Published in final edited form as:

Behav Brain Res. 2012 January 1; 226(1): 189–196. doi:10.1016/j.bbr.2011.09.010.

A trigeminal conditioned stimulus yields fast acquisition of cerebellum-dependent conditioned eyeblinks

Andrew J. Carrel, Svitlana Zbarska, Gary D. Zenitsky, and Vlastislav Bracha*

Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

Abstract

Classical conditioning of the eyeblink response in the rabbit is a form of motor learning whereby the animal learns to respond to an initially irrelevant conditioned stimulus (CS). It is thought that acquired conditioned responses (CRs) are adaptive because they protect the eye in anticipation of potentially harmful events. This protective mechanism is surprisingly inefficient because the acquisition of CRs requires extensive training – a condition which is unlikely to occur in nature. We hypothesized that the rate of conditioning in rabbits could depend on CS modality and that stimulating mystacial vibrissae as the CS could produce CR acquisition faster than the traditional auditory or visual stimulation. We tested this hypothesis by conditioning naïve rabbits in the delay paradigm using a weak air-puff CS (vCS) directed to the ipsilateral mystacial vibrissae. We found that the trigeminal vCS yields significantly faster CR acquisition. We next examined if vCS-evoked CRs are dependent on the intermediate cerebellum in the same fashion as CRs evoked by the traditional auditory CS. We found that vibrissal CRs could be abolished by inactivating the cerebellar interposed nuclei (IN) with muscimol. In addition, injections of picrotoxin in the IN shortened the onset latency of vibrissal CRs. These findings suggest that the tone and vCS-evoked CRs share similar cerebellar dependency.

Keywords

classical conditioning; cerebellum; rabbit; mystacial vibrissae; interposed nuclei

1. Introduction

Classical conditioning of the eyeblink reflex in rabbits has proven to be a successful model for the study of associative and motor learning. This model has many benefits, including precise control of experimental variables and an extensive knowledge of circuitry controlling the acquisition and expression of conditioned responses (CRs) [1–6].

During eyeblink conditioning, an initially irrelevant conditioned stimulus (CS, e.g., an audible tone) is paired with an aversive, eyeblink reflex-evoking unconditioned stimulus (US, e.g., airpuff to the cornea). After repeated paired presentations of the two stimuli, subjects acquire conditioned responses (CRs), which are expressed in anticipation of the upcoming US. CRs are considered to be adaptive responses that protect the eye from

© 2011 Elsevier B.V. All rights reserved

*Corresponding author. Tel.: +1 515 294 6278; fax: +1 515 294 2315. vbracha@iastate.edu (V. Bracha)..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

potentially harmful events. Consistent with this notion, CRs are adaptively timed with the response peak occurring close to the onset of the US [7–10]. Taking into account the protective function of CRs, their acquisition in rabbits is surprisingly slow. The most commonly used CS modalities (auditory and visual) generally produce asymptotic learning curves after four to six training days [11–18]. Such slow learning would likely have limited practical significance under natural conditions. The relatively slow rate of eyeblink conditioning has implications not only for a rabbit's fitness, but also for experimenters. A faster learning rate would be highly desirable, especially in studies designed to interfere with learning by using repeated drug microinjections into specific parts of eyeblink circuits. Since failure of a single microinjection would negatively affect experiments requiring repetitive treatments, shortening the number of training sessions required for CR acquisition would significantly improve the experimental design.

For rabbit eyeblink conditioning, domesticated breeds (New Zealand White, Dutch-belted) of the European Rabbit (*Oryctolagus cuniculus*) serve as the research model. We hypothesized that slow CR acquisition in rabbits could be accelerated with a different CS modality. Under natural conditions, the eye of the rabbit likely encounters harmful objects during locomotion through a complex environment. European Rabbits are crepuscular animals native to open forest scrub habitats [19], and therefore, approaching objects should be reliably signaled by vision. Indeed, a number of mammalian species (e.g. humans, horses, dogs and cats) display robust and naturally learned, visual menace eyeblinks. However, rabbits don't develop visual menace-triggered blinks, and their rate of CR acquisition to arbitrary visual stimuli is relatively slow [13], similar to the traditional auditory CS. Another potentially effective “natural” CS could be derived from the vibrissal somatosensory input. Rabbits are endowed with an elaborate vibrissal system and the long and far-projecting whiskers represent an excellent early-warning system for detecting objects approaching their eyes. The vibrissal system in rabbits is very sensitive and its stimulation can elicit reflexive alpha eyeblink responses [20]. Importantly, previous studies have demonstrated that stimulation of mystacial vibrissae could be used in eyeblink conditioning [20–23].

In this study, we examined whether vibrissal stimulation could be optimized to yield accelerated CR acquisition. We found that a simple vibrissal airpuff CS (vCS) yields robust and fast CR acquisition both in naïve and previously trained animals. In the second part of this study, we tested whether vCS-evoked CRs depend on cerebellum-related circuits. Here we report that, similar to traditional auditory CS-triggered CRs, vCS-evoked CRs are abolished by inactivating the cerebellar interposed nuclei (IN) with muscimol and their latencies are shortened by elevating IN activity with picrotoxin.

