Eyeblink Conditioning During an Interstimulus Interval Switch in Rabbits (Oryctolagus cuniculus) Using Picrotoxin to Disrupt Cerebellar Cortical Input to the Interpositus Nucleus

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The role of the cerebellar cortex in eyeblink classical conditioning remains unclear. Experimental manipulations that disrupt the normal function impair learning to various degrees, and task parameters may be important factors in determining the severity of impairment. This study examined the role of cerebellar cortex in eyeblink conditioning under conditioned stimulus–unconditioned stimulus intervals known to be optimal or nonoptimal for learning. Using infusions of picrotoxin to the interpositus nucleus of the rabbit cerebellum, the authors pharmacologically disrupted input from the cerebellar cortex while training with an interstimulus interval (ISI)-switch procedure. One group of rabbits (Oryctolagus cuniculus) was 1st trained with a 250-ms ISI (optimal) and then switched to a 750-ms ISI (nonoptimal). A 2nd group was trained in the opposite order. 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.

Keywords: cerebellum, eyeblink classical conditioning, interstimulus interval, GABA, picrotoxin

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 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 to define the stimulus and timing parameters that promote optimal learning, as defined by the fastest rate of acquisition, the greatest percentage of CRs, and the most extinction-resistant learning. One important factor is the time between CS onset and US onset—the interstimulus 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 & Gormezano, 1971; Gormezano, Kehoe, & Marshall, 1983; Schneiderman, 1966; Schneiderman & Gormezano, 1964; Smith, 1968; Smith, Coleman, & Gormezano, 1969). It can thus be argued that an ISI of 200–500 ms is optimal for rabbit eyeblink conditioning, and longer or shorter ISIs are nonoptimal.

The neural substrates for eyeblink conditioning have been extensively summarized (see Christian & Thompson, 2003). Briefly, CS and US signals converge on cells in two regions of the cerebellum: the cortex and the deep nucleus. At the level of cortex, these signals ultimately converge on 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 used 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: (a) the cerebellar cortex can be ablated or electrolytically lesioned (e.g., Lavond & Steinmetz, 1989), (b) Purkinje cells can be selectively destroyed (Chen, Bao, Lockard, Kim, & Thompson, 1996; Nolan & Freeman, 2006), or (c) compounds that alter activity can be infused into discrete regions of the cerebellum (e.g., Bao, Chen, Kim, & Thompson, 2002). Although each of these approaches has its 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, & Madden, 1987). This procedure produces a rapid and reversible block of GABA-mediated Cl\(^{-}\) flux at the GABA\(_A\) receptor complex on interpositus cells. Although this manipulation has been presented as a reversible disconnection of cerebellar cortex (e.g., Garcia & 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 is based on 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, & Bracha, 2003; Bao et al., 2002; Garcia & Mauk, 1998; Ohyama & Mauk, 2001; Ohyama, Nores, Medina, Riusech, & Mauk, 2006). These data fit well with lesion (Perrett, Ruiz, & Mauk, 1993) and electrophysiological (Green & 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 whether there is a differential role for this region in learning the

Method

Subjects

Subjects were 40 New Zealand white rabbits (Oryctolagus cuniculus; Myrtle’s Rabbitry, Thompson Station, TN) weighing a minimum of 2.0 kg before use. All subjects were housed in stainless steel cages in the Animal Care Unit at the University of Kansas, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. On arrival, rabbits acclimated to the housing milieu for at least 1 week. All rabbits were housed individually with 24-hr access to food and water and a 12-hr light–dark cycle. The research presented here was approved by the Institutional Animal Care and Use Committee 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, Melville, NY) was applied to the eyes to prevent irritation, and the rabbit’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 four-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, stain-
less 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 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. Postoperative 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 started.

**Drug Infusions**

Before 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, St. Louis, MO; 200 μmol/L solution in phosphate-buffered saline). All drug infusions were delivered through the guide cannula with a 10-μl syringe (Hamilton; Reno, NV), 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 μl, injected at a rate of 0.25 μl/15 s. After the infusion was complete, the syringe was left in place for exactly 2 min. Rabbits were trained two at a time, and the classical conditioning session started after both rabbits were infused and placed in separate sound-attenuating chambers. The maximum time between the end of drug delivery and the beginning of the conditioning session was 7 min.

