention tests with muscimol (Mus Ret) and saline (Sal Ret) infusions into the CeA on separate sessions for the same rats depicted in A. Colors represent CS onset and group initial condition; red = muscimol, blue = saline. Black region represents US onset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bilaterally (Farley et al., 2016). A custom built 32-gauge infusion cannula was lowered into the guide and extended 0.5 mm ventrally beyond its tip. Infusion cannulae were removed 2 min after the end of the infusion.

2.5. Delay eye-blink conditioning

Experiment 1. Fig. 1A shows the onsets and offsets of the stimuli used in EBC. The sampling window for each trial was 1000 ms, consisting of a 300 ms pre-trial baseline period, 400 ms CS period, 25-ms US period, and 275-ms post-US period. The CS was a white LED (~10 lx). The mean inter-trial interval was 30 s ± 10 s. Each session consisted of ten 10-trial blocks including 9 paired CS-US trials and one CS-alone trial. Consecutive muscimol infusions were separated by 48 h, a practice used in previous studies with repeated muscimol infusions to minimize tolerance-like $GABA_A$-R adaptation (Farley et al., 2016; Freeman, Halverson, & Poremba, 2005). Pre-session infusions ended starting with session 6 and training continued until reaching a criterion of 2 consecutive sessions of 80% CRs or greater. Rats from both groups were then given 2 retention sessions with infusions of PBS or muscimol in a counterbalanced order.

Experiment 2. Rats were trained in the same EBC procedures as described in Experiment 1 except that the acquisition period with muscimol infusions continued for 15 sessions (Fig. 4A). On Session 16, the rats were given an infusion of the voltage-gate sodium channel blocker bupivacaine (1.2%, 0.2 µL bilaterally) to confirm that the deficit in acquisition was not specific to muscimol's mechanism of action or related to an alteration in $GABA_A$ receptors. They were then trained to the criterion and given a retention session, followed by an additional 'recovery' session to verify that there were no persistent effects of CeA inactivation.

2.6. Behavioral data collection and analysis

Integrated EMG activity for each trial was analyzed with a custom 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 (SR). CR and SR amplitude was measured from the mean of the pre-CS activity. Unconditioned responses (UR) were defined as activity that crossed the threshold after the offset of the US. Any trial where the pre-CS baseline signal crossed threshold was removed from analysis. Acquisition of EBC across sessions was analyzed with linear mixed effects modeling using R (version 3.4.1). The linear models included fixed effects of group (Experiment 1), session, a quadratic function (across sessions), and random effects for intercept, slope, and the quadratic function. A model simplification strategy was used to find the model that best fit the data (Crawley, 2007). We first started with the full model and then systematically removed random effects, one at a time, to determine if dropping the different random effects influenced the estimates of the model. To goal

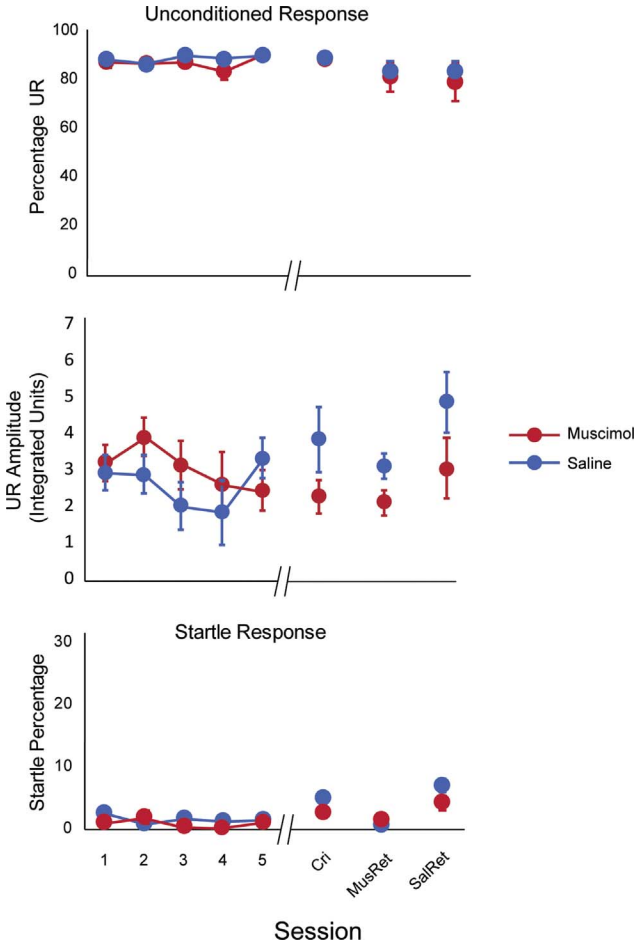

Fig. 3. Startle and unconditioned response (UR) percentage and UR amplitude across EBC training and testing sessions in the rats from Experiment 1 (experimental design described in Fig. 1). Response percentages were calculated from both CS-US paired trials and CS-alone trials. Cri = Criterion; MusRet = muscimol retention; SalRet = saline retention.