2. Material and methods

2.1. Subjects

Experiments were performed on 16 male New Zealand White Rabbits (Harlan; Indianapolis, IN) weighing 2.5–3.0 kg (3–4 months old at time of surgery). Rabbits were housed individually on a 12-hour light/dark cycle and provided food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health's “Principles of Laboratory Animal Care” (publication No. 86–23, revised 1985), the American Physiological Society's “Guiding Principles in the Care and Use of Animals,” and the protocol approved by Iowa State University's Animal Care and Use Committee.

2.2. Surgery

Surgery was performed using aseptic techniques on naïve rabbits anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg) and acepromazine (1.5 mg/kg). The

head was secured in a stereotaxic apparatus with lambda positioned 1.5 mm ventral to bregma. After exposing the skull and affixing three stainless steel anchor screws, a stainless steel injection guide tube (27-gauge thin-wall) was stereotaxically implanted 0.5 mm dorsal to the expected location of the left anterior IN ($(0.69x+4.8)-x$) rostral from lambda, x being the horizontal distance between bregma and lambda in mm; 5.3 mm lateral and 13.5 mm ventral to lambda). A 33-gauge stainless steel stylet was inserted into the guide tube between experiments to protect its patency. The guide tube, anchor screws, and a small Delrin (polyoxymethylene thermoplastic) block machined to accommodate an airpuff delivery nozzle and eyeblink sensor were secured in place with dental acrylic. All animals were treated with antibiotics for 5 days while recovering from surgery.

2.3. Training Procedures

Prior to surgery, rabbits were adapted to a restraint box (Plas-Labs Inc., Lansing, MI) inside a sound attenuating chamber for 30 min on two consecutive days. Following surgery, rabbits were given one additional day of box adaptation. Rabbits were assigned to one of three groups: the naïve, pseudoconditioning, or 2nd CS group. Animals in the naïve group were trained to a 450-ms weak airpuff CS directed at rows B and C of the left mystacial vibrissae (vCS). The strength of this CS was individually calibrated for each rabbit to an intensity that was just below the threshold for eliciting an alpha response. Initially, the CS intensity was calibrated using a Clippard MAR-1–6 pressure regulator (Clippard Instrument Laboratory, Cincinnati, OH). Later, we switched to another pressure regulator (Bellofram Corporation, Precision Pressure Regulator Type 10LR, Newell, WV) that could more reliably produce the lower CS pressures required in several subjects. Rabbits in the pseudoconditioning group were given an equal number of trials in each session (100 trials of each stimulus) of explicitly unpaired presentations of the vCS and US in a randomized order. Following pseudoconditioning, rabbits were given a retention test consisting of 40 CS-alone trials. The purpose of the retention test was to obtain a set of measurements which would be compatible with future experiments examining effects of drugs on vCS-evoked CR acquisition. Animals in the 2nd CS group were initially trained to a tone CS. The tone CS was an 85-db, 450-ms, 1-kHz tone, superimposed on a continuous 70-dB white noise background. After acquiring tone-evoked CRs, they were trained to the vCS. All experimental sessions were conducted using the standard delay classical conditioning paradigm until rabbits reached at least 90% CRs for 3 consecutive days. Both CS types co-terminated with a 36-psi (at the source), 100-ms air-puff unconditioned stimulus (US) directed to the left eye. The inter-stimulus interval was 350 ms and the intertrial interval varied pseudorandomly between 15 to 25 sec. Each training session consisted of 100 paired trials per day. During the sessions when the vCS was delivered, a discriminatory airpuff with an intensity of 6 psi was triggered every 7 seconds throughout the duration of the session. This discriminatory airpuff was used to ensure that the rabbit was responding to the vCS and not responding to the sounds of the solenoid or the air exiting the air nozzle.

2.4. Injection procedures

Microinjections targeted the IN ipsilateral to the vCS and US. They were delivered through a 33-gauge stainless steel injection needle that was connected to a 10- μ l Hamilton syringe (Hamilton Company, Reno, NV) via transparent Tygon tubing. The Tygon tubing was first filled with ultra-purified water, a small air bubble was pulled into the injection needle, and then the drug was drawn into the tubing. The bubble was used to monitor the volume of drug being injected relative to gradation marks located on the tubing. The injection needle was inserted in the intracranial guide tube before starting the experiment. Forty pre-injection trials were delivered in order to ensure there were no effects on CR incidence due to needle insertion. The injections were performed manually at a rate of 0.25 μ l/min and after their completion, training continued for an additional 100 trials. If rabbits had less than 85% CRs

in the pre-injection period, drug injections were not delivered. Muscimol (Enzo Life Sciences, Switzerland) and picrotoxin (PTX, Sigma-Aldrich, USA) were dissolved in artificial cerebrospinal fluid (aCSF) and the pH of the solution was adjusted to 7.4 ± 0.1 . Injections of picrotoxin (0.83–2.5 nmol) and aCSF were administered at locations where previous 0.5 μ l (1.75 nmol) injections of muscimol completely abolished conditioned eyeblinks. All rabbits were first injected with muscimol to determine the optimal depth for IN injections. Once the optimal site was determined, picrotoxin volume/concentration was individually titrated for each rabbit in order to produce the dose-dependent short latency responses as described in Parker et al. [24]. Following the final PTX experiment, each rabbit was injected with a control injection of an equal volume of aCSF. This order of injections was followed for each rabbit in order to show the effects of IN inactivation, disinhibition, and vehicle on CRs.

