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Eyeblink conditioning during an inter-stimulus interval switch in rabbits using picrotoxin to disrupt cerebellar cortical input to the interpositus nucleus

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Abstract

The role of the cerebellar cortex in eyeblink classical conditioning remains unclear. Experimental manipulations that disrupt the normal function of this region impair learning to various degrees and task parameters may be important factors in determining the severity of impairment. The present investigation was undertaken to study the role of cerebellar cortex in eyeblink conditioning under CS-US intervals known to be optimal or non-optimal for learning. Using discrete infusions of picrotoxin to the interpositus nucleus of the rabbit cerebellum, we pharmacologically disrupted input from the cerebellar cortex while training with an inter-stimulus interval (ISI) switch procedure. One group of rabbits was first trained with a 250 ms ISI (optimal) and then switched to a 750 ms ISI (non-optimal). A second group was trained in the opposite order. As expected, control rabbits learned the 250 ms ISI much faster than the 750 ms ISI. The most striking effect was that picrotoxin-treated rabbits initially trained with a 250 ms ISI learned comparably to controls, but those initially trained with a 750 ms ISI were severely impaired. These results suggest that functional input from cerebellar cortex becomes increasingly important for the interpositus nucleus to learn delay eyeblink conditioning as the ISI departs from an optimal interval of 250 ms.

Keywords

Cerebellum; Eyeblink Classical conditioning; ISI; GABA; Picrotoxin

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Introduction

Classical conditioning has proven to be an important tool for studying brain-behavior relationships, and significant progress has been made in the endeavor to understand the neural mechanisms that govern learning and memory. Indeed, arguably, we know more about the structures and functions that underlie basic associative memory than any other form of experience-dependent behavior. The eyeblink classical conditioning preparation has made critical contributions to our current knowledge base in this area.

A single eyeblink conditioning trial usually consists of an auditory conditioned stimulus (CS) that is presented to an organism shortly before a blink-eliciting unconditioned stimulus (US), such as a puff of air directed at the cornea. In the most basic procedure, delay conditioning, the CS and US overlap and coterminate. Initially, naïve subjects produce a reflexive, unconditioned blink response (UR) that follows US onset. After many trials, however, organisms associate the stimuli and a CS-triggered response actualizes in the form of a blink that begins before US onset. This is the classically-conditioned eyeblink response (CR). Under optimal conditions, well-trained organisms execute CRs on a high percentage of trials.

Rabbits are commonly used in eyeblink conditioning studies and extensive data have been collected in order to define the stimulus and timing parameters that promote optimal learning – as defined by the fastest rate of acquisition, the greatest percent CRs, and the most extinction-resistant learning. One important factor is the time between CS onset and US onset; the inter-stimulus interval (ISI). Classic studies have demonstrated that learning in delay eyeblink conditioning is most robust in rabbits when the ISI is 200-500 ms (Coleman and Gormezano, 1971; Gormezano, Kehoe, and Marshall, 1983; Schneiderman, 1966; Schneiderman and Gormezano, 1964; Smith, 1968; Smith, Coleman, and Gormezano, 1969). It can thus be argued that an ISI of 200-500 ms is optimal for rabbit eyeblink conditioning while longer or shorter ISIs are non-optimal.

The neural substrates for eyeblink conditioning have been extensively summarized (see Christian and Thompson, 2003). Briefly, CS and US signals converge upon cells in two regions of the cerebellum: the cortex and the deep nucleus. At the level of cortex, these signals ultimately converge upon Purkinje cells, which in turn inhibit cerebellar output cells located in the deep nucleus. Both the cortex and deep nucleus of the cerebellum have been studied extensively in an effort to delineate the neural mechanisms that underlie learning and memory in eyeblink conditioning. Significant progress has been made in that the cerebellar interpositus nucleus has been identified as the critical locus for all forms of eyeblink conditioning, but the function of cerebellar cortex remains poorly understood.

Various methodologies have been successfully employed in an effort to study the role of cerebellar cortex in eyeblink conditioning. One approach is to interfere with the cortico-nuclear connection, and this can be done three ways: 1) the cerebellar cortex can be ablated or electrolytically lesioned (e.g., Lavond and Steinmetz, 1989), 2) Purkinje cells can be selectively destroyed (Chen, Bao, Lockard, Kim, and Thompson, 1996; Nolan and Freeman, 2006), or 3) compounds that alter activity can be infused into discrete regions of the cerebellum (e.g., Bao, Chen, Kim, and Thompson, 2002). While each of these approaches has their advantages and drawbacks, we chose the last approach for this study because the drug effects are temporary and reversible.

Many cerebellar pharmacological perturbations take advantage of the fact that Purkinje cells represent the sole output of the cerebellar cortex, and they modulate neurons in the interpositus nucleus through release of γ -aminobutyric acid (GABA). Thus, temporary disruption of cerebellar cortico-nuclear function can be accomplished by infusing the noncompetitive GABA receptor antagonist, picrotoxin, into the interpositus nucleus (Mamounas, Thompson,

and Madden, 1987). This procedure produces a rapid and reversible block of GABA-mediated Cl^- flux at the GABA_A receptor complex on interpositus cells. While this manipulation has been presented as a reversible disconnection of cerebellar cortex (e.g. Garcia and Mauk, 1998), alternative pharmacological manipulations that may represent a more complete disconnection of cerebellar cortex have been described (Bao et al., 2002). Thus, picrotoxin infusion to the interpositus nucleus may best be characterized as a disruption of the cerebellar cortico-nuclear connection, rather than a complete decortication of the cerebellum.

