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Inactivation of the interpositus nucleus blocks the acquisition of conditioned responses and timing changes in conditioning-specific reflex modification of the rabbit eyeblink response

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Abstract

Conditioning-specific reflex modification (CRM) of the rabbit eyeblink response is an associative phenomenon characterized by increases in the frequency, size, and peak latency of the reflexive unconditioned eyeblink response (UR) when the periorbital shock unconditioned stimulus (US) is presented alone following conditioning, particularly to lower intensity USs that produced minimal responding prior to conditioning. Previous work has shown that CRM shares many commonalities with the conditioned eyeblink response (CR) including a similar response topography, suggesting the two may share similar neural substrates. The following study examined the hypothesis that the interpositus nucleus (IP) of the cerebellum, an essential part of the neural circuitry of eyeblink conditioning, is also required for the acquisition of CRM. Tests for CRM occurred following delay conditioning under muscimol inactivation of the IP and also after additional conditioning without IP inactivation. Results showed that IP inactivation blocked acquisition of CRs and the timing aspect of CRM but did not prevent increases in UR amplitude and area. Following the cessation of inactivation, CRs and CRM latency changes developed similarly to controls with intact IP functioning, but with some indication that CRs may have been facilitated in muscimol rabbits. In conclusion, CRM timing and CRs both likely require the development of plasticity in the IP, but other associative UR changes may involve non-cerebellar structures interacting with the eyeblink conditioning circuitry, a strong candidate being the amygdala, which is also likely involved in the facilitation of conditioning. Other candidates worth consideration include the cerebellar cortex, prefrontal and motor cortices.

Keywords

Classical eyeblink conditioning; Reflex modification; Fear conditioning; Stress; Interpositus nucleus; Cerebellum

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Conflicts of interest

The authors have no conflicts of interest to disclose.

1. Introduction

Since its first detailed description by Pavlov in the 1920s (Pavlov, 1927), classical conditioning has traditionally been characterized as the emergence of a conditioned response (CR) against the backdrop of an unconditioned response (UR) once considered to be automatic, fixed, and relatively invariant. Almost a century later, classical conditioning studies still tend to focus mainly on the CR; however, there is a much greater understanding and appreciation of the dynamic qualities of the UR, which can be modified by nonassociative phenomena such as sensitization and habituation (Boulis and Sahley, 1988; Gormezano and Kehoe, 1975; Hawkins, Cohen, & Kandel, 2006; Thompson, 2009) and importantly, can also be modified by associative factors during conditioning (Canli, Detmer, & Donegan, 1992; Schreurs, Oh, Hirashima, & Alkon, 1995; Weisz and McInerney, 1990). Studying conditioning-related changes in the UR alongside development of the CR is highly relevant for disorders of abnormal fear conditioning like post-traumatic stress disorder (PTSD) in which symptomology can include not only CRs to cues associated with trauma but also exaggerated reflexive responding to innately stressful stimuli (i.e. URs).

Classical conditioning of the rabbit nictitating membrane response, or eyeblink conditioning, is one paradigm for which associative changes in the UR have been well characterized, starting first with studies documenting changes in the UR in the presence of the conditioned stimulus (CS) and continuing with later and ongoing work detailing changes in the UR when tested in the absence of the CS (for review, see Burhans, Smith-Bell, & Schreurs, 2008). Earlier studies examining changes in the UR following presentation of the CS described increases in UR amplitude that rapidly developed at the start of conditioning, termed reflex facilitation (Ison and Leonard, 1971; Weisz and McInerney, 1990; Young, Cegavske, & Thompson, 1976) and decreases in UR amplitude when preceded by the trained CS, known as conditioned diminution (Donegan, 1981; Kimble and Ost, 1961). For the past two decades, ongoing work in our laboratory has extensively detailed changes to the UR that are measured when the unconditioned stimulus (US) is presented alone without the CS, referred to as conditioning-specific reflex modification (CRM) (Burhans et al., 2008; Schreurs and Burhans, 2015; Schreurs, 2003). In a typical experiment, rabbits are presented with varying intensities of a periorbital shock (0.1–2.0 mA) presented alone prior to and following classical delay eyeblink conditioning in which a tone CS is paired with a 2.0 mA periorbital shock US. After conditioning, increases in UR frequency, amplitude, and area, in addition to increases in response latency are observed, particularly to lower intensity shocks that elicited little to no responding prior to conditioning. This exaggerated responding is deemed “conditioning-specific” because the same changes are not observed in rabbits receiving explicitly unpaired presentations of the CS and US (Buck, Seager, & Schreurs, 2001; Schreurs et al., 1995; Schreurs, Shi, Pineda, & Buck, 2000). CRM-like changes during eyeblink conditioning have also been observed by others in rabbits and rodents (Gruart and Yeo, 1995; Servatius, Brennan, Beck, Beldowicz, & Coyle-DiNorica, 2001; Wikgren and Korhonen, 2001), and with conditioning of galvanic skin responses in humans (Morrow, 1966). It has also been reported that during trace eyeblink conditioning, combat veterans with PTSD show increased UR amplitude on US-alone trials, compared to veterans without PTSD (Burriss, Ayers, & Powell, 2007), suggesting CRM-like changes may be part of a

PTSD phenotype, which also includes increases in the amplitude of the CR (Handy et al., 2018).

Earlier work delineating the behavioral laws governing CRM in the rabbit eyeblink conditioning paradigm demonstrated commonality with the CR, leading to the theory that CRM may be a CR that has generalized from the CS to the US (Burhans et al., 2008; Schreurs, 2003). The first indication came from observations that the topography of the UR following conditioning closely resembled the CR on paired CS-US trials, with a latency shift to the right and a more complex, sometimes dual peaked profile where the first and second peaks coincided with the timing of CS and US presentations, respectively (Schreurs et al., 1995). Other evidence came from studies demonstrating that the strength of CRM was influenced by the same factors that influenced the strength of conditioning. For example, CRM got stronger with additional days of conditioning, with the greatest changes seen after six, rather than one or three daily sessions (Schreurs et al., 1995). In addition, stronger CRM occurred as the aversiveness of the US utilized during conditioning increased, such as when periorbital shock was used instead of corneal airpuff (Buck et al., 2001) or when shock intensity was amplified (Seager, Smith-Bell, & Schreurs, 2003). However, other findings showed that a dichotomy can exist between CRs and CRM, suggesting that the CR generalization hypothesis does not completely explain the nature of CRM. For example, training rabbits to produce multiphasic CRs by conditioning with a tone paired with two sequential USs did not produce a multiphasic UR that paralleled the CR topography, and it was shown that CRs could be extinguished without CRM and vice versa if rabbits were given CS-alone or US-alone presentations, respectively (Schreurs et al., 2000). It was only when both treatments were combined using explicitly unpaired CS/US presentations that both CRs and CRM were extinguished simultaneously (Burhans, Smith-Bell, & Schreurs, 2015; Schreurs et al., 2000). In addition, CRs and CRM have been shown to differentially respond to systemic serotonergic, glutamatergic, and noradrenergic manipulations (Burhans, Smith-Bell, & Schreurs, 2013, 2017, 2018). Meta analysis studies examining larger pools of behavioral data have also demonstrated that conditioning levels do not strongly predict individual susceptibility to CRM (Smith-Bell and Schreurs, 2017; Smith-Bell, Burhans, & Schreurs, 2012). Overall, these findings suggested that although CRs and CRM may have some commonality, they also have distinctions, suggesting some overlapping but possibly also distinct neural underpinnings. As there is evidence that CRs and URs can be differentiated as far downstream as the eyelid motoneurons (Trigo et al., 1999), there are many places along the eyeblink conditioning pathway where CR and CRM generation may diverge.