2.5. Data recording and analysis

Movements of the eyelids were recorded by a wide field-of-view infrared sensor that measures the amount of infrared light reflected from the eye and peri-orbital region [25]. The output of the sensor was amplified, digitized (25 kHz, 12-bit A/D converter), and stored on a custom-made data acquisition system. An infrared video system installed in the experiment chamber was used to monitor behavior of the rabbits and the positioning of the infrared sensor.

Eyeblink data were acquired starting 250 ms before the onset of the CS and continued 800 ms beyond the US onset for a total of 1400 ms in each trial. Eyeblink responses were analyzed offline for the presence of alpha responses, CRs, and URs, each defined by their onset latencies. An alpha response was classified as any response up to 40 ms after CS onset, a CR as an eyelid movement within 41 ms after CS onset up to the US onset, and a UR as any response to the US. Trials containing spontaneous eyeblinks before CS onset were removed from further analyses. The threshold for eyelid movements was set to 5 standard deviations of the baseline signal noise, which corresponded to approximately a 0.15 mm decrease in eyelid aperture. Mean CR incidence and latency were calculated for consecutive blocks of 10 trials. The data were pooled from individual rabbits and statistically analyzed using repeated measures ANOVA followed by individual and simultaneous contrast analyses. All group data were reported as mean $\pm$ standard error of the mean, with an alpha level = 0.05 for declaring significance.

2.6. Histology

After all experiments were concluded, rabbits were deeply anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (12 mg/kg), and acepromazine (3 mg/kg). Injection sites were marked by injecting 0.75–1.0 μ l of tissue marking dye. Rabbits were transcardially perfused with 1 L of phosphate buffered saline followed by 1 L of tissue fixative (10% neutral buffered formalin). Excised brains were stored in a solution of 30% sucrose and 10% formalin. Brains were sectioned coronally in 50- μ m slices on a freezing microtome. Subsequently, sections were mounted on gelatin-coated slides, dried, and stained with luxol blue and neutral red. Injection site locations were identified using bright light microscopy and plotted on standardized sections of the rabbit cerebellum.

3. Results

3.1. General observations

The vCS was a very effective conditioned stimulus and all rabbits acquired vCS-evoked CRs quickly. One of the features of the vCS is that it can evoke URs when sufficiently strong. The intensity of the vCS was individually adjusted for each rabbit at the onset of training to

a sub-threshold level for UR expression. The initial sub-threshold vCS intensity varied from 2–20 psi between rabbits. Due to conditioning-related reflex facilitation [26], this threshold was not constant. As the training progressed, in some animals the initial vCS intensity began to evoke small URs. The transition of these URs into developing CRs formed a two-peak response, reminiscent of the multi-component CRs described in mice [27]. If the vCS UR was observed, the intensity of vCS was further decreased to the sub-threshold level. This procedure ensured that all rabbits developed long-latency, single component CRs to the vCS. The vCS-evoked CRs were unilateral in all animals. Absence of responses to discriminative masking hisses of the air confirmed that acquired CRs were specific to the somatosensory component of the CS.

A total of 15 rabbits were included in the injection experiments. After histological analysis, three rabbits were excluded from the results due to misplaced implants. The histological reconstruction of IN injection sites for the rabbits included in the analyses of pharmacological effects revealed that all rabbits had their injection cannulae either directly in or proximal to the IN (Fig. 1).

3.2. The rate of CR acquisition to the vCS and to the tone CS

Rabbits trained with the vCS acquired CRs in a quick and steady fashion. Figure 2 shows an individual example from each of the first three days of training with paired vCS-US trials from one of the rabbits in the naïve group. Over the three days of training, rabbits develop substantial, properly-timed CRs whose amplitude peaked at the onset of the US. At the group level, the rate of acquisition in naïve rabbits ($n = 4$) trained with the vCS was considerably faster than naïve rabbits trained to a tone CS ($F_{3,18} = 3.668$, $p = 0.03$) (Fig. 3A). Unlike the tone group, rabbits in the naïve vCS group exhibited a fair number of CRs on Day 1 of training and during Day 2 of training already had a mean CR incidence of $79.2 \pm 17.8\%$. On Day 3, rabbits reached an asymptotic level of conditioned responding, producing a mean CR incidence of $90.0 \pm 5.8\%$. By comparison, rabbits assigned to the naïve tone group ($n = 4$) showed a mean CR incidence of $5.6 \pm 4.3\%$ on Day 2 and $47.3 \pm 11.4\%$ on Day 3 while not reaching an asymptotic level of responding until Day 6, exhibiting a mean CR incidence of $90.1 \pm 0.96\%$. These data show that a weak airpuff to the ipsilateral mystacial vibrissae produces faster CR acquisition than the more commonly used auditory CS modality.