To our knowledge, only one study has examined the effects of picrotoxin infusions to the interpositus nucleus on acquisition of eyeblink CRs in naïve animals. Bao et al (2002) found that picrotoxin alone, or in combination with the GABA agonist, muscimol, prevented acquisition (but not retention) of CRs. The authors argue that the primary memory trace for eyeblink conditioning occurs in the interpositus nucleus, and relies upon patterned inhibition from the cortex where secondary memory traces exist for shaping CR timing and amplitude. In accordance with this idea, numerous studies have found that picrotoxin infusions to the interpositus nucleus of previously trained animals can result in the emergence of improperly timed, short latency eyeblink CRs (Aksenov, Serdyukova, Irwin, and Bracha, 2004; Bao et al., 2002; Garcia and Mauk, 1998; Ohyama and Mauk, 2001; Ohyama, Nores, Medina, Riusech, and Mauk, 2006). These data fit well with lesion (Perrett, Ruiz, and Mauk, 1993) and electrophysiological (Green and Steinmetz, 2005) studies, which suggest that the anterior lobe of the cerebellar cortex regulates proper CR timing via precisely timed inhibition and disinhibition of the interpositus nucleus.

It is of interest to us that cerebellar cortex may contribute so critically to CR timing and we are interested in determining if there is a differential role for this region in learning the CR at different ISIs. Recent evidence from aging rabbits (Woodruff-Pak, Seta, Roker, and Lehr, 2007) and developing rats (Brown, Pagani, and Stanton, 2006) suggests so. Because higher doses of picrotoxin appear to block initial acquisition (Bao et al, 2002), we decided to use a lower dose that has a demonstrated efficacy in unmasking short latency responses in well trained rabbits for a period of time that is approximately equal to the length of our training session (Garcia and Mauk, 1998).

We undertook the present investigation, in part, to determine if cerebellar cortex plays a dissociative role in learning eyeblink conditioning when optimal or non-optimal ISIs are used. To this end, we examined the effects of picrotoxin-induced disruption of the cerebellar cortico-nuclear function on initial acquisition with a 250- or a 750 ms ISI. We hypothesized that normal input from cerebellar cortex to interpositus nucleus may become more important for CR learning and timing as task demands are increased in eyeblink conditioning (e.g., learning a non-optimal ISI). Additionally, we switched the ISI mid-training, making it longer or shorter, and examined the ability of the animal to alter the latency of the CR. Considering the importance of cerebellar cortex in CR timing, we further hypothesized that adapting the CR to the new ISI would be problematic in the absence of normal modulatory input from cerebellar cortex.

Methods

Subjects

Subjects were 40 New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN) weighing a minimum of 2.0 kg prior to use. All subjects were housed in stainless steel cages at The University of Kansas in the Animal Care Unit, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Upon arrival, rabbits acclimated to the housing milieu for at least one week. All animals were housed individually with 24-h access to food and water and a 12/12-h light/dark cycle. The research presented here was approved by the IACUC at The University of Kansas.

Surgery

A guide cannula for drug infusion was chronically implanted into the left interpositus nucleus of the cerebellum. Surgery was performed under aseptic conditions. Anesthesia was induced with xylazine (6-8 mg/kg IM) and ketamine (80-120 mg/kg IM). Once anesthetized, puralube ophthalmic ointment (Pharmaderm, Melleville, NY) was applied to the eyes to prevent irritation, and the animal's head was shaved, cleaned, and secured in a standard stereotaxic apparatus. Anesthesia was maintained throughout the surgical procedure with administration of 30 mg/kg ketamine (IM) every 30-45 min.

The skull was exposed with a midline incision, three anchor screws were inserted into the bone, and the head was positioned such that bregma was 1.5 mm higher than lambda in the dorsal-ventral plane. A 4-pin socket assembly, which served as a ground, was wired to two of the anchor screws and cemented in place. Next, a hole was drilled over the interpositus nucleus and filled with bone wax. Using a stereotaxic arm, a 22-gauge, stainless steel guide cannula was lowered into the brain (0.7 mm anterior, 5.2 mm left lateral, and 14.0 mm ventral to lambda) and cemented into place. A stainless steel insect pin (00) was then inserted into the cannula to prevent clogging, and an 18 mm diameter PVC pipe was cemented around the cannula in order to protect it from bending or breaking.

After cannula implantation, a base was formed on the skull with dental cement. A single bolt was cemented into the anterior portion of the base so that an air nozzle could be secured to the head during the training phase. The scalp was then sutured around the base and an antiseptic ointment was applied to the incision site. Rabbits were carefully monitored until they fully recovered from anesthesia. Post-operative treatment included buprenorphine (0.05-0.1 mg/kg SC) twice each day for 2 days or meloxicam (0.2-0.4 mg/kg SC) daily for 2 days. One week of recovery was allowed before training was started.

Drug Infusions

Prior to each of the classical conditioning sessions described below, rabbits were infused with either phosphate buffered saline (0.9% NaCl, 0.1 M phosphate buffer, 7.2 pH) or picrotoxin (Sigma-Aldrich; 200 μ M solution in phosphate-buffered saline). All drug infusions were delivered through the guide cannula with a 10 μ l syringe (Hamilton; Reno, NV, USA), which was equipped with a stopper on the needle to ensure that the blunted tip did not go more than 0.5 mm beyond the tip of the guide cannula. The total volume for all infusions was 2.0 μ l, injected at a rate of 0.25 μ l/15 s. After the infusion was complete, the syringe was left in place for exactly 2 minutes. Animals were trained two-at-a-time and the classical conditioning session started after both animals were infused and placed in separate sound-attenuating chambers. The maximum time between the end of drug delivery and beginning of the conditioning session was 7-min.

At the end of the study, all animals underwent retention testing in which muscimol (Sigma, 6.0 nM) was infused to the interpositus nucleus just prior to a single eyeblink conditioning session. Because muscimol infusions in this region are known to abolish the eyeblink CR (Krupa, Thompson and Thompson, 1993), the purpose of this test was to ensure that previous infusions reached the critical region of the interpositus nucleus.

Behavioral Training

Naïve rabbits were first adapted to the Plexiglas restraint apparatus and the conditioning chamber for approximately 45 minutes on each of the two days prior to initial training in eyeblink classical conditioning. The conditioning chamber housed a speaker for delivering the tone CS, an air nozzle and tube assembly for delivering the US, and wires for recording eyeblink EMG activity. On the second day of adaptation, two stainless steel wires were implanted under

local anesthetic (lidocaine IM) into the *Orbicularis oculi* muscle above the left eye for recording blink-related EMG activity.