Because the positive relationship between CRM strength and aversiveness of the US suggested a fear conditioning component to CRM (Buck et al., 2001), the first study to directly investigate the neural substrates of CRM examined the role of the amygdala (Burhans and Schreurs, 2008), a critical component of the neural circuitry of learned fear (Maren, 2001; Phelps and LeDoux, 2005). The central nucleus of the amygdala (CE) was specifically targeted because of its previously established role in eyeblink reflex facilitation (Choi, Lindquist, & Brown, 2001; Weisz, Harden, & Xiang, 1992; Whalen and Kapp, 1991), characterized as an increase in UR amplitude following CS presentation that is thought to be one of the earliest signs of the development of the CR (Weisz and McInerney, 1990). The

main finding of our study was that muscimol inactivation of the CE during eyeblink conditioning slowed the rate of acquisition of CRs, which were eventually acquired to the same level as controls, but did not block the development of CRM. In contrast, inactivation of the CE during testing for CRM specifically blocked the frequency, area, and amplitude changes that are characteristic of CRM. These findings established that the CE appears to be important for the expression of CRM, but left open the question of what neural substrates may be responsible for CRM acquisition.

Because of the overlap between some features of CRs and CRM, a strong neural candidate for CRM acquisition is the interpositus (IP) nucleus of the cerebellum, which is a major site for the integration of CS and US inputs with outputs controlling the generation of the rabbit eyeblink CR (Gonzalez-Joekes and Schreurs, 2012; Gould, Sears, & Steinmetz, 1993; Ostrowska, Zguczynski, & Zimny, 1992; Steinmetz and Sengelaub, 1992; Thompson, 2013). There is general consensus that the IP is necessary for the acquisition of eyeblink conditioning, as indicated by IP lesion and inactivation studies showing severe impairments to CR acquisition and electrophysiological studies demonstrating the development of learning-related neuronal activity in the IP closely associated with the execution of the CR (*for review, see* Christian and Thompson, 2003; Freeman and Steinmetz, 2011; but see also Ammann, Marquez-Ruiz, Gomez-Climent, Delgado-Garcia, & Gruart, 2016; López-Ramos, Houdek, Cendelín, Vožeh, & Delgado-García, 2018). Of particular relevance is work showing that CRM-like increases in the amplitude of the UR in well-trained rabbits is blocked or disfacilitated by IP inactivation, suggesting a role of the IP in CRM expression (Wikgren and Korhonen, 2001).

The goal of the following study was to examine whether the IP is necessary for CRM acquisition by testing whether CRM would develop if the IP was temporarily inactivated with the gamma aminobutyric acid-A (GABA_A) agonist muscimol during eyeblink conditioning. As it was expected that IP inactivation would block acquisition of CRs, the current study was also another test of the CR/CRM dichotomy, examining whether CRM could develop in the absence of a learned and performed conditioned eyeblink response. Following conditioning under IP inactivation and initial testing for CRM, conditioning was allowed to proceed without inactivation followed by another test for CRM, in order to confirm that deficits in CR acquisition and any resulting deficits in CRM could be overcome once IP functioning was restored.

2. Methods

2.1. Subjects

The subjects were 20 male, New Zealand White rabbits (*Oryctolagus cuniculus*), 2–3 months of age, weighing approximately 1.8–2.3 kg upon delivery from the supplier (Charles River, Saint-Constant, Canada). The rabbits were housed in individual cages on a 12 h light-dark cycle and given ad libitum access to food and water. They were maintained in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health, and the research was approved by the West Virginia University Animal Care and Use Committee. The experiment was run in six replications, with each run having 2–4 rabbits.

2.2. Surgical procedure

After a minimum period of six days for adaptation to living conditions, rabbits underwent aseptic stereotaxic surgery for implantation of a chronic guide cannula targeted at the right IP, ipsilateral to the trained eye. The rabbits were anesthetized using a subcutaneous injection (0.6 mg/kg) of a mixture of ketamine HCl (83.3 mg/ml) and xylazine (16.7 mg/ml) with additional 0.25–0.5 ml injections of ketamine (100 mg/ml) given every 30–60 min to maintain anesthesia. Rabbits also received a single subcutaneous dose of the long lasting analgesic buprenorphine-SR (0.1 mg/kg) prior to the start of surgery in addition to localized injection of bupivacaine (2.0 mg/kg), distributed along the incision site. Rabbits were given supplemental oxygen (1–2%), and vital signs were continually monitored by a surgical assistant throughout the procedure.

After aligning the skull with lambda 1.5 mm lower than bregma, three holes were drilled, two for skull screws and one directly above the right IP using the following coordinates in reference to lambda: AP = –0.5 AP (anterior to lambda) and ML = –5.1, based on the atlas of Lavond and Steinmetz (2003). The holes for skull screws were placed on opposite sides of the skull, anterior to the hole above IP, and were fitted with small stainless steel screws (Small Parts Inc., Logansport, IN) that were partially screwed into the skull. In early replications of the experiment, surgical complications due to brain hemorrhaging during drilling of the hole above the IP led to modifications in the drilling technique that significantly reduced the mortality risk. Drilling above IP was done slowly in stages, with frequent stops to place sterile bone wax into the hole to minimize skull bleeding, allowing better visualization of the drill site. The depth of drilling was reduced such that a very thin layer of bone remained, which was subsequently removed manually with a bone curette, leaving the dura and vessels below the skull intact. A stainless steel 22 gauge guide cannula, designed to custom fit an internal 28 gauge injection cannula with a 1.5 mm projection (Plastics One Inc., Roanoke, VA), was lowered slowly (DV = –13.0 from lambda skull surface). Sterile bone wax was used to fill in any empty space within the hole surrounding the cannula, and the cannula was then secured by anchoring to the skull screws with dental acrylic. The guide was fitted with a protective dummy cannula of the same length that was removed for infusions. Following suturing, the surgical incision site was treated with triple antibiotic ointment and for three consecutive days thereafter.

2.3. Apparatus

The apparatus and recording procedures for eyeblink conditioning have been detailed elsewhere by Schreurs and Alkon (1990) who modeled their apparatus based on those described by Gormezano (Coleman and Gormezano, 1971; Gormezano, 1966). Briefly, rabbits were restrained in a Plexiglas box placed inside a sound-attenuating, ventilated chamber (Coulbourn Instruments, Allentown, PA; Model E10–20). Inside the chamber, a stimulus panel containing a speaker and houselight (10-W, 120 V) was mounted at a 45° angle 15 cm anterior and dorsal to the rabbit's head. An exhaust fan created a constant ambient noise level of 75 dB inside the chamber. Periorbital electrical stimulation was delivered by a programmable two-pole stimulator (Coulbourn Instruments, Model E13–35) via stainless steel Autoclip wound clips (Stoelting, Wood Dale, IL) that were positioned 10 mm ventral and 10 mm posterior to the dorsal canthus of the right eye. Stimulus delivery,

data collection, and analysis were all accomplished using the LabVIEW software system (National Instruments, Austin, TX).