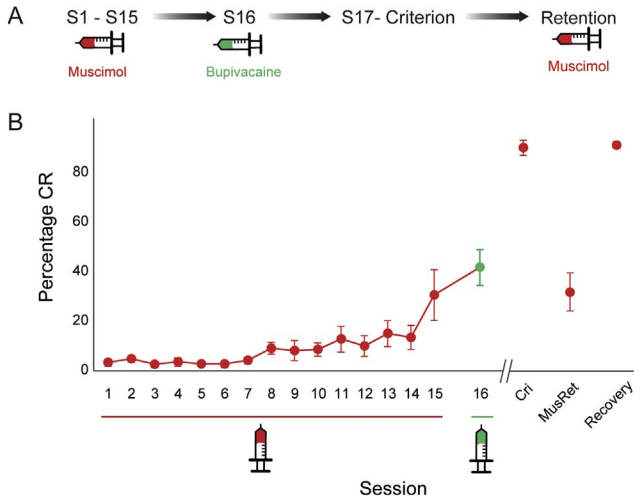

Fig. 4. (A) Timeline for Experiment 2. Rats were given delay eyeblink conditioning with an LED CS (400 ms) paired with a periorbital electrical stimulus (25 ms) for 15 training sessions, muscimol (syringe) was administered before each session. On the 16th session, the rats were given an infusion of bupivacaine into the CeA. The rats were then trained to a criterion of 80% CRs for two consecutive sessions, followed by retention tests with muscimol and saline infusions on separate sessions (order of infusion type counterbalanced). (B) Percentage of CRs across sessions in rats given muscimol (red) prior to Sessions 1–15, and bupivacaine prior to Session 16. The rats then received muscimol (MusRet) and saline (SalRet) infusions into the CeA prior to retention sessions (order of infusion type counterbalanced). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was to use the simplest model structure for the data. Retention session data were analyzed using a repeated measures ANOVA with infusion type (PBS vs. muscimol) as a within subjects variable and group as the between subjects variable (Experiment 1). A paired samples *t*-test was used to analyze the CR percentage data for the criterion and muscimol retention sessions for Experiment 2. The alpha level for all statistical tests was 0.05. All reported behavioral data were from both paired and CS-alone trials.

2.7. Histology (Experiments 1 and 2)

At completion of training, animals were anesthetized with sodium pentobarbital and perfused with 120 mL 0.1 M PBS and 10% buffered formalin. Brains post-fixed for 24 h and were cryoprotected for 48 h, then sliced in 50 µm thick coronal sections. Sections were mounted and stained with thionin and analyzed for cannula placement.

2.8. Anterograde and retrograde tract tracing (Experiment 3)

2.8.1. Surgery

Due to subnuclei of the amygdala having disparate projections throughout the brain, we utilized iontophoresis to make discrete injections of 2.5% solution of Phaseolus vulgaris leucoagglutinin (PHA-L). After the skull was exposed, a craniotomy was made from the sagittal sinus to the cortex over the medial CeA (CeM). A small slit was made in the dura mater to allow passage of a borosilicate glass micropipette (I.D. ~10 µm). Iontophoresis parameters were adjusted bearing in mind two primary constraints: (1) make a small enough injection to limit the deposit of lectin exclusively within the CeM, and (2) deliver enough tracer for reliable uptake and axonal transport. Injection currents were stepped from 4 µA (n = 6), 7 µA (n = 11), and 13 µA (n = 5) across animals (7 s on/off for 15 min). PHA-L was targeted to the right CeM (AP: −1.92, ML: +3.7, DV: −8.3) in 22 rats. The coordinates for the anterograde tracer were adjusted relative to the cannula placements (Experiments 1 and 2) based on the preliminary results of the retrograde tracing experiments showing projection neurons primarily in the rostromedial CeA. Micropipettes remained in place for an additional 5 min after injection. The surgical site was sutured and animals recovered from surgery in their home cage while placed on a heating pad.

A 2% solution of Fluoro-Gold (FG, Fluorochrome, LLC) was used for all retrograde tracing. For retrograde analysis of the vLGN afferent projections, 100 nL of FG was pressure injected (Pneumatic PicoPump, World Precision Instruments) with a glass micropipette (I.D. ~25 µm) to the vLGN (AP: −4.56, ML: +4.1, DV: −5.5) in 3 rats. For retrograde analysis of the MGm afferent projections, the same technique was applied with a slightly smaller injection volume (90 nL) to the MGm (AP: −6.00, ML: +3.1, DV: −3.0) in 14 rats. The survival time following surgery for all neuroanatomy experiments was 14 days.