A classical conditioning session consisted of 84 trials organized into 7 blocks of 12 trials. Each block consisted of 9 paired CS-US presentations, 2 CS-only presentations, and 1 US-only presentation. Each session, therefore, consisted of 63 paired, 14 CS-alone, and 7 US-alone trials. The CS consisted of a 1 kHz, 85 dB tone, and the US consisted of a 3 psi puff of air directed at the cornea. Trials were separated by a 30 (± 5) second intertrial interval. Both a long and a short inter-stimulus interval (ISI) were incorporated into the design of the present experiment. The short ISI (250 ms) consisted of a 350 ms CS that overlapped and coterminated with a 100 ms airpuff US. The long ISI (750 ms) consisted of an 850 ms CS that overlapped and coterminated with a 100 ms air-puff US. CS and US intensities remained constant across all groups.

Rabbits were randomly assigned to one of two ISI-switch groups. One group, 250 $\rightarrow$ 750 ($n = 21$), was first trained for 10 sessions with a 250 ms ISI, then switched to a 750 ms ISI and trained for an additional 10 sessions. The second group, 750 $\rightarrow$ 250 ($n = 19$), received training in the opposite order. Thus, all groups received 20 days of training. Within each ISI-switch group, rabbits were further divided into one of two groups; a picrotoxin group or a saline control group.

Data Acquisition and Planned Analyses

Acquisition of eyelid EMG data was accomplished with a Micro 1401 unit (Neuralynx, Inc., Bozeman, MT, USA) that was interfaced with a computer running Spike2 software (CED Ltd, London, UK). For each session, individual trials were extracted from the continuous EMG feed beginning 500 ms prior to CS onset and continuing for a total of 2000 ms. All EMG activity was amplified, rectified, and stored for subsequent offline analysis. Datamunch software (King and Tracy, 1999) was used to analyze the data.

For each trial, baseline EMG amplitude was established by calculating the average voltage across the first 400 ms of the pre-CS period. A trial was discarded if the amplitude of the EMG exceeded 3.0 times the average baseline amplitude during the bad trial window, which extended from 100 ms prior to CS onset to 25 ms following CS onset. Responses were recorded as blinks if the amplitude of the EMG exceeded 2.5 times the average baseline amplitude. Responses were scored as CRs if the blink occurred anywhere from 26 ms after CS onset to US onset, or to the end of the 1500 ms trial on CS-only trials. URs were scored if a blink was initiated after US onset.

Our method for scoring short latency CRs was determined in the analysis phase of the experiment. Previously, Ohyama et al (2006) defined the short latency CR as any blink that occurred prior to 200 ms after CS onset. This criterion had to be altered for the present study because the ISIs that we used in training were much shorter or longer than their 500 ms ISI and we required a window that could be used with both 250 and 750 ms ISIs. Most importantly, we required a window that accurately detected short latency responses without rejecting normally timed CRs as defined by the control rabbits. Thus, we used a rather restricted definition for short latency CRs: Those behavioral responses that had onsets that occurred 26-80 ms after CS onset.

During each session, several behavioral response parameters were measured, including percent CR (percentage of paired or CS-alone trials that resulted in a CR), CR amplitude (average deflection from baseline), CR onset latency (time from CS onset to the point at which a CR was executed), and CR peak latency (time from CS onset to maximum eyelid CR closure). For each of these response parameters, session-wide averages were computed. Percent paired CR

was computed for CS-US trials, and percent CS-alone trials was computed for test trials in which only a tone CS was presented. Because the topography of CRs can be contaminated by the presence of the UR on paired trials, CR amplitude and peak latency measures are computed for CS-alone trials only, except where indicated. Mixed-design ANOVAs were used to analyze response parameters. For each ISI switch group (250→750 or 750→250), drug (saline control or picrotoxin) served as the between subjects factor, and session (1-10 or 11-20) served as the repeated measure.

Histology

At the end of the experiment, to estimate the spread of picrotoxin, all subjects received infusions of 0.25% cresyl violet dye (2.0 μ l) to the interpositus nucleus. After a minimum period of 15 minutes following dye injection, rabbits were euthanized with an overdose of Euthasol (4.0 cc, i.v.), and the infusion site was marked in some animals by passing electrical current (500 μ A DC for 7-10 s) through the cannula. Rabbits were then immediately perfused as described below.

Transcardial perfusion was accomplished with a peristaltic pump. Approximately 1 liter of 0.9% saline was circulated, followed by a similar volume of 10% formalin (37% formaldehyde solution in 0.9% saline solution). Following perfusion, brains were removed and stored in 10% formalin/30% sucrose solution for at least one week prior to sectioning. In preparation for sectioning, the cerebellum was isolated, embedded in albumin-gelatin, and frozen. The cerebellum was positioned coronally on a microtome stage and sliced into 80 μ m sections. Slices were saved in a bath of 0.2 M phosphate-buffered solution, and mounted on gelatin-subbed slides from a bath of 0.2 M acetate buffer solution. In order to identify neural structures, and to provide contrast to cresyl violet infusions, most sections were stained with neutral red [Sigma-Aldrich (N7005, > 90%)]. In some cases, however, every-other slice was stained with cresyl violet and photomicrographs were taken of sections where cresyl violet infusions could be easily identified. Additionally, in the subset of brains that were marked by passing current through the cannula, slides were counter-stained with potassium ferrocyanide.

Inclusion Criteria

Animals were included in analyses if they met the following three criteria: 1) histology revealed a cannula mark or cresyl stain in the region of the interpositus nucleus, 2) the interpositus nucleus was not damaged, and 3) muscimol infusions in retention resulted in CR abolition, which we initially defined as less than 20% CR on all paired trials (but in actuality, all animals included in the analyses had less than 10 CRs after muscimol infusion). With regard to the control group, acquisition data from Sessions 1-20 were included as long as the interpositus nucleus was not found to be damaged.