At the end of the study, all rabbits underwent retention testing in which muscimol (Sigma/Aldrich, 6.0 nM) was infused to the interpositus nucleus just before a single eyelid conditioning session. Because muscimol infusions in this region are known to abolish the eyelid CR (Krupa, Thompson, & 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 min on each of the 2 days before initial training in eyelid 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 eyelid electromyogram (EMG) activity. On the 2nd 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 seven blocks of 12 trials. Each block consisted of nine paired CS–US presentations, two CS-only presentations, and one 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-s (±5 s) intertrial interval. Both a long and a short interstimulus interval (ISI) were incorporated into the design of the experiment. The short ISI (250 ms) consisted of a 350-ms CS that overlapped and coterminated with a 100-ms air puff 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→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→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) that was interfaced with a computer running Spike2 software (CED Ltd., London, England). For each session, individual trials were extracted from the continuous EMG feed beginning 500 ms before CS onset and continuing for a total of 2,000 ms. All EMG activity was amplified, rectified, and stored for subsequent offline analysis. The data were analyzed with custom software.

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 before CS onset to 25 ms after 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 1,500-ms trial on CS-only trials. Unconditioned blink responses 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 before 200 ms after CS onset. This criterion had to be altered for this study because the ISIs that we used in training were much shorter or longer than Ohyama et al.’s 500-ms ISI, and we required a window that could be used with both 250- and 750-ms ISIs. Most important, 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 percentage of 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, sessionwide averages were computed. Percentage of paired CR was computed for CS–US trials, and per-
percentage of 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 unconditioned blink response on paired trials, CR amplitude and peak latency measures were 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 µl) to the interpositus nucleus. After a minimum period of 15 min following dye injection, rabbits were euthanized with an overdose of Euthasol (4.0 cc iv), and the infusion site was marked in some rabbits by passing electrical current (500 µA 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 L 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 1 week before 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-µm 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. 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 counterstained with potassium ferrocyanide.

**Inclusion Criteria**

Rabbits were included in analyses if they met the following three criteria: (a) histology revealed a cannula mark or cresyl stain in the region of the interpositus nucleus, (b) the interpositus nucleus was not damaged, and (c) 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 rabbits 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 rabbits 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 posttraining 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, 9 control and 9 picrotoxin; 750→250, 8 control and 8 picrotoxin. 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 percentage of paired CRs, a 2 (drug treatment: control vs. picrotoxin) × 10 (training session) repeated measures analysis of variance (ANOVA) was conducted. This analysis revealed a significant main effect for training session, F(9, 144) = 67.73, p < .001, but there were no differences between the drug treatment conditions, and no interaction. The same analysis was performed on the dependent measure of percentage of CS-only CRs. Similarly, this analysis revealed a significant main effect for training session, F(9, 144) = 50.16, p < .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 > .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 > .05) between control (onset: M = 175.42, SD = 49.76; peak: M = 241.43, SD = 51.1) and picrotoxin (onset: M = 175.00, SD = 63.43; peak: M = 230.50, SD = 63.98). To graphically summarize these 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 four 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 four 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 four sessions. This format is used for all histograms that appear in this article.

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

When the ISI was switched to 750 ms, both control and picrotoxin groups exhibited an immediate decline in performance that was slowly recovered over training (Figure 3D). This initial de-
crease in performance after switching from a short to a long ISI has been demonstrated previously (Coleman & Gormezano, 1971). A mixed design ANOVA on percentage of paired CRs revealed a significant main effect for training session, $F(9, 144) = 3.443, p < .005$. Additionally, the drug treatment effect approached significance ($p = .088$); the interaction was not significant. When the same analysis was conducted using the dependent measure of percentage of CS-only CRs, a significant main effect for training session was revealed, $F(9, 144) = 5.521, p < .001$, 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 percentage of paired CRs, when the ISI is shifted from 250 to 750 ms, but this effect was not significant. This may be because of either 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 ($ps > .05$).