2.8.2. Anterograde tracing histology

Rats were anesthetized with sodium pentobarbital and perfused with 120 mL 0.1 M PBS and the low-high pH method of fixation (sequential fixation with ice-cold sodium acetate buffered paraformaldehyde, pH 6.5, and ice-cold borate buffered paraformaldehyde, pH 10–11). Brains post-fixed for 12 h before being cryoprotected for 24 h in 20% sucrose in 0.02 M KPBS at 4 °C. Five, one-in-five series of 30-µm thick coronal sections were obtained using a freezing microtome. Immunolocalization of PHA-L transport was visualized using an biotin-avidin peroxidase protocol (Sawchenko, Cunningham, Mortrud, Pfeiffer, & Gerfen, 1990) or an indirect immunofluorescence approach. Endogenous peroxidase was neutralized by treating tissue for 10 min with 0.3% hydrogen peroxide. Free-floating sections were incubated in primary rabbit antiserum (VectorLabs, AS-2300) for 48 h (1:1000 in KPBS, +0.3% Triton X-100 + 2% normal goat serum). PHA-L primary

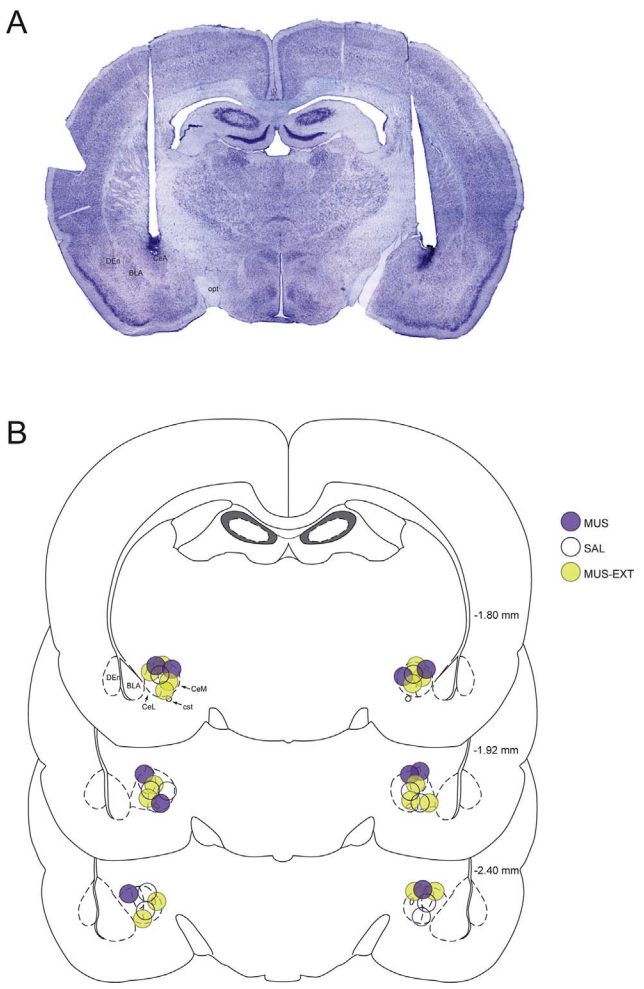

Fig. 5. (A) Thionin stained coronal section showing an example of bilateral cannula placements in the CeA. (B) Reconstruction of the cannula placements for all rats in Experiments 1 and 2. Each dot depicts the location of the tip of the infusion cannula.

antiserum was reacted with Vectastain Elite (Vector Labs) reagents and the reaction product was developed using a nickel-enhanced glucose oxidase method (Gerfen & Sawchenko, 1984). For immunofluorescence, series of sections were subject to the same primary rabbit antiserum incubation as above, followed by a 2 h incubation with a biotinylated goat anti-rabbit secondary antibody (VectorLabs, BA-1000; 1:500 in KPBS, +0.3% Triton X-100 + 2% normal rabbit serum) and a final 2 h incubation with the fluorophore conjugate Alexa Fluor streptavidin 568 (Life Technologies; 1:500 in KPBS, +0.3% Triton X-100). Sections were mounted and cover-slipped with Vectashield (H-1000, VectorLabs) for analysis using epifluorescence microscopy.

2.8.3. Retrograde tracing histology

Rats were perfused with ice-cold borate buffered (pH 9.5) 4% paraformaldehyde. Brains were post-fixed overnight and were placed in cryoprotectant for 24 h. Five, one-in-five series of 30-μm thick coronal sections were obtained using a freezing microtome. One series was mounted and coverslipped, and the other was stained with thionin, dehydrated with increasing concentrations of alcohol, cleared, and coverslipped with Permount. Retrograde transport and labeling of Fluoro-Gold epifluorescence was visualized using an ultraviolet excitation filter. Cytoarchitecture was verified with an adjacent thionin stained series in all neuroanatomy experiments.