Results

Histology

Figure 1 depicts cannula placements for all rabbits included in the analyses of sessions 1-20 for all conditions. Of the 40 original animals trained, 34 met our requirements for inclusion in the analyses. Two of the excluded rabbits, both control, never learned the CR and were found to have suffered damage to the interpositus nucleus. Additionally, 4 rabbits treated with picrotoxin were excluded because a post-training muscimol infusion did not effectively abolish CRs, and cresyl violet stain was not present in the interpositus nucleus. As a result, all analyses include the following numbers of rabbits: 250→750: control ($n = 9$), picrotoxin ($n = 9$); 750→250: control ($n = 8$), and picrotoxin ($n = 8$). Figure 2 depicts stained coronal brain sections that provide examples of cannula placement in the interpositus nucleus and cresyl violet spread in the region.

The 250→750 ms ISI switch: Effect of picrotoxin on learning and performance of the eyeblink CR

Figure 3 depicts eyeblink CR performance in the 250→750 ms ISI switch group. Control and picrotoxin-treated rabbits acquired the CR comparably when initially trained with a 250 ms ISI (Figure 3A). Using the dependent measure of percent of paired CRs, a 2 (drug treatment: control v. picrotoxin) $\times$ 10 (training session) repeated measures ANOVA was conducted. This analysis revealed a significant main effect for training session [$F(9, 144) = 67.739, p < 0.001$], but there were no differences between the drug treatment conditions, and no interaction. The same analysis was performed on the dependent measure of percent of CS-only CRs. Similarly, this analysis revealed a significant main effect for training session [$F(9, 144) = 50.16, p < 0.001$]. We performed these same statistical analyses on CR amplitude, CR peak latency, and CR onset latency, and there were no statistical differences between control and picrotoxin on any of these behavioral measures ($ps > 0.05$). Thus, when the initially-trained ISI is 250 ms, picrotoxin infusions to interpositus nucleus do not affect acquisition or performance of the CR across training sessions.

Timing of CR onset and peak did not differ significantly ($ps > 0.05$) between control (onset: $M = 175.42$, $s.d. = 49.76$; peak: $M = 241.43$, $s.d. = 51.1$) and picrotoxin (onset: $M = 175.00$, $s.d. = 63.43$; peak: $M = 230.50$, $s.d. = 63.98$). In order to graphically summarize this data, we constructed histograms that depict the frequency with which CRs began and peaked, within 10-ms-long bins, from CS onset through the end of the trial. For control (Figure 3B) or picrotoxin (Figure 3C) groups, these data were collapsed across the last 4 sessions with the 250 ms ISI. Because of the disparity, in some groups, between the total number of CRs executed by control and picrotoxin rabbits, we normalized the data by calculating CR onset or peak frequency relative to the total number of CRs executed. This was accomplished by dividing the number of CR trials with onsets or peaks that fall within each 10 ms bin by the total number of CRs executed over the last 4 sessions. Thus, the frequency of CRs that occur in each bin is relative to the total number of CRs executed by all rabbits in that group over the last 4 sessions. This format is used for all histograms that appear in this paper.

Figure 3C depicts the presence of a few short latency CRs in picrotoxin-treated rabbits, but their occurrence was relatively infrequent. We will address the issue of short-latency responses below in the Discussion section.

When the ISI was switched to 750 ms, both control and picrotoxin groups exhibit an immediate decline in performance that is slowly recovered over training (Figure 3D). This initial decrease in performance after switching from a short to a long ISI has been demonstrated previously (Coleman and Gormezano, 1971). A mixed design ANOVA on percent paired CRs revealed a significant main effect for training session [$F(9, 144) = 3.443, p < 0.005$]. Additionally, the drug treatment effect approached significance ($p = 0.088$); the interaction was not significant. When the same analysis was conducted using the dependent measure of percent CS-only CRs, a significant main effect for training session was revealed [$F(9, 144) = 5.521, p < 0.001$], but there was no effect of drug, and no interaction. Thus, picrotoxin may have a slightly detrimental effect on eyeblink conditioning performance, as measured by percent paired CR, when the ISI is shifted from 250 to 750 ms, but this effect was not significant. This may be due either to the relatively low CR rate in both groups, or the variability in response frequency exhibited by control rabbits within sessions. Again, we examined all other behavioral measures and found no significant differences between control and picrotoxin ($ps > 0.05$).

Figure 3 (E-F) depicts CR timing histograms for the 750 ms ISI. Onset and peak latency of the CR did not differ significantly between control (onset: $M = 505.67$, $s.d. = 198.62$; peak: $M = 668.22$, $s.d. = 166.11$) and picrotoxin (onset: $M = 517.30$, $s.d. = 188.46$; peak: $M = 646.20$, $s.d. = 175.91$).

In summary, when the initially-trained ISI was optimal for learning, pharmacological disruption of cerebellar cortico-nuclear function had little effect on eyeblink conditioning, even after the ISI was switched to a longer, non-optimal interval.

The 750→250 ms ISI switch: Effects of picrotoxin on learning and performance of the eyeblink CR

Figure 4 depicts eyeblink CR performance in the 750→250 ms ISI switch group. As compared to control, learning was profoundly impaired in picrotoxin-treated rabbits initially trained with a 750 ms ISI (Figure 4A). Using the dependent measure of percent paired CRs, a 2 (drug) x 10 (training session) repeated measures ANOVA was conducted. This analysis revealed significant main effects for training session [$F(9, 126) = 24.297, p < 0.001$] and drug [$F(1, 14) = 75.867, p < 0.005$], as well as a session x drug interaction [$F(9, 126) = 4.104, p < 0.001$]. Independent samples *t*-tests confirmed that control outperformed picrotoxin on training sessions 5-10 [respectively: $t(14) = 2.91, 2.45, 3.95, 2.24, 3.15$, and 2.38], *ps* listed in Figure 4 caption. These analyses were repeated for the dependent measure of percent of CS-only CRs. Significant main effects were revealed for training session [$F(9, 126) = 128.044, p < 0.001$] and drug treatment [$F(1, 14) = 120.428, p < 0.001$], and there was a significant session x drug interaction [$F(9, 126) = 4.831, p < 0.001$]. Independent samples *t*-tests indicated that control outperformed picrotoxin on training sessions 4-10 [respectively: $t(14) = 2.44, 3.13, 2.74, 4.39, 2.25, 5.07$, and 3.23], *ps* listed in Figure 4 caption. In essence, the CS-alone trial data were similar to the paired trial data, which is important. This indicates that during training with the 750 ms ISI, late CRs were not present in the animals that were injected with picrotoxin as sampling for CRs is taken to the end of the US period.