3.3. vCS as a second CS

In addition to testing the rate of CR acquisition to the vCS in naïve rabbits, we also tested the rate of acquisition in rabbits ($n = 4$) using the vCS as a second CS for a group of rabbits that had already been well trained to a tone CS. Using the vCS as a second CS resulted in acquisition that was numerically faster than vCS in naïve rabbits. The mean CR incidence on Day 2 of 2nd conditioning was already $91.4 \pm 4.7\%$ (Fig. 3B).

3.4. Pseudoconditioning

To test whether vCS-evoked eyeblinks are a product of an associative process, a group of rabbits ($n = 4$) was trained with explicitly unpaired vibrissal conditioned stimuli and corneal airpuff unconditioned stimuli. During the three days of pseudoconditioning (Fig. 4), the rabbits did not exhibit any signs of acquiring conditioned responses (maximum CR incidence, $2.57 \pm 1.95\%$, Day 3) relative to the rate of spontaneous eyeblinks. However, pseudo-conditioned rabbits did exhibit normal US-evoked eyeblinks. Following pseudoconditioning, a retention test (40 CS-alone trials) was presented to test for any CRs acquired during the three days of pseudoconditioning. The mean CR incidence for the retention test was $0.63 \pm 0.62\%$. Following pseudoconditioning, the rabbits were trained with paired vCS-US trials. By means of stimulus pre-exposure, pseudoconditioning may

have delayed the onset of learning typically seen on Day 1 during vCS conditioning. Otherwise, rabbits rapidly acquired CRs to the vCS, reaching a mean CR incidence of $95.5 \pm 0.5\%$ by Day 3 of training (Fig. 4). The pseudoconditioning results showed that the vCS-evoked CRs are learned, associative responses.

3.5. Cerebellar dependency of vCS-evoked CRs

The previous results showed that vibrissal stimulation produces faster acquisition than a tone CS and that CRs exhibited by the vCS are learned, associative responses. To further characterize these responses, we tested whether they are cerebellum-dependent. Specifically, we examined whether inactivating or activating deep cerebellar nuclei would affect vCS-evoked CRs in a way similar to previously reported effects of these manipulations on tone-evoked CRs [24, 28–30].

The first pharmacological test was an injection of the GABA agonist muscimol in the IN. Figure 5A shows an individual example of a muscimol injection in the IN. During the pre-injection trials, the rabbit exhibited adaptively timed, large amplitude vCS-evoked CRs. The vCS-evoked CRs were abolished following the muscimol injection and this effect was maintained for the rest of the experimental session. At the group level, when compared to vehicle injections, muscimol (1.75 nmol) abolished CRs in the post-injection trials ($F_{1, 11} = 957.03$, $p < 0.0001$) (Fig. 6A). The onset of behavioral effects from the injection was rapid as the mean CR incidence was significantly reduced in the initial post-injection block of 10 trials compared to pre-injection CR incidence (contrast- $t_{11} = 3.35$, $p = 0.006$) (Fig. 6A). Muscimol reduced the mean CR incidence from $93.3 \pm 2.25\%$ during the last pre-injection block of trials to $0.83 \pm 0.83\%$ by the fourth post injection block and maintained its effect for the duration of the experiment. In addition to effects on eyeblinks, muscimol injections also visibly decreased UR amplitude and increased the aperture of the palpebral fissure.

After establishing an effective injection site in the IN with muscimol and testing its effects on CRs, an injection of the GABA antagonist PTX was administered at the same location. Injections of PTX (0.83–2.5 nmol) produced several distinct effects on CRs. First of all, the selected amount of PTX visibly shortened CR latencies (Fig. 5B). This effect was significant at the group level when compared to pre-injection trials ($F_{1, 11} = 37.24$, $p < 0.0001$). The mean CR latency was reduced from 171.4 ± 12.6 ms in the last pre-injection block of trials to 72.2 ± 14.4 ms by the fourth and to 63.3 ± 5.5 ms by the sixth post-injection block of trials (Fig. 6B). PTX injections had no effect on CR incidence (Figs. 5B and 6A).

As previously reported by Parker et al [24], CR latency-shortening doses of PTX increased overall excitability of rabbits, increased tonic eyelid closure, and instead of producing isolated eyeblink movements, rabbits frequently reacted to the CS and US with a whole-head withdrawal away from the air nozzle.

The pharmacological data presented here provide clear evidence that CRs generated by the vCS modality are dependent on the intermediate cerebellum and that, similar to auditory CS-evoked CRs, the incidence and latency of the CRs can be manipulated by up- or down-regulating neuronal activity in the IN.

4. Discussion

The objective of the present study was to determine whether a vibrissal CS could produce fast CR acquisition. The results show that using a weak airpuff CS to the ipsilateral mystacial vibrissae does generate faster CR acquisition than the most commonly used auditory stimulation. In addition, we found that vCS-triggered CRs and auditory-triggered CRs share similar cerebellar dependency.