To measure eyeblink responses, nictitating membrane responses (NMRs) were transduced by a potentiometer (Novotechnik US Inc., Southborough, MA; Model P2201) connected at one end, via a freely moving ball and socket joint, to an L-shaped lever containing a hook that attached to a 6-0 nylon loop that was sutured into but not through the nictitating membrane (NM). At the other end, the potentiometer was connected to a 12-bit analog-to-digital converter (5-ms sampling rate, 0.05-mm resolution), and individual A/D outputs were stored on a trial-by-trial basis for subsequent analysis.

2.4. Procedure

One week following recovery from surgery, rabbits were first acclimated to restraint by being placed in restrainers for 30 min while under close supervision. Rabbits then received one training session per day in the following order: adaption, US pretest, three sessions classical delay eyeblink conditioning following muscimol or saline infusion, US posttest (Post1), six additional sessions classical delay conditioning, and a second US posttest (Post2). There was a 48 h break between the third session of delay and Post1 to allow sufficient time for clearance of muscimol from the brain. Comparisons of Post1 with Pretest served as the initial evaluation of CRM following inactivation of the IP with muscimol, while comparisons with Post2 measured CRM after conditioning was allowed to proceed without IP inactivation.

For adaptation, subjects were prepared for delivery of the periorbital shock US and NMR recording and then adapted to the training chambers for an amount of time equivalent to subsequent training sessions (80 min). For pretest and posttests, subjects received 80 trials of US presentations with an average inter-trial interval (ITI) of 60 s (range 50–70 s). Each US presentation was one of 20 combinations of periorbital shock intensity (0.1, 0.3, 0.5, 1.0, or 2.0 mA) and duration (10, 25, 50, or 100 ms), and these 20 unique USs were presented in four separately randomized blocks with the restriction that the same intensity or duration could not occur more than three times in succession. For delay eyeblink conditioning, each session consisted of 80 trials of paired presentations of a 400 ms, 1 kHz, 82 dB CS that coterminated with a 100 ms, 2 mA US (300 ms interstimulus interval). The CS-US presentations were presented with an average ITI of 60 s.

CRs were defined as any extension of the NM exceeding 0.5 mm that was initiated following CS onset but prior to US onset. For US testing, a UR was defined as any extension of the NM exceeding 0.5 mm that was initiated within 300 ms following US onset. The definition of the UR was based on prior observations that responses to the US following CS-US pairings had onset latencies within the same range as CRs (Schreurs et al., 2000). Amplitude of the response was calculated as the maximum extension of the NM in millimeters. Onset latency of the response was the latency in ms from stimulus onset to when the NM rose 0.1 mm above baseline while peak latency was the latency in ms from stimulus onset until maximum NM extension occurred. Area of the response was calculated as the total area of the response curve (arbitrary units, au) from stimulus onset until the end of trial (trial length = 2000 ms). For URs during US testing, two additional measures were calculated in order to

overcome the statistical limitations of empty data cells produced by subthreshold responses to periorbital shock, particularly at the lower intensities and durations. These measures, magnitude of the response amplitude and magnitude of the response area, included the amplitudes and areas of all NMRs above baseline regardless of whether the 0.5 mm criterion was met (Garcia, Mauk, Weidemann, & Kehoe, 2003). A significant pre- to post test increase in any of the UR response measures as a function of classical conditioning is a defining feature of CRM. To increase the sensitivity for detection of CRM and to follow the convention of previous CRM studies, data were collapsed at the five US intensities across duration and CRM analyses were focused on the first 20 trial US sequence where the strongest CRM is observed (Schreurs et al., 2000). To examine the shape and timing of NMRs during US tests, response topographies were generated at each US intensity by averaging across rabbits and across US durations within each experimental group.

Unless described otherwise, experimental group data were analyzed by repeated measures analysis of variance (ANOVA, SPSS 24), with p values corrected using the procedures of Huynh-Feldt for violations of the sphericity assumption. Effect sizes are reported as Partial Eta Squared (η^2), values of which represent small (< 0.06), medium (0.06–0.14), or large effects (> 0.14) (Cohen, 1973). Planned and follow-up comparisons were Bonferroni corrected for the number of comparisons.

2.5. Temporary inactivation of the interpositus nucleus

One hour before the start of each of three days of delay conditioning, rabbits were restrained, and either the GABA_A agonist muscimol (Tocris Bioscience) dissolved in 0.9% saline (pH 7.4) or vehicle (0.9% saline, pH 7.4) was infused into the right IP, ipsilateral to the trained eye. For all infusions, the dummy cannula was first removed, and 0.50 μ l of muscimol (1 μ g/ μ l) or vehicle was infused at a rate of 0.25 μ l/min through an injection cannula that when inserted, projected 1.5 mm from the tip of the guide cannula. Each injection cannula was attached to a 10 μ l Hamilton microsyringe via polyethylene tubing, and the rate of infusion was controlled by an infusion pump (KD Scientific, Holliston, MA) that was modified to allow multiple rabbits to be infused simultaneously. Following infusion, the injection cannula remained in place for three minutes and was then replaced with the dummy cannula. Rabbits were returned to transport containers and monitored until they were prepared for behavioral training.

2.6. Histology

After the completion of training, rabbits were anesthetized with a solution containing ketamine HCl (83.3 mg/ml) and xylazine (16.7 mg/ml), followed by a lethal dose of Euthasol (sodium pentobarbital, 390 mg/ml) and transcardial perfusion with 0.9% saline followed by 10% formalin. The brains were post-fixed in 10% formalin and prior to sectioning, cryoprotected in a 15% sucrose, 2% formalin solution for a minimum of 24 h. Coronal sections were cut at 40 μ m using a freezing microtome. Sections were placed on slides and digitally imaged while wet (1.5X) with an Olympus BX51 microscope equipped with a computer assisted camera and software (monochrome Olympus FVII, cellSens Standard v1.13). The digital images were then used to determine the placement of the guide

cannula, verified by consulting two rabbit brain atlases (Lavond and Steinmetz, 2003; McBride and Klemm, 1968).

3. Results

3.1. Histology

Due to anesthesia complications, one rabbit did not complete surgery beyond drilling of holes for the anchor screws and IP cannula. For this rabbit, the guide cannula was not lowered, drilled holes were filled with sterile bone wax, and the incision was sutured closed. The rabbit was designated a surgical sham control and was trained alongside rabbits with IP cannula, receiving the same handling, including a mock setup for infusions. In the early replications of the experiment, three rabbits suffered brain hemorrhaging during surgery and subsequently did not recover. One additional rabbit did not adapt well to restraint during the acclimation period and was removed from the study.

A reconstruction of the location of the guide cannula tips for the remaining 15 rabbits receiving muscimol (black squares, $n = 8$) or saline (white circles, $n = 7$) into the IP is illustrated in Fig. 1. The majority of placements were typically in the dorsal portion of the IP or less than 1.0 mm above the IP, indicating that the injection cannula, which extended 1.5 mm beyond the guide tip, would likely have been within the IP. A scaled rendering of the injection tip length when inserted into the guide is also depicted in Fig. 1. The IP cannula stereotaxic coordinates and muscimol concentration and volume chosen for this study is within range of other protocols for muscimol inactivation of the IP in the rabbit literature. Many studies have utilized IP infusion volumes of 1.0 μl (Bracha, Webster, Winters, Irwin, & Bloedel, 1994; Krupa, Thompson, & Thompson, 1993; Ramnani and Yeo, 1996) and demonstrated some spread to the overlying cortex (Krupa et al., 1993), but volumes as small as 0.1 μl have been shown to be effective and well-contained within the deep nuclei (Krupa and Thompson, 1997; Robleto and Thompson, 2008). As our infusion volume (0.5 μl) falls in between these published reports, we cannot rule out some spread to the overlying cortex, but it should be significantly less than that found for 1.0 μl volumes. We have also previously established that fluorescent muscimol (BODIPY TMR-X muscimol conjugate, Molecular Probes) under the concentration, volume, and infusion rate used in the current study produces a radial spread around 1 mm in the amygdala (Burhans and Schreurs, 2013).