We next examined CR amplitude across sessions 1-10. Both control and picrotoxin demonstrated increased amplitudes across training [$F(9, 126) = 13.983, p < 0.001$]. Additionally, there was a significant main effect for drug [$F(1, 14) = 6.381, p < 0.05$] and a session x drug interaction [$F(9, 126) = 3.386, p < 0.005$]. Thus, picrotoxin-treated rabbits are slower to learn the CR, and to a lower asymptotic level of performance, as compared to controls rabbits. Despite this, there were no differences in CR timing between control (onset: $M = 489.79$, *s.d.* = 162.39; peak: $M = 688.26$, *s.d.* = 128.13) and picrotoxin (onset: $M = 444.64$, *s.d.* = 238.04; peak: $M = 605.90$, *s.d.* = 225.50), *ps* > 0.05. Timing histograms for CR onset and peak with a 750 ms ISI are depicted in Figure 4B for control and Figure 4C for picrotoxin.

When the ISI is switched from 750 to 250 ms, percent CR performance initially declines, but control rabbits quickly compensate while picrotoxin-treated rabbits require significant training before they reach performance levels comparable to that of control (Figure 4D). A 2×10 repeated measures ANOVA using the dependent measure of percent paired CRs revealed significant main effects for session [$F(9, 126) = 15.686, p < 0.001$] and drug [$F(1, 14) = 5.992, p < 0.05$]. The session x drug interaction was not significant. Independent samples *t*-tests confirmed that control outperformed picrotoxin on sessions 13, 15, 16, 18, and 19 [respectively: $t(14) = 2.91, 2.45, 3.95, 2.24, 3.15$, and 2.38], *ps* < 0.05. The same analysis was conducted on percent CS-only CRs, and significant main effects were revealed for training session [$F(9, 126) = 10.585, p < 0.001$] and drug treatment [$F(1, 14) = 5.796, p < 0.05$]. Again, the session x drug interaction was not significant. Independent samples *t*-tests confirmed that control outperformed picrotoxin on sessions 12, 13, 15, 16, and 19 [respectively: $t(14) = 2.27, 2.15, 2.36, 2.43$, and 2.39], *ps* < 0.05.

We next examined CR amplitude across sessions 11-20. While control and picrotoxin groups both increased CR amplitudes across training [$F(9, 126) = 2.385, p < 0.05$], neither the drug effect nor the session x drug interaction effect was significant. Additionally, there were no significant differences in measures of CR timing between control (onset: $M = 158.55$, *s.d.* 49.37; peak: $M = 251.44$, *s.d.* = 54.32) and picrotoxin (onset: $M = 176.74$, *s.d.* = 51.69; peak:

$M = 244.89$, $s.d. = 67.63$), $ps > 0.05$. Timing histograms for CR onset and peak with a 250 ms ISI are depicted in Figure 4D for control and Figure 4E for picrotoxin.

Taken together, these results indicate that pharmacological disruption of the cerebellar cortico-nuclear connection impairs eyeblink conditioning when the initially-trained ISI is not optimal for learning, and these impairments continue, even after the ISI is switched to an optimal interval. However, after extended training at the 250 ms ISI, picrotoxin-treated rabbits eventually attain performance levels that are comparable to control rabbits.

Discussion

We pharmacologically disrupted the cerebellar cortico-nuclear connection with picrotoxin infusions into the interpositus nucleus and examined delay eyeblink conditioning performance in rabbits trained with ISIs that are known to be optimal or non-optimal for learning. When the initially-trained ISI was 250 ms (optimal), CR acquisition was comparable for control and picrotoxin-treated rabbits. However, when the initially-trained ISI was 750 ms, picrotoxin treatment severely impaired the rabbits' ability to learn the task, as compared to controls. These findings support our hypothesis that cerebellar cortical modulation of the interpositus nucleus plays a greater role in eyeblink conditioning when the ISI is not optimal for learning (> 500 ms).

Data from control rabbits corresponds well with a substantial body of previous research demonstrating that eyeblink CR learning is most robust with an ISI that is approximately 200-500 ms (Coleman and Gormezano, 1971; Gormezano, Schneiderman, Deaux, and Fuentes, 1962; Gormezano et al., 1983; Schneiderman, 1966; Schneiderman, Fuentes, and Gormezano, 1962; Schneiderman and Gormezano, 1964; Smith, 1968; Smith et al., 1969). Naïve, control rabbits that were trained with a 250 ms ISI (Figure 3A) learned faster and produced a higher overall percentage of CRs than those trained with a 750 ms ISI (Figure 4A). Also, after 10 training sessions, we switched the ISI, making it shorter or longer, and observed an initial decline in performance in both ISI-switch groups (Figure 3B, 4B). In each case, in the training sessions following the ISI switch, double-peaked CRs were often observed, and the CR peak associated with the initially-trained ISI diminished over subsequent training. Thus, after the ISI switch, the CR peak associated with ISI-1 (Sessions 1-10) was extinguished as the CR peak associated with ISI-2 (Session 11-20) was acquired (data not shown). Rabbits switched from 750 to 250 ms ISI acquired the new CR peak more rapidly than those switched from 250 to 750 ms. Similar performance trends have been reported by others who have studied the ISI switch in both humans (Boneau, 1958; Ebel and Prokasy, 1963; McAllister, 1953; Prokasy, Ebel, and Thompson, 1963) and rabbits (Coleman and Gormezano, 1971; Leonard and Theios, 1967; Prokasy and Papsdorf, 1965). Thus, data from our control rabbits provide further support for the idea that ISIs longer than 500 ms do not produce optimal eyeblink CR learning in rabbits.