Using an auditory or visual CS, rabbits typically acquire an asymptotic level of CRs after about four to six days [11–18]. In agreement with previous studies [20–22], we confirmed that stimulation of the vibrissae could be used as a CS in eyeblink conditioning. Here, we show that an airpuff that is just sub-threshold in respect to evoking unconditioned eyeblinks is a very effective CS that supports fast acquisition of CRs. A naïve-trained group of rabbits showed well-timed, adaptive blinks while reaching the asymptotic level of CR incidence in three days and in fact, most of the animals exhibited a near-asymptotic level of performance by the second day of training (Figs. 2B and 3A). In addition, rabbits trained with the vCS as a second CS reached a CR incidence of greater than 90% in just two days. This accelerated conditioning in animals trained with the vCS as a second CS is consistent with the previously reported effects of extended box adaptation [31] and cross-modal “savings” [32–34].

Along with an increase in CR incidence over the course of training, the vCS-evoked CRs showed an increase in amplitude and became more time-locked with the peak of the response coinciding with the onset of the US (Fig. 2). In addition, their topography was similar to tone-evoked CRs. The relatively long latency (Fig. 6B) and adaptive topography of vCS-evoked CRs indicated that they are an outcome of associative learning. However, assessing the potential involvement of non-associative learning required an explicit test. The possibility that the acquisition of responses to the vCS was a result of some non-associative aspect of learning (e.g., sensitization) was eliminated by the pseudoconditioning experiment. The absence of CRs during the three days of the pseudoconditioning control experiment, along with the pseudoconditioning group exhibiting a similar learning curve to the naïve group following the pseudoconditioning training, provides evidence that vCS-evoked CRs are an outcome of associative learning.

Faster acquisition of CRs to the vCS does not necessarily prove that the vCS is the most effective sensory modality for eyeblink conditioning in rabbits. For example, it is known that the rate of conditioning also depends on CS intensity. Scavio and Gormezano [35] reported that rabbits acquire CRs faster with a loud 86-dB tone CS than with a soft 65-dB CS. Thus, the loud 85-dB auditory CS is now the most commonly used CS intensity. Even though an 85-dB tone is fairly loud, one can't exclude that even louder auditory CS intensities would produce a CR acquisition rate that is comparable to vCS training. However, it is difficult to conceive a common situation in the rabbit's environment where auditory stimuli louder than 85 dB would reliably predict localized danger to the eye. In contrast to this, the vCS intensity used in this study seems to be well within the range of frequently encountered intensities of the rabbit's natural facial stimulation. In fact, several subjects were vCS-trained with 2 psi. This source pressure in our delivery system produces a very weak airpuff and its impact on the vibrissal pad produced barely visible hair deflections. Yet, it yielded fast learning. Since commonly encountered intensities of the vCS yield CR acquisition faster than infrequently occurring strong auditory CS intensities, it appears that the eyeblink conditioning system in the rabbit is comparably better tuned to the facial cutaneous input.

One could argue that even vCS conditioning is too slow to provide rabbits with effective eye protection. After all, most of our rabbits generated a high CR incidence on the second day of conditioning, and by that time they had been exposed to more than 100 conditioning trials! Requiring this many trials to acquire protective responses seems inefficient, especially when compared to other forms of biologically-tuned forms of learning, such as conditioned taste aversion, where a one-trial exposure to the CS and US is sufficient for robust learning [36]. Before drawing conclusions from this comparison, it is important to realize that not all training parameters in typical laboratory eyeblink conditioning sessions are designed to maximize the rate of learning. For example, it is known that fewer than a dozen trials can

yield the first CR when rabbits are trained in 1-trial/day sessions (for review see [37]). It appears that the number of training sessions rather than the cumulative number of trials better describes the rate of eyeblink conditioning. From this perspective, even eyeblink conditioning to a tone CS could be viewed as providing rabbits with some adaptive advantage. Why then do typical eyeblink conditioning sessions run 100, seemingly redundant trials? This “sub-optimal” arrangement is deliberate. Yes, it inflates the cumulative number of trials required for learning, but this cost is outweighed by the numerous advantages offered by repeated sampling. Aside from the possible need to introduce probe-CS or US-alone trials, multiple trials control for potentially large per-trial differences between individual animals, including spontaneous responses, chance events (e.g., postural movements), and the onset and duration of drug effects. Without repeated trials, spurious sampling errors would contaminate the measurements of intervention effects on CR incidence, eyeblink kinematics, and neuronal activity in neurophysiological experiments. The only other option the investigator would be left with is to add many more animals to the experiment.

Why do rabbits learn to respond to the vibrissal CS faster than to auditory or visual conditioned stimuli? It is likely that during evolution neuronal circuits controlling eyeblink conditioning were honed by species-specific selective pressures. European rabbits are prey animals and their visual and auditory systems play a pre-eminent role in teleceptive predator detection. As a consequence, arbitrary visual and auditory stimuli evoke a strong orienting response, part of which is the opposite of an eyeblink–eyelid opening. This competing response has to be habituated first before the animal learns to blink and this process could delay CR acquisition. We presume that the importance of teleception in predator detection is also reflected in the fact that rabbits don't display visual menace eyeblink responses. The menace eyeblinks are naturally learned conditioned eyeblinks [38] and if evoked by distant objects, they could interfere with continuity of visual input that is required for effective predator avoidance. Thus, neuronal circuits that control visual menace eyeblinks are present, but not optimized in this species. On the other hand, vibrissae and guard hairs are reliable predictors of nearby and imminently harmful objects, especially during locomotion when the head and eye frequently approach stationary objects, such as vegetation or walls of a rabbit's burrow. In this context, it is not surprising that mammals respond to strong stimulation of the extra-ocular trigeminal region with unconditioned eyeblinks and that eyeblink conditioning circuits in rabbits are well primed for vibrissal sensory input.