3.2. Muscimol inactivation of the IP during delay conditioning

Responding during delay conditioning and US tests in the surgical sham rabbit was statistically equivalent to rabbits with IP cannula receiving saline infusions, verified by One-way ANOVAs conducted for all CR and UR parameters for each session of delay conditioning and US testing. The rabbits were therefore combined to create a single control group ($n = 8$). The average percentage of CRs (% CRs) to the tone CS across the first three sessions of delay eyeblink conditioning (S1–S3) for controls and rabbits receiving muscimol is shown on the left side of Fig. 2. Inactivation of the IP with muscimol completely blocked the acquisition of delay conditioning whereas controls averaged 35.5% CRs (± 11.2 SEM) on the first session of conditioning and over 90% CRs on subsequent sessions. These observations were confirmed by a significant Session by Group interaction [$F(2,28) = 34.79$,

$p < 0.001$; $\eta p^2 = 0.71$]. Controls had significantly higher % CRs across all three sessions (S1: $p < 0.05$; S2, S3: p 's < 0.001), and within group comparisons indicated that responding in controls increased from the first to second session ($p < 0.05$) and remained at a similar high level thereafter.

To assess sensory effects of muscimol on the UR without confounding effects of the CR, URs for muscimol rabbits were compared with controls on trials where a CR did not occur. Analysis was focused on the first session as the incidence of URs in the absence of a CR was low and in some cases zero in controls as training proceeded beyond the first session. There were no differences between groups for the amplitude, area, or peak latency of the UR, but there was a significant effect of Group for onset latency [$F(1,14) = 7.33$, $p < 0.05$; $\eta p^2 = 0.34$] as a result of muscimol rabbits having longer UR onset latencies than controls.

3.3. Delay conditioning without muscimol inactivation of the IP

The right side of Fig. 2 shows mean % CRs during the six additional sessions of delay conditioning without IP infusions of muscimol or saline (S4–9). Muscimol rabbits appeared to learn at a similar rate as controls did during the first three sessions, although they did have a higher level of CRs during the first session without muscimol ($49.6\% \pm 10.6$ SEM) compared to controls during the first session with infusions ($35.5\% \pm 11.2$ SEM). Analysis of sessions S4–9 indicated a significant Session by Group interaction [$F(5,70) = 18.94$, $p < 0.001$; $\eta p^2 = 0.58$], with between group comparisons indicating that muscimol rabbits had significantly lower CRs than controls during session S4 only ($p < 0.01$). Within group comparisons indicated that while controls remained plateaued at a high level of CRs throughout sessions S4–9, muscimol rabbits showed increased CRs from S4 to S5 ($p < 0.01$) and remained at the same level thereafter. Analysis comparing sessions S1–6 for controls with S4–9 for muscimol rabbits indicated a significant effect of Session [$F(5,70) = 55.51$, $p < 0.001$; $\eta p^2 = 0.80$] with no effects involving Group, suggesting that muscimol rabbits learned similarly to controls once muscimol inactivation of IP was removed.

Focused analysis of controls during session S1 with muscimol rabbits during session S4 were conducted to explicitly examine whether there was any evidence of savings from the three days of conditioning under IP inactivation. One-way ANOVA for % CRs averaged across the entire session did not indicate any effects of Group. The sessions were also analyzed divided into eight, 10-trial blocks (see Fig. 3), and again, results did not reveal any significant group effects. A final analysis was conducted to examine the number of trials before the first CR emerged, which averaged $42.2 (\pm 9.2$ SEM) trials for controls and $19.9 (\pm 5.8$ SEM) trials for muscimol rabbits (Fig. 3, boxed insert). One-way ANOVA revealed a significant difference between groups [$F(1,14) = 4.84$, $p < 0.05$; $\eta p^2 = 0.26$]. Importantly, however, no muscimol rabbit demonstrated any CRs during the first five trials of session S4. The earliest CR exhibited by a muscimol rabbit was on trial 6 and by a control was on trial 9. These findings suggested that although muscimol rabbits did not exhibit savings at the very start of S4, there may have been some facilitation of learning due to past experience.

The latency, amplitude, and area of CRs were also compared for sessions S1–6 in controls versus S4–9 in muscimol rabbits, in addition to focused comparisons of session S1 in controls with S4 in muscimol rabbits divided into trial blocks. There were no effects

involving Group, demonstrating that once CRs were allowed to emerge in rabbits formerly receiving IP inactivation, they developed similar timing, size, and shape compared to controls.

3.4. Conditioning-specific reflex modification following IP inactivation

Analysis of baseline responsivity to the varying intensities of the US presented during Pretest revealed that one control rabbit exhibited extremely large responses to the lower US intensities. After being mathematically determined to be an outlier (Hoaglin and Iglewicz, 1987) for magnitude of the amplitude (mAmp) at the 0.1 and 0.3 mA intensities and magnitude of the area (mArea) at the 0.1, 0.3, and 0.5 mA intensities, the rabbit was removed from analyses for CRM. However, this rabbit did remain in analyses of delay conditioning because responding to the 1.0 mA intensity and the 2.0 mA intensity used as the US during delay conditioning were within normal range across all response parameters. The UR topographies in Fig. 4 show the timing, shape, and size of the UR to the five intensities presented during Pretest, the first posttest following three days of IP infusions (Post1), and the second posttest following six additional sessions of delay conditioning without IP infusions (Post2) in the seven remaining control and eight muscimol rabbits. Data for UR parameters are also represented in bar graphs for the UR latency measures (Fig. 5) and for the mAmp, mArea, and frequency of the UR (% URs), the latter not being discernible from topographies (Fig. 6).

To determine the effect of muscimol inactivation of the IP on the development of CRM, responding at Pretest was compared with Post1. Examination of the UR topographies in Fig. 4 suggests that the latency shift of the UR to the right, a defining feature of CRM, was present in controls but was strikingly absent in muscimol rabbits. However, both groups did appear to show some increases in area and amplitude from Pretest to Post1 at intermediate intensities, another hallmark of CRM. The size increases were somewhat heterogeneous between groups with controls showing the greatest change at the 0.3 mA intensity while muscimol rabbits demonstrated greater changes at the 0.5 and 1.0 mA intensities. For % URs (Fig. 6, top row), Pretest to Post1 increases at the intermediate 0.3 and 0.5 mA intensities were more apparent in controls whereas muscimol rabbits had a small increase at 0.5 mA but a decrease at 0.3 mA.

For UR latency measures (see Fig. 5), analyses focused on the intensities at which a majority of rabbits responded (0.3, 0.5, 1.0, and 2.0 mA). For onset latency, there was a significant US Test by Group interaction [$F(1,11) = 6.49$, $p < 0.05$; $\eta p^2 = 0.37$], with corrected comparisons indicating that only controls showed a Pretest to Post1 increase in latency ($p < 0.01$). Similarly, for peak latency, there was also a US Test by Group interaction [$F(1,11) = 8.44$, $p < 0.05$; $\eta p^2 = 0.43$], and again, only controls demonstrated Pretest to Post1 increases in latency ($p < 0.01$). Between group comparisons for both measures demonstrated that controls had greater latencies at Post1 than muscimol rabbits (onset: $p < 0.05$; peak: $p < 0.001$), but the groups did not differ at Pretest. There were no significant interactions of US Test, Group, and US Intensity. These results confirm a clear lack of a UR latency shift for CRM in rabbits receiving muscimol inactivation of the IP during the first three days of delay conditioning.