Normal input from the cerebellar cortex to the interpositus nucleus appears to be differentially important for eyeblink CR acquisition with optimal versus non-optimal ISIs. When we initially trained rabbits with a 250 ms ISI, control and picrotoxin-treated rabbits had comparable rates of CR acquisition and asymptotic levels of performance as measured by percent CR (Figure 3A). Also, there were no differences on measures of CR amplitude or latency. In contrast, significant learning impairments were observed in picrotoxin-treated rabbits initially trained with a 750 ms ISI. Indeed, after 10 sessions of training, these rabbits never fully acquired the CR to the same level as controls (Figure 4A). Furthermore, the rarely executed CR was low in amplitude and highly variable in latency (Figure 4C). These results suggest that modulatory input from cerebellar cortex becomes increasingly important for delay eyeblink conditioning if the initially-trained ISI is longer than that which is optimal for learning.

One of the questions that we addressed in the present study was how pharmacological disruption of the cerebellar cortico-nuclear connection affects the ability of rabbits to learn a new ISI after an abrupt switch from previous training with a longer or shorter ISI. After fixed ISI training, we switched the ISI and found that picrotoxin-treated rabbits generally produced lower CR percentages than control rabbits. In the 250→750 switch condition (Figure 3D), this effect was not quite statistically significant. In the 750→250 ISI switch condition (Figure 4D), the cause of this impairment is unclear, but one likely explanation is that the relatively reduced CR performance was due to the fact that picrotoxin-treated rabbits never fully learned the CR prior to the ISI switch (Figure 4A). These results may indicate that modulatory input from cerebellar cortex is important for learning and adapting the CR under changing conditions. Alternatively, cerebellar cortex may be important for new learning after previous training has occurred in eyeblink conditioning (Garcia, Steele, and Mauk, 1999; Perrett and Mauk, 1995; Yeo, Lobo, and Baum, 1997).

Given the growing body of evidence that suggests a role for cerebellar cortex in CR timing (and specifically the role of the anterior lobe), we were interested in evaluating the ability of picrotoxin-treated rabbits to adapt their CR latency over training. The frequency histograms in Figures 3 and 4 indicate that rabbits in all conditions seem to learn adaptively-timed CRs before and after the ISI switch. In this respect, the overall performance of picrotoxin-treated rabbits indicated that normal input from cerebellar cortex is not critical for CR timing in the interpositus nucleus. However, we noted considerable variability in response timing in picrotoxin-treated rabbits. And, we did observe that a few rabbits developed short latency CRs, as previously described by Garcia and Mauk (1998). Given the literature describing the effects of picrotoxin infusions in the interpositus nucleus (Bao et al., 2002; Garcia and Mauk, 1998; Ohyama et al., 2006), we were surprised by the infrequency and variability with which short latency CRs were observed in the present study.

There are two technical explanations for why this discrepancy may have occurred. First, picrotoxin infusions may not have the potential to be effective throughout the entire session. To test for this possibility, we examined the behavior of individual animals who expressed short latency CRs to determine if these picrotoxin-mediated responses were present at the end of the session. Figure 5 depicts blink activity over the last 10 paired trials in a single session for one rabbit that was first trained with a 250 ms ISI (Figure 5A) and one animal that was first trained with a 750 ms ISI (Figure 5B). In each case, short latency CRs were robustly expressed through the end of the conditioning session, as defined by the presence of short-latency responses (see Garcia & Mauk, 1998). These data indicate that picrotoxin has the potential to be effective throughout the duration of a classical conditioning session. A second reason why our results may differ from those described previously is that our criterion for scoring short latency CRs was much more restrictive. (e.g., Garcia and Mauk, 1998). We used the performance of the control animals with the 250 and 750 ms ISIs to define the window of time during which a normal CR could be seen, and we scored short latency CRs when any response onset preceded that window. This resulted in a short-latency CR criterion defined as any response that occurred 26-80 ms after CS onset; a window of time that is much narrower than previous studies. This may ultimately account for a lower number of responses that meet the criterion for short latency CR.

Other reasons why our results may differ from those described previously may lie in the methodology. For example, short latency CRs have been unmasked primarily in CR retention studies that follow a cerebellar cortex lesion (Perrett, Ruiz, and Mauk, 1993), or disruption of cerebellar cortical input to the interpositus nucleus (Garcia and Mauk, 1998). In these studies, a CR has been established through training, and removal of cerebellar cortex, or disrupting interpositus activity, presumably disrupts expression of previously established plasticity in the form of parallel fiber – Purkinje cell long-term depression, which ultimately modulates CRs

via well-timed disinhibition of the interpositus nucleus. Interestingly, it has been demonstrated that overtraining in eyeblink conditioning prior to disruption of cortico-nuclear function can rescue CR timing, and endow the interpositus with plasticity sufficient to execute a well-timed CR (Christian and Thompson, 2005). Similarly, while we examined the effect of cerebellar disruption over the course of acquisition, the extensive training that the animals received may allow for timing-related plasticity to develop in the interpositus, even in the absence of normal input from the cerebellar cortex. Thus, the variability in response timing that we observed in picrotoxin-treated rabbits suggests that the cerebellar cortex is important for consistently producing well-timed CRs in initial acquisition, but the ability of animals to accurately time most CRs with disrupted cortical input to the interpositus nucleus suggests that compensatory mechanisms may be at play. In essence, our hypothesis that cerebellar cortico-nuclear disruption would impair the rabbits' ability to adapt the latency of the CR over training, including after an ISI switch, was not conclusively supported.