3. Results

3.1. Experiment 1. CeA inactivation effects on EBC acquisition and retention

Rats in the PBS group showed acquisition of EBC within the first 5 sessions, whereas the muscimol group showed no evidence of learning (Figs. 1B and 2A). Rats in the muscimol group were able to acquire EBC after the cessation of muscimol infusions. Rats in both acquisition groups showed a substantial drop in CR percentage during the retention session with muscimol infusions into the CeA relative to the retention session with PBS infusions (Figs. 1B and 2B). The results of Experiment 1 are very similar to the results of a previous study which showed that CeA inactivation severely impaired acquisition and retention of EBC with an auditory CS (Farley et al., 2016).

The CR percentage data for sessions 1–5 in Experiment 1 were analyzed using linear mixed effects modeling with fixed effects of group, session, and a quadratic function (across sessions) and random effects for intercept, slope, and the quadratic function. A model simplification strategy was used to find the model that best fit the data (Crawley, 2007), with the final model including a random effect for intercept. There was a highly significant Group X Quadratic Session interaction, $t(36) = 3.838$, $P = 0.000482$. The interaction indicated that the PBS group showed a significantly greater increase in CR percentage across sessions with a negatively accelerated curve relative to the muscimol group (Fig. 1B).

Testing data were analyzed using a repeated measures ANOVA with infusion type (PBS vs. muscimol) as a within subjects variable and group as the between subjects variable (Fig. 1B). The group effect was not significant, indicating that prior acquisition training with PBS or muscimol had no effect on testing after criterion. However, an effect of infusion type indicated that muscimol reduced CR% during retention testing $F(1, 8) = 58.574$, $p = 0.000$. The interaction of group and infusion type was not significant.

No significant group or session effects were found for UR percentage or amplitude (Fig. 3). Startle percentage and amplitude were not analyzed statistically due to the low frequency of events within and between subjects.

3.2. Experiment 2. CeA inactivation effects on EBC acquisition with extended training

Rats given muscimol inactivation of the CeA for 15 training sessions began to show increased CRs at around session 8 and progressively increase the percentage of CRs to reach > 30% CRs by session 15 (Fig. 4). Bupivacaine (voltage-gated sodium channel blocker) was infused into the CeA for session 16 to confirm that the deficit in acquisition was not specific to muscimol's mechanism of action or related to an alteration in $GABA_A$ receptors. The effect of bupivacaine did not differ substantially from the previous muscimol session or the subsequent muscimol retention session, suggesting that the modest learning during extended training is not due to $GABA_A$ receptor downregulation following repeated muscimol infusions.

The CR percentage data for Experiment 2 were analyzed using linear mixed effects modeling with fixed effects for session and a quadratic function for session and random effects for intercept, slope (sessions), and the quadratic function (across sessions). After using a model simplification strategy, the model that best fit the data had the full random effects structure. The quadratic session effect was significant, $t(7.5) = 4.222$, $p = 0.00335$, indicating a positively accelerated increase in CR percentage across sessions.

A paired samples *t*-test was used to analyze the CR percentage data for the PBS and muscimol test sessions for Experiment 2. There was a significant difference in CR percentage between the PBS and muscimol testing sessions, $t(7) = 6.489$, $p = 0.000$, indicating that CR percentage was reduced with muscimol infusion into the CeA.