For mAmp (see Fig. 6, middle row), there was a significant US Test by Intensity by Group interaction [$F(4,52) = 2.63$, $p < 0.05$; $\eta p^2 = 0.17$], with corrected planned comparisons indicating that this was primarily a result of the large Pretest to Post1 increase for controls at the 0.3 mA intensity (trend: $p = 0.075$). Although muscimol rabbits demonstrated increases in amplitude at the 0.5 and 1.0 mA intensities, the comparisons were not significant. CRM for mArea in both groups (see Fig. 6, bottom row) was indicated by a significant US Test by Intensity interaction [$F(4,52) = 5.90$, $p < 0.01$; $\eta p^2 = 0.31$] with no effects involving Group. Although there were Pretest to Post1 increases at the 0.3, 0.5, and 1.0 mA intensities, the corrected planned comparisons at each intensity, however, did not reach significance. Overall, these findings suggest both groups showed some increases in the size (amplitude and area) of the UR, representative of a moderate amount of CRM following three days of delay conditioning (Schreurs et al., 1995).

Analysis of Pretest compared to Post1 for % URs (see Fig. 6, top row) revealed a US Test by Group [$F(1,13) = 7.44$, $p < 0.05$; $\eta p^2 = 0.36$] as well as a US Test by Intensity by Group interaction [$F(4,52) = 4.45$, $p < 0.05$; $\eta p^2 = 0.26$]. For the two way interaction, controls but not muscimol rabbits demonstrated increased UR frequency at Post1 compared to Pretest ($p < 0.05$). For the three way interaction, there was a trend for controls to have a Pretest to Post1 increase at only the 0.3 mA intensity ($p = 0.075$), but no Pretest to Post1 changes for muscimol rabbits. The lack of significant Pretest to Post1 increases in muscimol rabbits may have been, in part, the result of larger %URs at Pretest, causing possible ceiling effects. Although muscimol rabbits demonstrated greater %URs compared to controls at the 0.1, 0.3, 0.5, and 1.0 mA intensities, analyses focused on Pretest responding did not reveal any statistically significant differences between groups.

3.5. Conditioning-specific reflex modification following conditioning without IP inactivation

After six additional days of delay conditioning without infusions of muscimol into the IP, muscimol rabbits developed a shift to the right in the peak latency of the UR at Post2, similar to that seen in controls at Post1 and sustained at Post2 (see Fig. 4). Rabbits in both groups continued to show increases in the area and amplitude of the UR, which appeared to be even larger than the increases observed at Post1.

For analyses comparing Pretest with Post2, there were no effects involving Group, confirming that after additional training without IP inactivation, rabbits previously receiving muscimol developed CRM to levels that were similar to controls. CRM in both groups was confirmed by significant interactions of US Test and Intensity for peak latency [$F(3,36) = 10.86$, $p < 0.001$; $\eta p^2 = 0.48$], mAmp [$F(4,52) = 12.97$, $p < 0.001$; $\eta p^2 = 0.50$], and mArea [$F(4,52) = 8.04$, $p < 0.001$; $\eta p^2 = 0.38$] (see Figs. 5 and 6). For peak latency, there were significant Pretest to Post2 increases at the 0.3 and 0.5 mA intensities (p 's < 0.01) and also at 1.0 mA ($p < 0.05$). There were significant Pretest to Post2 increases for mAmp at 0.3 mA ($p < 0.05$) and for mArea at 0.3 mA ($p < 0.05$) and at 0.5 mA (trend, $p = 0.06$). For onset latency, there was a main effect of Test [$F(1,12) = 22.68$, $p < 0.001$; $\eta p^2 = 0.65$] reflecting an overall Pretest to Post2 increase across intensities. There was also a significant interaction of US Test and Intensity for % URs [$F(4,52) = 3.15$, $p < 0.05$; $\eta p^2 = 0.20$], reflecting Pretest

to Post2 increases at intermediate intensities (0.3, 0.5, 1.0 mA), although comparisons at each individual intensity were not significant. Further confirming a lack of difference in CRM between controls and muscimol rabbits, there were no significant between group comparisons at Post2 for any UR measures.

Analyses of Post1 compared to Post2 yielded group effects for latency measures but not for mAmp or mArea, further indicating that the latency shift was the main CRM measure affected by muscimol inactivation of the IP during delay conditioning. For onset latency, there was a significant US Test by Intensity by Group interaction [$F(3,36) = 3.79$, $p < 0.05$; $\eta p^2 = 0.24$] that was a result of significant Post1 to Post2 increases for muscimol rabbits at 0.3 and 1.0 mA (p 's < 0.05) while onset latency remained unchanged for controls. For peak latency, there was a significant US Test by Group interaction [$F(1,12) = 16.11$, $p < 0.002$; $\eta p^2 = 0.57$] with corrected comparisons indicating that only muscimol rabbits showed a latency increase from Post1 to Post2 ($p < 0.001$).

Indicative of an increase in the size and shape of CRM from Post1 to Post2 in both groups, there was a significant US Test by Intensity interaction for mAmp [$F(4,52) = 6.32$; $\eta p^2 = 0.33$] and a strong trend for a main effect of Test for mArea [$F(1,13) = 4.41$, $p = 0.056$; $\eta p^2 = 0.25$]. Corrected comparisons indicated that for CRM amplitude, there was a Post1 to Post2 increase at the 0.3 mA intensity ($p < 0.050$), and the main effect of Test for mArea reflected an overall Post1 to Post2 increase across intensities.

4. Discussion

For rabbits receiving muscimol inactivation of the IP during the first phase of delay eyeblink conditioning, acquisition of CRs was completely blocked, but there was some suggestion for facilitation of learning once conditioning continued without inactivation. Although some aspects of CRM developed under IP inactivation, including increases in area and amplitude of the UR, the latency shift seen in controls was completely abolished. After conditioning proceeded without inactivation, rabbits formerly receiving muscimol demonstrated a level of CRM indistinguishable from controls, which importantly included a clear latency shift, with increases in both UR onset and peak latency. In summary, these results showed that the IP is necessary for both the acquisition of eyeblink CRs and CRM, where for the latter, it plays a crucial role in conditioning-induced changes in the timing of the UR.

4.1. Role of the IP in the acquisition of associative changes in the timing of the UR

The major finding of this study was that the IP is required for the timing aspect of CRM, a shift in latency of the UR to the right, which is more pronounced at US intensities two-to eight-fold smaller than the intensity utilized during conditioning. Because this effect manifested during CRM testing in the absence of muscimol in the IP, it does not represent a performance deficit, but rather points to a role of the IP in the acquisition of the conditioning-induced change in UR latency. In addition, the ability of rabbits to learn once IP inactivation ceased argues against any lasting IP damage from cannula placements or repeated muscimol infusions. Overall, these findings support the idea that the latency shift in CRM depends on the development of plasticity in the IP or plasticity that the IP induces in downstream targets that initiate or modulate the UR.