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: $M = 505.67$, $SD = 198.62$; peak: $M = 668.22$, $SD = 166.11$) and picrotoxin (onset: $M = 517.30$, $SD = 188.46$; peak: $M = 646.20$, $SD = 175.91$).

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, nonoptimal 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 with control rabbits, learning was profoundly impaired in picrotoxin-treated rabbits initially trained with a 750-ms ISI (Figure 4A). Using the dependent measure of percentage of paired CRs, a 2 (drug) $\times$ 10 (training session) repeated measures ANOVA was conducted. This analysis revealed significant main effects for training session, $F(9, 126) = 24.297, p < .001$, and drug, $F(1, 14) = 75.867, p < .005$, as well as a Session $\times$ Drug interaction, $F(9, 126) = 4.104, p < .001$. Independent samples $t$ tests confirmed that control outperformed picrotoxin on training Sessions 5–10, $t(14) = 2.91, 2.45, 3.95, 2.24, 3.15$, and $2.38$, respectively ($ps$ are listed in Figure 4’s caption). These analyses were repeated for the dependent measure of percentage of CS-only CRs. Significant main effects were revealed for training session, $F(9, 126) = 128.044, p < .001$, and drug

![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 (millimeters) from lambda. Rabbits excluded from analyses not shown.](image-url)
treatment, $F(1, 14) = 120.428, p < .001$, and there was a significant Session $\times$ Drug interaction, $F(9, 126) = 4.831, p < .001$. Independent samples $t$ tests indicated that control outperformed picrotoxin on training Sessions 4–10, $t(14) = 2.44, 3.13, 2.74, 4.39, 2.25, 5.07,$ and 3.23, respectively ($p$ values are listed in Figure 4’s 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 rabbits 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, $F(9, 126) = 13.983, p < .001$. Additionally, there was a significant main effect for drug, $F(1, 14) = 6.381, p < .05$, and a Session $\times$ Drug interaction, $F(9, 126) = 3.386, p < .005$. Thus, picrotoxin-treated rabbits were slower to learn the CR, and to a lower asymptotic level of performance, as compared with control rabbits. Despite this, there were no differences in CR timing between control (onset: $M = 489.79, SD = 162.39$; peak: $M = 688.26, SD = 128.13$) and picrotoxin (onset: $M = 444.64, SD = 238.04$; peak: $M = 605.90, SD = 225.50$; $p$ values > .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 was switched from 750 to 250 ms, percentage of CR performance initially declined, but control rabbits quickly compensated, whereas picrotoxin-treated rabbits required significant training before they reached performance levels comparable to those of control rabbits (Figure 4D). A $2 \times 10$ repeated measures ANOVA using the dependent measure of percentage of paired CRs revealed significant main effects for session, $F(9, 126) = 15.686, p < .001$, and drug, $F(1, 14) = 5.992, p < .05$. The Session $\times$ Drug interaction was not significant. Independent samples $t$ tests confirmed that control rabbits outperformed picrotoxin-treated rabbits on Sessions 13, 15, 16, 18, and 19, $t(14) = 2.91, 2.45, 3.95, 2.24, 3.15,$ and 2.38, respectively, $p$ values < .05. The same analysis was conducted on percentage of CS-only CRs, and significant main effects were revealed for training session, $F(9, 126) = 10.585, p < .001$, and drug treatment, $F(1, 14) = 5.796, p < .05$. Again, the Session $\times$ Drug interaction was not significant. Independent samples $t$ tests confirmed that control rabbits outperformed picrotoxin-treated rabbits on Sessions 12, 13, 15, 16, and 19, $t(14) = 2.27, 2.15, 2.36, 2.43,$ and 2.39, respectively, $p$ values < .05.

We next examined CR amplitude across Sessions 11–20. Although control and picrotoxin groups both increased CR amplitudes across training, $F(9, 126) = 2.385, p < .05$, neither the drug effect nor the Session $\times$ Drug interaction effect was significant. Additionally, there were no significant differences in measures of CR timing between control rabbits (onset: $M = 158.55, SD = 49.37$; peak: $M = 251.44, SD = 54.32$) and picrotoxin-treated rabbits (onset: $M = 176.74, SD = 51.69$; peak: $M = 244.89, SD = 67.63$), $p$ values > .05. Timing histograms for CR onset and peak with a 250-ms ISI are depicted in Figure 4E for control rabbits and Figure 4F for picrotoxin-treated rabbits.