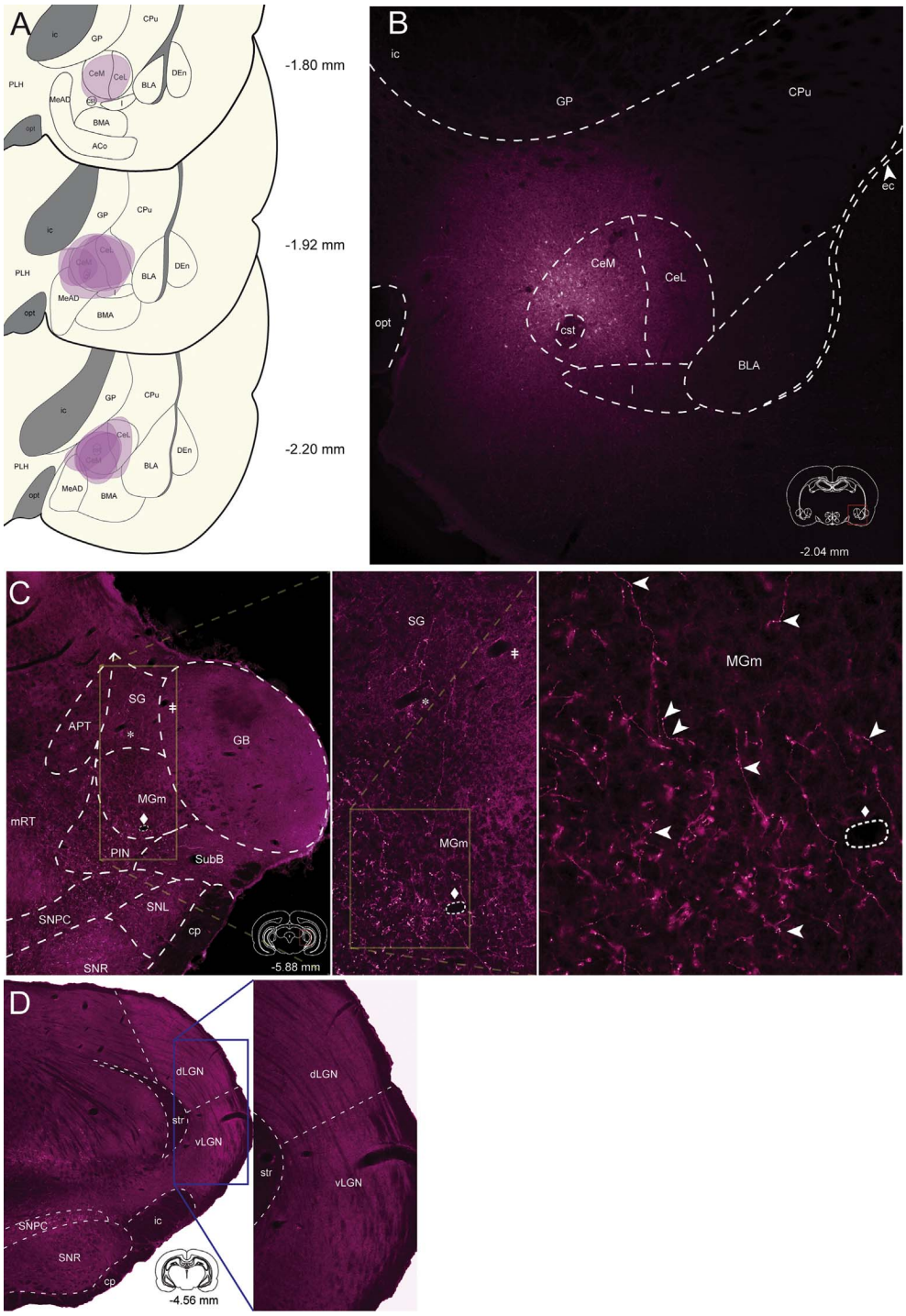

Fig. 6. Anterograde tracing of (CeA) and thalamic circuits. (A) Schematic montage of PHAL iontophoretic injection sites targeted at the CeM for the 9 animals classified as on-target placements. (B) Pseudo-colored photomicrograph of PHAL injection site centered on the CeM. (C) Left: Labeled varicosities in multiple areas of the auditory thalamus, including the SG, MGm, PIN, however absent in the geniculate body (GB). Middle: 10× zoom on SG and MGm. Right: 20× zoom of the MGm varicosities and terminals of CeM neurons. (D) Labeling was absent in the LGN. Cytoarchitecture was verified with an adjacent thionin stained series. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

No significant effects were found for UR percentage or UR amplitude. Startle percentage and amplitude were not analyzed statistically due to the low frequency of events.

3.3. Cannula placements for experiments 1 and 2

Cannula placements were verified with light microscopy. Fig. 5 displays an example of bilateral cannula placements and a composite drawing depicting the cannula placements for all of the rats is displayed in Fig. 5. All of the rats included in the analysis had bilateral cannula placements within the CeA. Two rats were removed from the analyses due to misplaced cannulas.

3.4. Experiment 3. Anterograde and retrograde labeling of CeA efferent projections

Thalamic relays upstream of the BPN, such as the MGm and vLGN are required for EBC when the CS is auditory or visual, respectively (Campolattaro et al., 2007; Halverson & Freeman, 2006, 2010a, 2010b; Halverson et al., 2009, 2010, 2008, 2015; Steinmetz et al., 2013). We performed neuroanatomical tract tracing experiments to address whether the CeA sends projections to thalamic areas that relay CS information to the BPN. On-target injections of PHA-L within the CeM (n = 9) were characterized by their placement dorsal to the commissural stria terminalis and ventral to the globus pallidus (Fig. 6). The descending pathway of PHA-L-labeled elements conformed to what has