Considering that one of the most CR-like features of CRM is the increase in peak latency which is reminiscent of the CR timing to CS-US presentations (Schreurs et al., 2000), it is possible that a similar mechanism that controls the timing of the CR may also control the learning-induced change in timing of the UR. Two sites of plasticity in the cerebellum have been proposed to mediate the development and timing of the eyeblink CR -the cerebellar cortex and the deep cerebellar nuclei, specifically the IP (for review, see Freeman, 2015). One type of plasticity at the cortical level is thought to involve long-term depression of parallel fiber-Purkinje cell synapses coactive with climbing fiber inputs, which represents convergence of the CS and US input pathways, respectively. Recent work also proposes that conditioning-induced decreases in Purkinje cell firing may involve an intrinsic timing mechanism that depends on activation of metabolic glutamate receptors (Johansson, Hesslow, & Medina, 2016). Regardless of how it is initiated, this depression/pause in Purkinje cell firing inhibits its major GABAergic projection onto IP neurons, which in turn, disinhibits the IP output projections to downstream areas involved in generating the CR. This mechanism is thought to enable precise timing of the CR, which is well-supported by electrophysiological studies examining the CR-related firing patterns of cortical Purkinje cells and IP neurons (Berthier and Moore, 1990; Halverson, Khilkevich, & Mauk, 2015; Jirenhed, Bengtsson, & Hesslow, 2007; McCormick and Thompson, 1984b; Rasmussen, Jirenhed, Wetmore, & Hesslow, 2014). However, it is thought that the IP can also develop plasticity independently from the cerebellar cortex, possibly via long-term potentiation at mossy fiber-IP synapses (Ohyama, Nore, Medina, Riusech, & Mauk, 2006). The main support for this second form of plasticity comes from studies demonstrating that disconnecting the cortical input to the IP, either through cortical lesions/inactivation or by blocking the cortical inputs into the IP with GABA antagonists, does not abolish CRs, but rather reveals abnormally timed, short-latency CRs that are dependent on the IP (Aksenov, Serdyukova, Irwin, & Bracha, 2004; Bao, Chen, Kim, & Thompson, 2002; Garcia and Mauk, 1998; Garcia, Steele, & Mauk, 1999; McCormick and Thompson, 1984a; Ohyama et al., 2006). Because CRM involves the development of longer latency URs similar in timing to normal CRs, it seems most likely that CRM of UR timing involves plasticity in IP that is initiated by cortical input. The use of the GABA_A agonist muscimol in our study essentially shuts down the IP from developing any learning-related plasticity, whether it is initiated by cortical inputs or direct mossy fiber inputs. Therefore, to definitively answer the question as to what type of plasticity in the IP is behind the change in UR timing, it would be necessary to repeat this experiment without completely silencing the IP, but rather disconnecting it from cortical input by using a GABA antagonist like picrotoxin (Aksenov et al., 2004; Bao et al., 2002; Garcia and Mauk, 1998; Ohyama et al., 2006).

If associative changes in the timing of the UR did utilize a similar mechanism as timing for the CR, this would suggest that after conditioning, the US presented alone is capable of activating parts of the CR pathway. Sensory information about the US is relayed from the trigeminal nuclei to the inferior olive (IO), reaching both the cerebellar cortex and IP via climbing fibers (Brodal, Walberg, & Hoddevik, 1975; Sugihara, Wu, & Shinoda, 1996, 2001; Van Ham and Yeo, 1992). Studies have shown that as eyeblink conditioning proceeds and the CR emerges, US-elicited responding in the IO decreases, the mechanism of which is thought to be a cerebellar nuclear-olivary negative feedback loop (Hesslow and Ivarsson,

1996; Medina, Nores, & Mauk, 2002; Najac and Raman, 2015; Nicholson and Freeman, 2003; Sears and Steinmetz, 1991). The decrease in IO responsivity to the US has been shown to be specific to CS-US pairings and does not occur when the US is presented alone (Sears and Steinmetz, 1991). This suggests that this inhibitory feedback loop would not be activated to US presentations during CRM testing and opens up the interesting possibility that following conditioning, US-alone presentations, particularly those of a reduced magnitude, may in fact generate potentiated responding in the IO. Since the IO climbing fiber input is thought to represent a teaching or error detection signal to the cerebellum (Albus, 1971; Ito, 1984; Marr, 1969; Medina et al., 2002), unexpected presentation of an unsignaled US may amplify or change the timing of the climbing fiber signals into the cerebellum, resulting in changes to the UR. This idea, however, assumes that the IO to cerebellum pathway somehow becomes favored over the shorter, spinal motor reflex pathway activated by the periorbital US. However, another possibility is more direct associative modification of the reflex pathway within the spinal trigeminal complex, shown to develop learning-related activity during eyeblink conditioning (Richards, Ricciardi, & Moore, 1991). However, this trigeminal associative activity appears to be driven by the IP and red nucleus (Clark and Lavond, 1996), therefore making it unlikely to be the site where associative changes to the UR originate.

Relating our findings to the literature, at least one other eyeblink conditioning study has suggested a role for the IP in associative changes in the UR. Wikgren and Korhonen (2001) found that cooling probe inactivation of the IP reduced UR amplitude during US-alone presentations only in rabbits that previously received paired, but not unpaired CS/US presentations prior to IP manipulation. These UR effects were not found during paired CS-US trials in trained subjects, further implicating the IP in expression of CRM-like changes that manifest when the US is presented alone after conditioning. Also possibly of relevance, earlier work by Welsh and Harvey (1989) demonstrated that post-training IP lesions that effectively abolished the CR also affected the size and latency of the UR, specifically to lower intensity US-alone presentations, although the authors did not interpret those effects as associative. Of additional importance, reflex facilitation, a type of associative change in the UR that manifests as increased amplitude in the presence of the CS, still develops in rabbits with IP lesions even though CRs are not acquired (Wikgren, Ruusuvirta, & Korhonen, 2002). Taken together with these past findings, our current results clarify that the IP's role in changes to the size of the UR may be limited to expression rather than acquisition since increases in the area and amplitude of the UR still developed in rabbits with IP inactivation. Interestingly, past work by Gruart and Yeo (1995) showed that post-training lesions of the cerebellar cortex enhanced CRM-like increases in UR amplitude during US-alone presentations of varying intensity, suggesting that expression of CRM size changes may be modulated by cerebellar cortical inputs into the IP. Further work is warranted to examine whether plasticity at the level of the cerebellar cortex is involved in acquisition of the non-timing aspects of CRM.

4.2. Alternative non-cerebellar substrates for associative changes in the size of the UR

Since IP inactivation did not prevent the acquisition of associative changes in the amplitude and area of URs, other possibly non-cerebellar substrates should be considered as sites of

plasticity underlying these aspects of CRM. A role for the amygdala in the modulation of cerebellar learning is well-established (Farley, Radley, & Freeman, 2016; Lee and Kim, 2004; Neufeld and Mintz, 2001; Shors and Mathew, 1998; Weisz et al., 1992; Whalen and Kapp, 1991), but our prior work showed that the central nucleus (CE) of the amygdala is crucial for the expression but not acquisition of amplitude, area, and frequency increases in CRM (Burhans and Schreurs, 2008). In agreement with these findings, work in rats has shown that blocking memory consolidation in the CE with protein synthesis inhibitors had no effect on eyeblink conditioning even though CE lesion or inactivation typically impairs or slows the acquisition of eyeblink CRs (Steinmetz, Ng, & Freeman, 2017). These findings therefore suggest plasticity in the CE is not required for amygdalar modulation of CRs or CRM. Alternatively, other amygdalar nuclei that output to the CE may be involved, such as the lateral or basolateral nuclei, which have been implicated as sites of acquisition-related plasticity that can initiate plasticity in other brain areas involved in maintenance of those learned responses (Chau and Galvez, 2012; Chavez, McGaugh, & Weinberger, 2009; Johansen, Cain, Ostroff, & LeDoux, 2011; Maren, Yap, & Goosens, 2001; McIntyre, Power, Roozendaal, & McGaugh, 2003; Poremba and Gabriel, 1999).