Because of the fast rate of CR acquisition, vCS conditioning could be well suited for studies examining effects of drug microinjections on CR acquisition. Typically, experiments of this kind require sequential days of drug injections before each daily training session [16, 17, 30]. Shortening the CR acquisition period would reduce the number of injections and thus the likelihood of injection failure invalidating an experiment. The potential of the vCS for eyeblink conditioning studies is further enhanced by our demonstration that vCS-evoked CRs are cerebellum-dependent. The cerebellar dependency is a hallmark feature of tone and light-evoked CRs in the rabbit. It has been repeatedly shown that inactivating the IN with either lidocaine or muscimol abolishes previously acquired tone or light CS-evoked CRs [28, 39–43]. Similar to these findings, vCS evoked CRs are abolished when the IN is infused with muscimol (Figs. 5A and 6A). Muscimol is a GABA-A agonist which hyperpolarizes neurons, blocking their task-related responses and also their spontaneous activity [28]. On the other hand, picrotoxin is known to reduce task-related modulation of IN neuronal activity and to dramatically increase their spontaneous firing rate [28]. This effect translates into a dose-dependent effect on the expression of tone CS-evoked CRs. At lower doses, IN PTX shortens CR latencies [24, 44] and at higher doses it abolishes CRs [24]. Similar to the auditory CS, the vCS-evoked CRs have shorter latencies following PTX injections (Figs. 5B and 6B). Surprisingly, we were not able to abolish vCS-evoked responses with higher doses

of PTX (data not shown). The most likely explanation for this finding is that high PTX dose-associated responses were not CRs, but rather facilitated unconditioned responses to the vCS. IN PTX is known to facilitate trigeminal unconditioned eyeblinks [24] and it is plausible that the originally sub-threshold intensity of the vCS became supra-threshold following the drug injection. Similar facilitation of tone CS-evoked URs by PTX has been previously reported in naïve rabbits [45].

It is clear that vCS-evoked CRs share similar cerebellar dependency with tone-evoked CRs. Cerebellar involvement in the control of vCS CRs offers a possible explanation to the enhanced speed of learning. Most of the present concepts assume that a significant portion of plastic changes responsible for CR acquisition occur in the cerebellum [18, 30, 46, 47]. Cerebellar learning requires sensory information which is brought via cerebellar mossy fibers from the pontine nuclei and via climbing fibers from the inferior olive. Auditory as well as trigeminal CS information reaches the cerebellum via projections to the pontine nuclei [48–51]. In addition to pontine projections, trigeminal sensory nuclei, including their parts that receive vibrissal information [52, 53], are known to project directly to the cerebellar cortex [51]. It is possible that these direct projections, together with the pontine input, could facilitate CR acquisition. In addition, information processing in the cerebral cortex could also contribute significantly to vCS conditioning [23].

Overall, the presented data demonstrate that the vCS yields fast acquisition of associative, classically conditioned responses in rabbits and that these CRs share similar cerebellar dependency with tone CS-evoked CRs. These features make the vCS well suited for future investigations of this form of motor learning.

Acknowledgments

This work was supported by National Institutes of Health grant R01 NS036210.

References

- [1]. Bracha V, Bloedel JR. The multiple-pathway model of circuits subserving the classical conditioning of withdrawal reflexes. *Acquisition of Motor Behavior in Vertebrates*. 1996;175–204.
- [2]. Bracha V, Zbarska S, Parker K, Carrel A, Zenitsky G, Bloedel JR. The cerebellum and eye-blink conditioning: learning versus network performance hypotheses. *Neuroscience*. 2009; 162:787–96. [PubMed: 19162131]
- [3]. De Zeeuw CI, Yeo CH. Time and tide in cerebellar memory formation. *Curr Opin Neurobiol*. 2005; 15:667–74. [PubMed: 16271462]
- [4]. Christian KM, Thompson RF. Neural substrates of eyeblink conditioning: acquisition and retention. *Learn Mem*. 2003; 10:427–55. [PubMed: 14657256]
- [5]. Mauk MD, Donegan NH. A model of Pavlovian eyelid conditioning based on the synaptic organization of the cerebellum. *Learn Mem*. 1997; 4:130–58. [PubMed: 10456059]
- [6]. Steinmetz JE. Brain substrates of classical eyeblink conditioning: a highly localized but also distributed system. *Behav Brain Res*. 2000; 110:13–24. [PubMed: 10802300]
- [7]. Smith MC. CS-US interval and US intensity in classical conditioning of the rabbit's nictitating membrane response. *Journal of comparative and physiological psychology*. 1968; 66:679–87. [PubMed: 5721496]
- [8]. Coleman SR, Gormezano I. Classical conditioning of the rabbit's (*Oryctolagus cuniculus*) nictitating membrane response under symmetrical CS-US interval shifts. *Journal of comparative and physiological psychology*. 1971; 77:447–55. [PubMed: 5118253]
- [9]. Lepora NF, Porrill J, Yeo CH, Dean P. Sensory prediction or motor control? Application of marr-albus type models of cerebellar function to classical conditioning. *Front Comput Neurosci*. 2010; 4:140. [PubMed: 21031161]