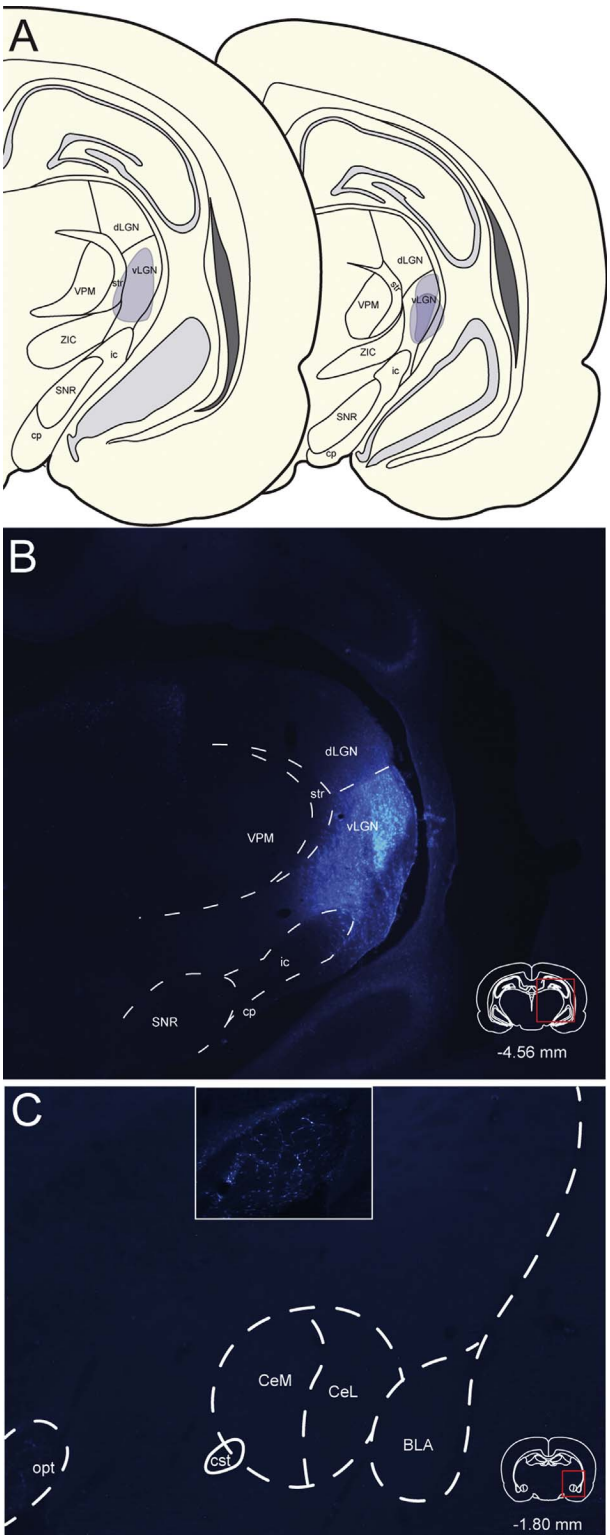

Fig. 7. Retrograde tracing showing no CeM to vLGN projection. (A) Schematic panels showing spread of Fluorogold injection site targeted to the vLGN. (B) Representative fluorescent micrograph of Fluorogold injection site. (C) Fluorescent micrograph of rostral amygdaloid complex absent of labeled somata. Inset: axonal transport of Fluorogold in the optic tract indicating successful uptake in the vLGN. Cytoarchitecture was verified with an adjacent thionin stained series.

been previously described and was exclusively ipsilateral (Hopkins & Holstege, 1978; Krettek & Price, 1978; Rizvi, Ennis, Behbehani, & Shipley, 1991; Rosen, Hitchcock, Sananes, Miserendino, & Davis, 1991). Labeled fibers departed medially from the injection site to the lateral region of the hypothalamus, forming varicosities and a small terminal field. The main fiber bundle proceeded caudally at the subthalamic nucleus, forming the dorsal boundary of the substantia nigra pars compacta. The vLGN, a visual relay to the BPN, did not contain labeled fibers or varicosities (Fig. 6). As the PHA-L labeled fibers progressed to the caudal diencephalon, labeled fibers turned mediodorsally from the pars compacta to the lateral aspect of the rostral periaqueductal grey. Varicosities were observed along this path including heavy labeling in the auditory thalamus, specifically, the posterior intralaminar nucleus (PIN), MGm, and portions of the SG (Fig. 6).

All anterograde findings with PHA-L were confirmed with Fluoro-Gold, a retrograde tracer. Fluoro-Gold injections into the vLGN (n = 3) confirmed no CeA ≥ vLGN projection (Fig. 7). Axonal transport of Fluoro-Gold was observed in the optic tract, demonstrating proper uptake in the vLGN. Lastly, Fluoro-Gold injections to the MGm confirmed a CeM → MGm projection emanating from rostral aspect of the CeM (n = 8; Fig. 8).

4. Discussion

Inactivation of the CeA impaired acquisition and retention of EBC with a visual CS (Figs. 1 and 2). In Experiment 1, there was no evidence of learning across five days of CeA inactivation, a finding consistent with the results of a previous study showing an acquisition deficit in EBC with an auditory CS (Farley et al., 2016). As in the Farley et al. (2016) study, there were no effects of CeA inactivation on unconditioned response percentage or amplitude. Experiment 2 demonstrated that rats with CeA inactivation could eventually learn with more extended training, exhibiting a significant increase in CR percentage across sessions (Fig. 4). Experiment 3 identified a monosynaptic projection from the CeA to the medial auditory thalamus (Figs. 6 and 8), but importantly, no projection to the vLGN (Fig. 7). The findings of this study indicate that similar mechanisms underlie CeA modulation of cerebellar learning for auditory and visual CSs.