A consensus is yet to be reached regarding the origin(s) of neural processes that regulate CR timing in eyeblink conditioning. The most parsimonious hypothesis holds that PCs regulate timing via coordinated disinhibition of the interpositus nucleus just prior to US onset (e.g., Mauk and Donegan, 1997). Some electrophysiological recording data from lobule HVI of cerebellar cortex do not fit this model, as PCs tend to increase their discharge with CS-US pairing (Berthier and Moore, 1986; Gould and Steinmetz, 1996; Kotani, Kawahara, and Kirino, 2003). However, Green and Steinmetz (2005) recently provided support for this hypothesis by demonstrating that PCs in the anterior lobe tend to decrease their firing rate with CS-US pairings. This suggests that important timing-related plasticity may be confined to a rather discrete region of cerebellar cortex (but see Nolan and Freeman, 2006).

While cerebellar cortex may play an important role in CR timing, our results argue that normal cerebellar cortical input to the interpositus nucleus is not critical for CR timing, at least in initial acquisition. Indeed, while timing-related neural activity may develop in cerebellar cortex, it does not necessarily follow that timing mechanisms originate in cerebellar cortex. It could be the case, for example, that CR timing relies on a distributed network of processes in regions of the brain that ultimately send important timing-related input to areas of the cerebellum where CS-US associations are made. Furthermore, while both the interpositus nucleus and the cerebellar cortex appear to be important for timing, our picrotoxin data argue that adaptive timing of CRs may be accomplished by the interpositus nucleus, even in the absence of normal modulatory input from cerebellar cortex. This compensatory mechanism may stand alone in the interpositus nucleus, or rely on timing-related input from extracerebellar structures, such as the hippocampus (Hoehler and Thompson, 1980; Port, Mikhail, and Patterson, 1985; Port and Patterson, 1985; but see Poulos and Thompson, 2004).

There exists a rather significant body of literature on the role of cerebellar cortex in eyeblink conditioning (see Christian and Thompson, 2003). The most frequent methodological approach has been to examine CR acquisition or retention after cerebellar cortex lesions. While this manipulation does not typically prohibit learning or block performance, a myriad of behavioral deficits have been observed, even with an optimal ISI, including severe impairments in CR acquisition (Lavond and Steinmetz, 1989; McCormick and Thompson, 1984). This is difficult to reconcile with the normal CR learning that we observed in picrotoxin-treated rabbits trained with the 250 ms ISI (Figure 3A-C). However, one possible explanation is that lesion and other chronic preparations may represent a more substantial disruption to the cerebellar network. The chronic effects of lesions often include loss of neurons in afferent and efferent structures and these losses can affect conditioning. For example, loss of cells in the inferior olive would affect US projections to the cerebellum potentially impairing acquisition. Thus, chronic preparations may have wider-spread effects on neural structure and function than intended. A feature of acute preparations, such as the drug infusion method used here, is that the effect is

temporary, and this approach allows one to physically alter neural function without compromising gross neural structure. Thus, chronic and acute preparations are fundamentally different, and, for this reason, it may be difficult to make direct comparisons between their effects on behavior. Taken together, however, data generated from the chronic and acute approaches should help delineate the role of cerebellar cortex in eyeblink conditioning.

While the literature on the subject is rather sparse, pharmacological perturbations of the cerebellar network may ultimately affect neural activity in the nucleus and produce a multitude of behavioral deficits, even with an optimal ISI. For example, various eyeblink conditioning deficits emerge when picrotoxin is infused to the interpositus nucleus during tests of CR retention with a 500 ms ISI (Attwell, Ivarsson, Millar, and Yeo, 2002; Garcia and Mauk, 1998; Medina, Garcia, and Mauk, 2001; Ohyama et al., 2006). These deficits include reductions in the CR latency, CR amplitude, and the percentage of CRs. Using a 250 ms ISI, Bao et al. (2002) reported that complete pharmacological decortication of the cerebellum – with infusions of muscimol then picrotoxin into the interpositus nucleus – blocked acquisition of CRs in naïve animals, but CR expression was retained in well-trained animals. Interestingly, CR expression in retention was critically dependent on the level of excitability in the nucleus. While the results of Bao et al (2002) may appear to contradict our findings, a resolution to the discrepancy is that cerebellar decortication produces more profound deficits than those observed during disruption of cerebellar cortico-nuclear activity. The critical difference may lie in the basal level of activity that is maintained in the interpositus nucleus.

Evidence for this idea comes from other studies that have employed acute drug injections into discrete regions of the cerebellar cortex. For example, Yeo and colleagues have found that CNQX (AMPA/kainite receptor antagonist) injections to cortical area HVI blocked both acquisition (Attwell, Rahman, and Yeo, 1999) and retention (Attwell, Rahman, Ivarsson, and Yeo, 2001) of the eyeblink CR with a 350 ms ISI. This effect makes sense in light of the fact that glutamate antagonists in the cerebellar cortex increase the spontaneous discharge of Purkinje cells (Häusser and Clark, 1997), and thus potentially increasing basal inhibition in the nucleus (Christian and Thompson, 2003). Thus, increased inhibition in the interpositus nucleus would be expected to prohibit normal acquisition and expression of CRs. Similarly, Mamounas et al (1987) reported temporary abolition of CRs following microinjections of picrotoxin to lobule HVI, which also has the effect of increasing Purkinje cell output (Häusser and Clark, 1997). When muscimol was infused into cortical lobule HVI (see Larsell, 1970), which could decrease Purkinje cell output, David Krupa (cited in Christian and Thompson, 2003) found that this manipulation did not disrupt CR acquisition with similar task parameters (i.e., 250 ms ISI). Taken together, these results may suggest that neurotransmitter systems in the cerebellum may work together to maintain a critical level of activity in the interpositus nucleus, and behavioral impairments observed in eyeblink conditioning may be related, in part, to the degree to which interpositus nucleus activity is altered.