Taken together, these results indicate that pharmacological disruption of the cerebellar cortico-nuclear connection impairs eyelink 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 those of 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 nonoptimal 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

*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 before 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. Conditioned response (CR) learning and timing for rabbits in the 250–750-ms interstimulus interval (ISI)-switch condition. A: Percentage of CR over the first 10 training sessions with a 250-ms ISI (all error bars indicate standard error of the mean). Circles indicate control rabbits (n = 9), triangles indicate picrotoxin-treated rabbits (n = 9), gray indicates paired conditioned stimulus–unconditioned stimulus (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: Percentage of CR over 10 sessions of training with a 750-ms ISI, after the switch from a 250-ms ISI (all error bars indicate standard error of the mean). M = muscimol infusion to interpositus nucleus on Session 21. E and F: 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. Conditioned response (CR) learning and timing for rabbits in the 750-→250-ms interstimulus interval (ISI)-switch condition. A: Percentage of CR over the first 10 training sessions with a 750-ms ISI (all error bars indicate standard error of the mean; statistical differences indicated for paired conditioned stimulus–unconditioned stimulus [CS–US] trials only). Circles indicate control rabbits \((n = 8)\), triangles indicate picrotoxin-treated rabbits \((n = 8)\), gray 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: Percentage of CR over 10 sessions of training with a 250-ms ISI, after the switch from a 750-ms ISI (all error bars indicate standard error of the mean). M = muscimol infusion to interpositus nucleus on Session 21. E and F: Relative frequency of CR onset and peak for control and picrotoxin groups, respectively, over the last 4 sessions with a 250-ms ISI. * \(p < .05\), ** \(p < .01\).
task, as compared with controls. These findings support our hypo-
thesis 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 & Gormezano, 1971; Gormezano et al., 1983; Gor-
mezano, Schneiderman, Deaux, & Fuentes, 1962; Schneiderman,
1966; Schneiderman, Fuentes, & Gormezano, 1962; Schneiderman &
Gormezano, 1964; Smith, 1968; Smith et al., 1969). Nai√e
control rabbits that were trained with a 250-ms ISI (Figure 3A)
learned faster and produced a higher overall percentage of CRs
compared with 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 (Figures 3D and 4D). 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 dimin-
ished 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 (Sessions 11–20) was acquired
(data not shown). Rabbis switched from the 750- to the 250-ms
ISI acquired the new CR peak more rapidly than those switched
from the 250- to the 750-ms ISI. Similar performance trends have
been reported by others who have studied the ISI switch in both
humans (Boneau, 1958; Ebel & Prokasy, 1963; McMullister, 1953;
Prokasy, Ebel, & Thompson, 1963) and rabbits (Coleman & Gor-
mezano, 1971; Leonard & Theios, 1967; Prokasy & 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 nonoptimal 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 percentage of CR (Figure
3A). Also, there were no differences on measures of CR amplitude
or latency. In contrast, significant learning impairments were ob-
served 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 control rabbits (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 this study was how
pharmacological disruption of the cerebellar cortico-nuclear con-
nection 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 percent-
ages than control rabbits. In the 250–750 ISI-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 rela-
tively reduced CR performance was because the picrotoxin-treated
rabbits never fully learned the CR before the ISI switch (Figure
4A). These results may indicate that modulatory input from cere-
bellar cortex is important for learning and adapting the CR under
changing conditions. Alternatively, cerebellar cortex may be im-
portant for new learning after previous training has occurred in
eyeblink conditioning (Garcia, Steele, & Mauk, 1999; Perrett &
Mauk, 1995; Yeo, Lobo, & 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 seemed 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 cere-
bellar 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 & Mauk, 1998; Ohyama et al., 2006), we were
surprised by the infrequency and variability with which short
latency CRs were observed in this 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 rabbits
that expressed short-latency CRs to determine whether 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 1 rabbit that was first trained with a 250-ms
ISI (Figure 5A) and 1 rabbit 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 ses-
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 & Mauk, 1998). We
used the performance of the control rabbits 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-
lateny CR criterion defined as any response that occurred 26–80
ms after CS onset, a window of time that is much narrower than in
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 et al., 1993) or disruption
of cerebellar cortical input to the interpositus nucleus (Garcia &
Mauk, 1998). In these studies, a CR has been established through
training, and removal of cerebellar cortex, or disrupting interposti-
tus activity, presumably disrupts expression of previously estab-
lished 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 before disruption of cortico-nuclear function can rescue CR timing and endow the interpositus with plasticity sufficient to execute a well-timed CR (Christian & Thompson, 2005). Similarly, although we examined the effect of cerebellar disruption over the course of acquisition, the extensive training that the rabbits 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 rabbits 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 has yet to be reached regarding the origin(s) of neural processes that regulate CR timing in eyeblink conditioning. The most parsimonious hypothesis holds that Purkinje cells regulate timing via coordinated disinhibition of the interpositus nucleus just before US onset (e.g., Mauk & Donegan, 1997). Some electrophysiological recording data from lobule HVI of cerebellar cortex do not fit this model, as Purkinje cells tend to increase their discharge with CS–US pairing (Berthier & Moore, 1986; Gould & Steinmetz, 1996; Kotani, Kawahara, & Kirino, 2003). However, Green and Steinmetz (2005) recently provided support for this hypothesis by demonstrating that Purkinje cells 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 & Freeman, 2006).