All of the previous studies examining the effects of CeA manipulations on acquisition of EBC used an auditory CS; these studies found a severe deficit in acquisition of EBC with lesions or inactivation of the CeA (Blankenship et al., 2005; Burhans & Schreurs, 2008; Farley et al., 2016; Lee & Kim, 2004; Pochiro & Lindquist, 2016). Only one study, that we know of, examined the effect of CeA manipulations on EBC with an visual stimulus (Siegel et al., 2015). This study examined the effect of CeA inactivation during post-asymptotic retention of trace EBC in mice trained with an LED CS. There could be differences in the extent to which visual trace conditioning is affected by CeA inactivation relative to delay conditioning. Moreover, the effect of CeA inactivation on acquisition of visual trace EBC was not examined in the Siegel et al. (2015) study. It was therefore crucial to assess the effect of CeA inactivation on *acquisition* of delay EBC with a visual CS and to compare the results with our previous study on CeA inactivation during acquisition of delay EBC with an auditory CS (Farley et al., 2016). In both studies, CeA inactivation resulted in a severe deficit in EBC acquisition across five training sessions (Fig. 1). The magnitude of the deficit in CR percentage was perhaps bigger with the visual CS than with the auditory CS, with ~10% CRs on Session 5 in the visual group and ~20% in the auditory group. However, the non-associative baseline level of responding was not assessed in these studies and might account for the difference in CR percentage between the muscimol group given a visual CS in the current study and the muscimol group given an auditory CS in the Farley et al. (2016) study. The results of Experiments 1 and 2 are consistent with the hypothesis that the CeA modulates cerebellar learning, regardless of the CS modality. The generality of this hypothesis awaits further studies examining the effect of CeA manipulations on EBC with additional CSs such as whisker stimulation or body vibration.

Previous studies found that rats given lesions of the CeA are severely impaired in EBC but show an increase in CR percentage within 4–6 sessions (Blankenship et al., 2005; Lee & Kim, 2004). This finding has