Other candidate sites for associative modification of the UR are cerebral cortical areas such as the motor cortex (MC) or medial prefrontal cortex (mPFC). Surprisingly, the MC has only recently been explicitly examined in eyeblink conditioning (Ammann et al., 2016; Pacheco-Calderon, Carretero-Guillen, Delgado-Garcia, & Gruart, 2012). These studies utilizing MC inactivation, stimulation, and single unit recordings in MC cells identified by antidromic activation via stimulation of the red or facial nucleus, offer a convincing account of a role for the MC in the acquisition and performance of delay eyeblink CRs. Small decreases in amplitude of the UR with MC inactivation were also reported, but to a much lesser extent than that seen for CRs; however, URs were only examined during CS-US presentations. Eyeblink studies on the mPFC have been more extensive, mostly focusing on the role of the mPFC in the more challenging hippocampal-dependent trace eyeblink conditioning paradigm (Oswald, Knuckley, Mahan, Sanders, & Powell, 2006; Siegel et al., 2015; Weiss and Disterhoft, 2011; Wu et al., 2017). However, recent work investigating the role of the mPFC in delay conditioning suggests it may play a modulatory role in expression and timing of the CR (Caro-Martin, Leal-Campanario, Sanchez-Campusano, Delgado-Garcia, & Gruart, 2015; Leal-Campanario, Delgado-Garcia, & Gruart, 2013) and can be critical for acquisition when using a reduced salience CS (Wu et al., 2012). Even more relevant, mPFC lesions in rabbits have been shown to impair the UR amplitude increase in reflex facilitation, without affecting acquisition of delay eyeblink conditioning (McLaughlin, Flaten, Chachich, & Powell, 2001). Even though reflex facilitation is measured in the presence of the CS, this study did show data for US-alone presentations that appeared to indicate reduced UR amplitude in lesioned rabbits, specifically in paired but not unpaired controls; however, statistics were not reported. In addition, mPFC-amygdala interactions may be particularly important as we have shown that the CE amygdala, which is known to be anatomically connected to mPFC along with other amygdalar nuclei (Buchanan, Thompson, Maxwell, & Powell, 1994), is crucial for expression of the UR size increase in CRM. The mPFC-amygdala interactions have also been implicated in stress modulation of eyeblink conditioning, although specifically in female rabbits (Maeng and Shors, 2013; Maeng,

Waddell, & Shors, 2010). Taken together, these findings suggest that the MC and mPFC are good candidate sites for mediating associative changes in the UR, specifically the amplitude and area changes in CRM that do not require the IP.

4.3. Role of the IP in acquisition of eyeblink CRs and possible modulation by the amygdala

The finding that inactivation of the IP blocked the development of CRs during delay eyeblink conditioning is in agreement with many electrolytic/excitotoxic lesion and reversible inactivation studies demonstrating that the IP is required for acquisition of delay conditioning across multiple species including rabbits (Clark, Zhang, & Lavond, 1992; Krupa et al., 1993; Lavond, Hembree, & Thompson, 1985; Lincoln, McCormick, & Thompson, 1982; Nordholm, Thompson, Dersarkissian, & Thompson, 1993; Pacheco-Calderon et al., 2012; Sears and Steinmetz, 1990; Weisz and LoTurco, 1988), rats (Freeman, Carter, & Stanton, 1995; Lee and Kim, 2004), and mice (Chen, Bao, Lockard, Kim, & Thompson, 1996; Porrás-García et al., 2010), with evidence extending to eyeblink studies in humans with cerebellar damages (Daum et al., 1993). However, of note, there is still some controversy regarding whether the IP is necessary for acquisition versus performance of eyeblink CRs and whether the cerebellar cortex can support acquisition in the absence of the IP in multiple species (Jimenez-Diaz, Navarro-Lopez, Gruart, & Delgado-Garcia, 2004; Sakamoto and Endo, 2010; Welsh and Harvey, 1991), including humans (Gerwig et al., 2003, 2010). In addition, recent work suggests that extracerebellar areas interacting with the IP such as the motor cortex and red nucleus may play a more active role in the acquisition and generation of CRs than traditionally thought (Ammann et al., 2016; Pacheco-Calderon et al., 2012), which opens up the possibility that the IP could also be modulating other brain areas capable of developing eyeblink conditioning-related plasticity. Although findings presented here support an important role for the IP in acquisition of eyeblink conditioning, we cannot rule out the possibility that longer latency CRs occurred beyond the 300 ISI between the CS and US. Following the convention of two decades of CRM studies, we did not incorporate CS-alone presentations during conditioning, which precludes us from detecting longer latency CRs unobscured by the UR. However, the lack of any initial savings during the second phase of conditioning without IP inactivation further supports the view that learning rather than performance was blocked.

Despite the absence of savings observed at the start of conditioning following IP inactivation, rabbits that had previously received muscimol did take significantly fewer trials to exhibit the first CR compared to control rabbits, suggesting that learning was facilitated by past experience. One potential mechanism for this facilitation is the before-mentioned modulation of cerebellar learning by the amygdala. The two stage theory of cerebellar learning posits that rapidly conditioned emotional/fear CRs mediated by the amygdala facilitate the motor CRs mediated and then maintained by the cerebellum (Lee and Kim, 2004; Lennartz and Weinberger, 1992; Mintz and Wang-Ninio, 2001). Supporting evidence for this theory comes from past studies showing that the CE can slow the rate of acquisition and is critical for the facilitation/enhancement of the eyeblink UR following CS presentation, thought to represent the earliest sign of a CR at the very beginning of learning (Choi et al., 2001; Pochiro and Lindquist, 2016; Weisz et al., 1992; Whalen and Kapp,

1991). In addition, fear preconditioning or prior exposure to acute stress such as tail shock or swim stress can facilitate subsequent eyeblink conditioning (Neufeld and Mintz, 2001; Servatius and Shors, 1994; Shors, 2001; Shors, Weiss, & Thompson, 1992), both also being dependent on an intact amygdala (Neufeld and Mintz, 2001; Shors and Mathew, 1998), but note that facilitatory effects may be specific to male subjects as female rats can show amygdalar-dependent impairment of eyeblink conditioning following stress (Waddell, Bangasser, & Shors, 2008). Since the current study utilized an aversive periorbital shock US, the amygdala would presumably be mediating the development of conditioned fear responses to the CS during the first phase of conditioning, regardless of the state of the IP. This fear pre-conditioning, including the prior stressful exposure to unsignaled shocks during Pretest and Post1, may consequently have led to the enhanced acquisition of eyeblink CRs observed here in male rabbits. Our previous work showing that muscimol inactivation of the CE delays eyeblink conditioning (Burhans and Schreurs, 2008) lends further support to a role for the amygdala in the facilitation of learning under the eyeblink conditioning parameters used for this study. In addition, we and others have shown the CE is critical for rabbit heart rate conditioning (conditioned bradycardia), a direct measure of autonomic conditioning that rapidly develops at the start of eyeblink conditioning (Burhans and Schreurs, 2013; Chachich and Powell, 1998; Kapp, Frysinger, Gallagher, & Haselton, 1979; McCabe, Gentile, Markgraf, Teich, & Schneiderman, 1992).