Although 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, although 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, although 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 & Thompson, 1980; Port, Mikhail, & Patterson, 1985; Port & Patterson, 1985; but see Poulos & Thompson, 2004).

There exists a rather significant body of literature on the role of cerebellar cortex in eyeblink conditioning (see Christian & Thompson, 2003). The most frequent methodological approach has been to examine CR acquisition or retention after cerebellar cortex lesions. Although this manipulation does not typically prohibit learning or block performance, a myriad behavioral deficits have been observed, even with an optimal ISI, including severe impairments in CR acquisition (Lavond & Steinmetz, 1989; McCormick & 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.

Although 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, & Yeo, 2002; Garcia &

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**Figure 5.** Picrotoxin infusions were effective throughout the entire training session. Each graph represents electromyogram activity recorded from the *Orbiculus oculi* muscle of a single rabbit over the last 10 paired (conditioned stimulus–unconditioned stimulus) trials in a single eyeblink conditioning session. Upward deflections indicate eyelid closure. This graph demonstrates that when short-latency conditioned responses are observed, they can persist throughout the entirety of a conditioning session. A: Data from a rabbit first trained with a 250-ms interstimulus interval (ISI). B: Data from a rabbit first trained with a 750-ms ISI.
Mauk, 1998; Medina, Garcia, & Mauk, 2001; Ohyama et al., 2006). These deficits include reductions in the CR latency, CR amplitude, and percentage of CRs. Using a 250-ms ISI, Bao et al. (2002) reported that complete pharmacological decortication of the cerebellum—with infusions of muscimol and 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. Although Bao et al.’s results 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 used acute drug injections into discrete regions of the cerebellar cortex. For example, Yeo and colleagues have found that CNQX (AMPA/kainate receptor antagonist) injections to cortical area HVI blocked both acquisition (Attwell, Rahman, & Yeo, 1999) and retention (Attwell, Rahman, Ivarsson, & 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 (Haüsser and Clark, 1997) and thus potentially increase basal inhibition in the nucleus (Christian & 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 into lobule HVI, which also has the effect of increasing Purkinje cell output (Haüsser & Clark, 1997). When muscimol was infused into cortical lobule HVI (see Larsell, 1970), which could decrease Purkinje cell output, David Krupa (1993; cited in Christian & 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 in 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, Green, Levin, & Meisler, 2006; Woodruff-Pak et al., 1985). 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 a short trace interval. They concluded that performance in trace conditioning was, in part, dependent on task parameters such as the duration of the CS and the trace periods. Thus, the hippocampus may play the same important role in learning nonoptimal task parameters in trace conditioning as the cerebellar cortex plays in learning nonoptimal 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 nonoptimal 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 on 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.

References


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