- [10]. Jirenhed DA, Hesslow G. Learning Stimulus Intervals-Adaptive Timing of Conditioned Purkinje Cell Responses. *Cerebellum*. 2011
- [11]. Clark RE, Zhang AA, Lavond DG. Reversible lesions of the cerebellar interpositus nucleus during acquisition and retention of a classically conditioned behavior. *Behav Neurosci*. 1992; 106:879–88. [PubMed: 1335267]
- [12]. Krupa DJ, Thompson RF. Reversible inactivation of the cerebellar interpositus nucleus completely prevents acquisition of the classically conditioned eye-blink response. *Learn Mem*. 1997; 3:545–56. [PubMed: 10456116]
- [13]. Welsh JP, Harvey JA. Acute inactivation of the inferior olive blocks associative learning. *Eur J Neurosci*. 1998; 10:3321–32. [PubMed: 9824445]
- [14]. Gruart A, Morcuende S, Martinez S, Delgado-Garcia JM. Involvement of cerebral cortical structures in the classical conditioning of eyelid responses in rabbits. *Neuroscience*. 2000; 100:719–30. [PubMed: 11036206]
- [15]. Chen G, Steinmetz JE. Microinfusion of protein kinase inhibitor H7 into the cerebellum impairs the acquisition but not the retention of classical eyeblink conditioning in rabbits. *Brain Res*. 2000; 856:193–201. [PubMed: 10677626]
- [16]. Attwell PJ, Rahman S, Yeo CH. Acquisition of eyeblink conditioning is critically dependent on normal function in cerebellar cortical lobule HVI. *J Neurosci*. 2001; 21:5715–22. [PubMed: 11466443]
- [17]. Nilaweera WU, Zenitsky GD, Bracha V. Inactivation of cerebellar output axons impairs acquisition of conditioned eyeblinks. *Brain Res*. 2006; 1122:143–53. [PubMed: 17067561]
- [18]. Kellett DO, Fukunaga I, Chen-Kubota E, Dean P, Yeo CH. Memory consolidation in the cerebellar cortex. *PLoS One*. 2010; 5:e11737. [PubMed: 20686596]
- [19]. Smith, AT.; Boyer, AF. *Oryctolagus cuniculus*. 2008. IUCN 2011. IUCN Red List of Threatened Species. Version 2011. <http://www.iucnredlist.org/apps/redlist/details/41291/0>. Downloaded 30 May 2011
- [20]. Schreurs BG, Jha B, Gormezano I. Classical-Conditioning of the Rabbits Nictitating-Membrane Response to a Piezoceramic Vibrotactile Cs. *Behav Res Meth Instr*. 1986; 18:359–62.
- [21]. Das S, Weiss C, Disterhoft JF. Eyeblink conditioning in the rabbit (*Oryctolagus cuniculus*) with stimulation of the mystacial vibrissae as a conditioned stimulus. *Behav Neurosci*. 2001; 115:731–6. [PubMed: 11439462]
- [22]. Troncoso J, Munera A, Delgado-Garcia JM. Classical conditioning of eyelid and mystacial vibrissae responses in conscious mice. *Learn Mem*. 2004; 11:724–6. [PubMed: 15537734]
- [23]. Leal-Campanario R, Delgado-Garcia JM, Gruart A. Microstimulation of the somatosensory cortex can substitute for vibrissa stimulation during Pavlovian conditioning. *Proc Natl Acad Sci U S A*. 2006; 103:10052–7. [PubMed: 16782811]
- [24]. Parker KL, Zbarska S, Carrel AJ, Bracha V. Blocking GABAA neurotransmission in the interposed nuclei: effects on conditioned and unconditioned eyeblinks. *Brain Res*. 2009; 1292:25–37. [PubMed: 19635470]
- [25]. Ryan SB, Detweiler KL, Holland KH, Hord MA, Bracha V. A long-range, wide field-of-view infrared eyeblink detector. *J Neurosci Methods*. 2006; 152:74–82. [PubMed: 16257057]
- [26]. Wikgren J, Ruusuvirta T, Korhonen T. Reflex facilitation during eyeblink conditioning and subsequent interpositus nucleus inactivation in the rabbit (*Oryctolagus cuniculus*). *Behav Neurosci*. 2002; 116:1052–8. [PubMed: 12492303]
- [27]. Boele HJ, Koekkoek SK, De Zeeuw CI. Cerebellar and extracerebellar involvement in mouse eyeblink conditioning: the ACDC model. *Front Cell Neurosci*. 2010; 3:19. [PubMed: 20126519]
- [28]. Aksenov D, Serdyukova N, Irwin K, Bracha V. GABA neurotransmission in the cerebellar interposed nuclei: involvement in classically conditioned eyeblinks and neuronal activity. *J Neurophysiol*. 2004; 91:719–27. [PubMed: 14573551]
- [29]. Ohya T, Nores WL, Medina JF, Riusech FA, Mauk MD. Learning-induced plasticity in deep cerebellar nucleus. *J Neurosci*. 2006; 26:12656–63. [PubMed: 17151268]
- [30]. Krupa DJ, Thompson JK, Thompson RF. Localization of a memory trace in the mammalian brain. *Science*. 1993; 260:989–91. [PubMed: 8493536]