One theory concerning the mechanism by which the amygdala may modulate cerebellar learning is the sensory gating hypothesis whereby the amygdala is thought to gate the CS sensory input to the cerebellum and enhance conditioning by amplifying the CS salience or by increasing attention to the CS (Farley et al., 2016; Taub and Mintz, 2010), a mechanism which has also been implicated in facilitating appetitive conditioning (Holland and Gallagher, 1999). Recent work by Farley and colleagues in rats has demonstrated that inactivation of the CE amygdala can impair the acquisition of eyeblink conditioning and can importantly block the development of learning-related neuronal activity in response to the CS in the IP (Farley et al., 2016). The proposed pathway through which the amygdala can modulate CS input is through connections with the pontine nuclei (Farley et al., 2016; Hopkins, 1975; Krettek and Price, 1978; Siegel et al., 2015; Tracy, Thompson, Krupa, & Thompson, 1998), a critical part of the eyeblink conditioning circuit relaying sensory input from the auditory CS to the IP and cerebellar cortex (Brodal and Jansen, 1946; Mihailoff, 1993; Steinmetz et al., 1987). Additional support for this pathway comes from studies demonstrating that the amygdala can influence CS-related activity in the pontine nuclei (Pochiro and Lindquist, 2016; Taub and Mintz, 2010). Based on these findings, the learning facilitation shown here may be the result of the amygdala progressively amplifying the CS signal input to the cerebellum via feedback connections to the pontine nuclei during the first three days of conditioning, the full benefit of which would manifest once IP inactivation ceased.

4.4. Role of the IP in expression of the UR

The lack of observed effects of IP inactivation on the incidence and size of the UR during conditioning is in agreement with many other studies that report no effects of IP lesions or inactivation on URs during paired CS-US trials and/or US-alone presentations (Krupa et al.,

1993; Weisz and LoTurco, 1988; Welsh and Harvey, 1991; Yeo, Hardiman, & Glickstein, 1985). However, some studies have reported decreases in UR size that are nonsignificant or transient (Chapman, Steinmetz, Sears, & Thompson, 1990; Freeman et al., 1995; Steinmetz, Lavond, Ivkovich, Logan, & Thompson, 1992), more severe (Bracha et al., 1994; Jimenez-Diaz et al., 2004; Pacheco-Calderon et al., 2012), or found to manifest to lower intensity US presentations (Welsh and Harvey, 1989). Of note, however, we did find effects of IP inactivation on the latency of the UR during conditioning. More specifically, we found that URs in rabbits receiving muscimol had significantly longer onset latencies than controls but peak latency was not affected. This effect was not transient and appeared to persist across all three days of conditioning under inactivation, although the incidence of URs unobserved by CRs in controls was too low during subsequent days to do statistical comparisons beyond the first session. UR latency measures are typically not examined in other studies or at least not explicitly reported, making our findings difficult to compare to the literature. However, Welsh and Harvey (1989) did report significant increases in UR peak latency and a slight increase in onset latency to lower intensity US-alone presentations following post-training electrolytic IP lesions. Another study examining URs in rabbits given explicitly unpaired CS/US presentations during muscimol inactivation of IP indicated no latency effects (Jimenez-Diaz et al., 2004). We did not include US-alone trials during conditioning with IP inactivation, but we did specifically limit our analyses of URs to the first day of conditioning, comparing to controls on only trials in which a CR did not occur. However, it is difficult to conclude whether we are observing a nonassociative, sensory effect of muscimol since the CS precedes the US presentation.

4.5. Conclusions

The major finding of the current study is that the IP is necessary for the acquisition of associative changes in the timing of the UR, one of the defining features of CRM that shares similarities to the timing of the CR. The development of the eyeblink CR and CRM timing therefore appear to share a common neural mechanism, as both appear to require plasticity in the IP during conditioning. However, other aspects of CRM that can develop independently of the IP and in the absence of CRs, such as increases in area and amplitude of the UR, further highlight a dichotomy that can exist between CRs and CRM. The larger picture is that CRM is not necessarily a unitary learning phenomenon but rather the manifestation of several associative changes in the UR that are likely mediated by a larger network of neural circuitry, involving both the cerebellum and other areas that can interact with and modulate the eyeblink circuitry, strong candidates being the amygdala, cerebellar cortex, and prefrontal and motor cortices.

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Fig. 1.

Reconstruction of the location of guide cannula tips in the right interpositus nucleus for rabbits in the saline (white circles) and muscimol (black squares) groups, shown in coronal drawings 1.5 to 0.0 mm anterior to Lambda. Also shown is a to-scale depiction of the injection-guide cannula assembly, which projected an additional 1.5 mm from the guide tip. On the right is an example coronal brain section taken from a rabbit in the muscimol group. FA: fastigial nucleus; DE: dentate nucleus; IP: interpositus nucleus.

Fig. 2.

The mean percentage ($\pm$ SEM) of conditioned responses (% CRs) during delay eyeblink conditioning in rabbits receiving pretraining infusions of saline (controls, white circles) or muscimol (black squares) into the interpositus nucleus during the first three sessions of conditioning (S1–S3) and no infusions during remaining sessions (S4–9). The X axis summarizes the experimental design and shows where unconditioned stimulus (US) testing sessions took place on days prior to (US Pretest), in between (US Post1), and following conditioning sessions (US Post2). The control group contains one rabbit that did not receive infusions.

Fig. 3.

The mean percentage ($\pm$ SEM) of conditioned responses (% CRs) during the first delay eyeblink conditioning session (S1) in controls (white circles) overlaid with the first session without inactivation of the interpositus nucleus (S4) in muscimol rabbits (black squares). Data shown are divided into blocks of 10 trials (B1–B8), and the right inset shows the average number of trials to the first instance of a CR.

Fig. 4.

Eyeblick unconditioned response topographies to unconditioned stimulus (US) presentations prior to delay eyeblink conditioning (Pretest, dotted black line), following three sessions of conditioning with (Muscimol) or without (Control) inactivation of the IP (Post1, solid gray line), and after an additional six days of conditioning without inactivation (Post3, solid black line). Data shown are for the first 20 trials of pre- and posttesting, averaged at each of five US intensities (0.1–2.0 mA) and collapsed across US duration.

Fig. 5.

Mean ($\pm$ SEM) unconditioned response onset (top row) and peak latencies (bottom row) to unconditioned stimulus (US) presentations prior to delay eyeblink conditioning (Pretest, white bar), following three sessions of conditioning with (Muscimol) or without (Control) inactivation of the interpositus nucleus (Post1, grey bar), and after an additional six days of conditioning without inactivation (Post3, black bar). Data shown are for the first 20 trials of pre- and posttesting, averaged at each of five US intensities (0.1–2.0 mA) and collapsed across US duration.

Fig. 6. Mean ($\pm$ SEM) unconditioned response frequency (% URs, top row), amplitude (MAmp, middle row), and area (MArea, bottom row) to unconditioned stimulus (US) presentations prior to delay eyeblink conditioning (Pretest, white bar), following three sessions of conditioning with (Muscimol) or without (Control) inactivation of the interpositus nucleus (Post1, grey bar), and after an additional six days of conditioning without inactivation (Post3, black bar). Data shown are for the first 20 trials of pre- and posttesting, averaged at each of five US intensities (0.1–2.0 mA) and collapsed across US duration. au: arbitrary units.