An equally viable and nonexclusive hypothesis is that ISI-learning-related plasticity is distributed throughout the brain, with the most critical and fundamental component being the interpositus nucleus. ISIs longer than 500 ms do not promote optimal learning in delay eyeblink conditioning, and one possibility is that normal learning requires input from other brain regions. If this is the case, then it is not unreasonable to extrapolate that higher brain regions may need to contribute more for learning increasingly difficult tasks. For example, trace eyeblink conditioning, which is characterized by a brief stimulus-free period between CS offset and US onset requires both the hippocampus (Solomon, Vander Schaaf, Thompson, & Weisz 1986) and the cerebellum (Woodruff-Pak, Lavond, & Thompson, 1985), but the cerebellar cortex does not appear to be important (Woodruff-Pak, Lavond, & Thompson, 1985; Woodruff-Pak, Green, Levin, & Meisler, 2006). Because there is no temporal overlap between the CS and US, the trace conditioning procedure may be a more difficult learning task than delay.

Walker and Steinmetz (2008) recently demonstrated that ibotenic acid lesions to hippocampus were more detrimental to acquisition and performance of trace eyeblink conditioning with a long trace interval, than with short. They concluded that performance in trace conditioning was, in part, dependent upon task parameters such as the duration of the CS and the trace periods. Thus, the hippocampus may play the same important role in learning non-optimal task parameters in trace conditioning as the cerebellar cortex plays in learning non-optimal task parameters in delay conditioning. In other words, the importance of hippocampus (trace conditioning) or cerebellar cortex (delay conditioning) may increase significantly as task demands become more difficult, and perturbing these regions, or their inputs to the interpositus nucleus, may restrict the ability of the organism to learn more difficult tasks without impairing learning in more basic tasks. Indeed, Clark, McCormick, Lavond, and Thompson (1984) suggested that different memory systems may be hierarchically organized, and that one may abolish higher forms of learning, while sparing more elementary association components. It will be interesting to further elucidate roles for various brain regions in eyeblink conditioning, and especially for the cerebellar cortex in short trace conditioning and the hippocampus in long delay conditioning. We may learn much about the neural substrates for learning and interval timing in eyeblink classical conditioning.

In summary, our picrotoxin data, along with trace conditioning data from Walker and Steinmetz (2008) suggest that learning eyeblink conditioning with non-optimal task parameters may require convergence of stimulus information in the interpositus nucleus from multiple brain regions, including cerebellar cortex and hippocampus. When this modulatory input is disrupted, it appears that CR acquisition occurs, albeit with deficiencies in CR amplitude, CR timing, rate of learning, and asymptotic level of responding. The magnitude of these deficiencies is likely dependent upon a complex interaction between the severity of the neural perturbation and the difficulty of the conditioning task. Future studies in eyeblink conditioning will need to examine more closely the relationship between task parameters, neurotransmitter systems, and the importance of basal activation in the interpositus nucleus.

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Figure 1. Schematic drawings of sections through the stereotaxic plane of the rabbit cerebellum depicting the placement of cannula tips for all control rabbits (left; $n = 17$) or picrotoxin treated rabbits (right; $n = 17$). Numbers indicate anterior distance (mm) from lambda. Animals excluded from analyses not shown.

Figure 2.

Cannula placements were identified by locating a marking lesion in the interpositus nucleus, and drug diffusion was estimated with discrete infusions of cresyl violet prior to euthanasia. Brain slices stained for cell bodies and iron deposits assisted in verifying correct cannula placement (left), and unstained slices were used to estimate the magnitude of drug infusion (right – dark field photomicrograph).

Figure 3.

CR learning and timing for rabbits in the 250→750 ms ISI switch condition. A: Percent CR over the first 10 training sessions with a 250 ms ISI (All error bars: S.E.M.). Circles indicate control ($n = 9$), triangles indicate picrotoxin ($n = 9$), grey indicates paired CS-US trials, and black indicates CS-alone trials. B and C: Relative frequency of CR onset and peak for control and picrotoxin groups, respectively, over the last 4 sessions with a 250 ms ISI. Dotted lines indicate time of US presentation. D: Percent CR over 10 sessions of training with a 750 ms ISI, after the switch from a 250 ms ISI (All error bars: S.E.M.). M = muscimol infusion to interpositus nucleus on Session 21. B and C: Relative frequency of CR onset and peak for control and picrotoxin groups, respectively, over the last 4 sessions with a 750 ms ISI.

Figure 4.

CR learning and timing for rabbits in the 750→250 ms ISI switch condition. A: Percent CR over the first 10 training sessions with a 750 ms ISI (all error bars: S.E.M.; statistical differences indicated for paired CS-US trials only: * indicates $p < 0.05$, ** indicates $p < 0.01$). Circles indicate control ($n = 8$), triangles indicate picrotoxin ($n = 8$), grey indicates paired CS-US trials, and black indicates CS-alone trials. B and C: Relative frequency of CR onset and peak for control and picrotoxin groups, respectively, over the last 4 sessions with a 750 ms ISI. Dotted lines indicate time of US presentation. D: Percent CR over 10 sessions of training with a 250 ms ISI, after the switch from a 750 ms ISI (All error bars: S.E.M.). M = muscimol infusion to

interpositus nucleus on Session 21. B and C: Relative frequency of CR onset and peak for control and picrotoxin groups, respectively, over the last 4 sessions with a 250 ms ISI.

A

Last 10 Paired Trials (250 ms ISI)

B

Last 10 Paired Trials (750 ms ISI)

Figure 5.

Picrotoxin infusions were effective throughout the entire training session. Each graph represents EMG activity recorded from the *Orbicularis oculi* muscle of a single rabbit over the last 10 paired (CS-US) trials in a single eyeblink conditioning session. Upward deflections indicate eyelid closure. This graph demonstrates that, when short latency CRs are observed, they can persist throughout the entirety of a conditioning session. A: data from a rabbit first trained with a 250 ms ISI. B: data from a rabbit first trained with a 750 ms ISI.