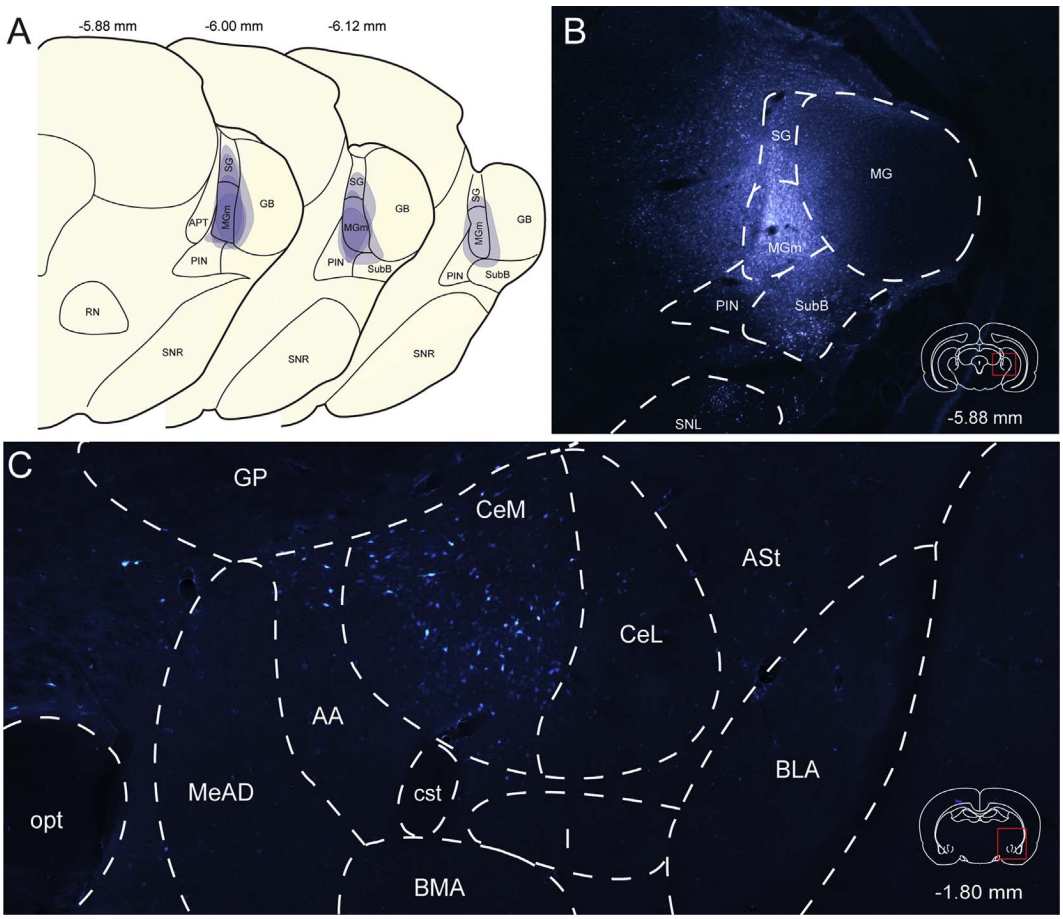

Fig. 8. Retrograde tracing verification of the CeM to MGm projection. (A) Schematic panels of Fluorogold injection sites in the MGm for the 8 on-target animals. (B) Fluorescent micrograph of an injection site showing uptake centered on the MGm. (C) Labeled somata in the rostral medial central amygdala (CeM). Cytoarchitecture was verified with an adjacent thionin stained series.

been interpreted as evidence that the CeA modulates cerebellar learning and is not part of the necessary circuitry for EBC. Experiment 1 and a previous study (Farley et al., 2016) found that muscimol inactivation resulted in virtually no learning across five days of EBC, suggesting that the CeA could play a necessary role in EBC rather than a modulatory role. The increase in CR percentage across EBC sessions in rats given repeated CeA inactivation in Experiment 2 was therefore crucial evidence supporting the modulation hypothesis. Thus, the cerebellum is capable of learning without CeA input, but the rate of learning is much slower. Moreover, when the cerebellum learns in the absence of CeA modulation, CRs are more resistant to CeA inactivation during retention sessions (retention sessions in Figs. 1 and 4). An alternative interpretation of the results could be that the efficacy of muscimol inactivation of the CeA decreased with repeated administration, despite the 48-h inter-infusion interval. This alternative explanation for the results of Experiment 2 seems unlikely, however, since bupivacaine, which has a different mechanism of action than muscimol, did not produce a more severe impairment. The results of the Experiment 2 therefore suggest the CeA modulates cerebellar learning and is not part of the necessary neural circuitry underlying EBC.

The differential projection pattern of the CeA to the auditory and visual thalamic areas necessary for EBC helps narrow down the potential pathways underlying CeA modulation of cerebellar learning. In a previous report we hypothesized that CeA modulation of cerebellar learning could plausibly occur through projections to the BPN or to the thalamic nuclei that send CS-related input to the BPN (Farley et al., 2016). Experiment 3 in the current study confirmed the projection from the medial CeA to the medial auditory thalamus but found no indication of a projection from the CeA to the vLGN. Since the learning rate of EBC is similar for auditory and visual EBC and the magnitude of the inactivation effect was equivalent, we conclude that CeA modulation is probably mediated by the same neural circuit mechanism. The most parsimonious interpretation of the current results is that the CeA modulates cerebellar learning with auditory and visual stimuli through its monosynaptic projection to the BPN. Accordingly, the CeA is thought to bypass the thalamus to directly modulate mossy fiber input to the cerebellum. It is possible, however, that auditory and visual EBC utilize different circuits, with visual EBC requiring the CeA to BPN projection and auditory EBC requiring the CeA to MGm projection. Determination of the circuitry for CeA modulation of cerebellar learning will necessitate selective manipulations of the projections from the CeA to the BPN and other targets.

The results of the current study indicate that the central amygdala facilitates cerebellar learning with either an auditory or visual CS. The amygdala plays a role in modulating cerebellar learning and is not part of the necessary neural circuitry underlying EBC. Amygdala modulation of cerebellar learning is hypothesized to facilitate CS sensory input to the BPN, and as a result, facilitates plasticity formation within the cerebellar cortex and interpositus nucleus (Farley et al., 2016; Pochiro & Lindquist, 2016; Taub & Mintz, 2010). This proposed CS facilitation can be conceptualized as a form of attention, which may be selective or non-selective (i.e., increased vigilance). Our current hypothesis is that the CeA plays a role in increasing attention selectively to the CS, based upon our previous finding that CS-elicited neuronal activity in the cerebellum is impaired by CeA inactivation but spontaneous neuronal activity is not affected (Farley et al., 2016). Moreover, there is extensive

evidence from studies of appetitive Pavlovian conditioning indicating the CeA plays a crucial role in increasing attention to a light or tone CS (Gallagher, Graham, & Holland, 1990; Holland & Gallagher, 1993). The specific pathway(s) by which the CeA facilitates cerebellar learning has not been identified, but our neuroanatomical evidence indicates that CeA projections to the BPN may be the primary pathway. Additional studies that selectively manipulate CeA projections to the BPN and other targets are necessary to definitively identify the modulatory pathway.

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