- [31]. Hardiman MJ, Ramnani N, Yeo CH. Reversible inactivations of the cerebellum with muscimol prevent the acquisition and extinction of conditioned nictitating membrane responses in the rabbit. *Exp Brain Res*. 1996; 110:235–47. [PubMed: 8836688]
- [32]. Kehoe EJ, Holt PE. Transfer across Cs-Us Intervals and Sensory Modalities in Classical-Conditioning of the Rabbit. *Anim Learn Behav*. 1984; 12:122–8.
- [33]. Holt PE, Kehoe EJ. Cross-Modal Transfer as a Function of Similarities between Training Tasks in Classical-Conditioning of the Rabbit. *Anim Learn Behav*. 1985; 13:51–9.
- [34]. Campolattaro MM, Freeman JH. Cerebellar inactivation impairs cross modal savings of eyeblink conditioning. *Behav Neurosci*. 2009; 123:292–302. [PubMed: 19331453]
- [35]. Scavio MJ, Gormezano I. CS intensity effects upon rabbit nictitating membrane conditioning, extinction, and generalization. *Pavlovian journal of biological sciences*. 1974; 9:25–34.
- [36]. Garcia J, Kimeldorf DJ, Koelling RA. Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science*. 1955; 122:157–8. [PubMed: 14396377]
- [37]. Kehoe EJ, Macrae M. Classical conditioning of the rabbit nictitating membrane response can be fast or slow: Implications for Lennartz and Weinberger's (1992) two-factor theory. *Psychobiology*. 1994; 22:1–4.
- [38]. Bracha V, Zhao L, Wunderlich DA, Morrissy SJ, Bloedel JR. Patients with cerebellar lesions cannot acquire but are able to retain conditioned eyeblink reflexes. *Brain*. 1997; 120(Pt 8):1401–13. [PubMed: 9278630]
- [39]. Welsh JP, Harvey JA. Pavlovian conditioning in the rabbit during inactivation of the interpositus nucleus. *J Physiol*. 1991; 444:459–80. [PubMed: 1822558]
- [40]. Bracha V, Webster ML, Winters NK, Irwin KB, Bloedel JR. Effects of muscimol inactivation of the cerebellar interposed-dentate nuclear complex on the performance of the nictitating membrane response in the rabbit. *Exp Brain Res*. 1994; 100:453–68. [PubMed: 7813683]
- [41]. Bracha V, Irwin KB, Webster ML, Wunderlich DA, Stachowiak MK, Bloedel JR. Microinjections of anisomycin into the intermediate cerebellum during learning affect the acquisition of classically conditioned responses in the rabbit. *Brain Res*. 1998; 788:169–78. [PubMed: 9554999]
- [42]. Attwell PJ, Ivarsson M, Millar L, Yeo CH. Cerebellar mechanisms in eyeblink conditioning. *Ann N Y Acad Sci*. 2002; 978:79–92. [PubMed: 12582043]
- [43]. Christian KM, Thompson RF. Long-term storage of an associative memory trace in the cerebellum. *Behav Neurosci*. 2005; 119:526–37. [PubMed: 15839799]
- [44]. Garcia KS, Mauk MD. Pharmacological analysis of cerebellar contributions to the timing and expression of conditioned eyelid responses. *Neuropharmacology*. 1998; 37:471–80. [PubMed: 9704988]
- [45]. Poulos AM, Nobuta H, Thompson RF. Disruption of cerebellar cortical inhibition in the absence of learning promotes sensory-evoked eyeblink responses. *Behav Neurosci*. 2009; 123:694–700. [PubMed: 19485576]
- [46]. Raymond JL, Lisberger SG, Mauk MD. The cerebellum: a neuronal learning machine? *Science*. 1996; 272:1126–31. [PubMed: 8638157]
- [47]. Medina JF, Garcia KS, Mauk MD. A mechanism for savings in the cerebellum. *J Neurosci*. 2001; 21:4081–9. [PubMed: 11356896]
- [48]. Halverson HE, Freeman JH. Medial auditory thalamic nuclei are necessary for eyeblink conditioning. *Behav Neurosci*. 2006; 120:880–7. [PubMed: 16893294]
- [49]. Halverson HE, Freeman JH. Medial auditory thalamic input to the lateral pontine nuclei is necessary for auditory eyeblink conditioning. *Neurobiol Learn Mem*. 2010; 93:92–8. [PubMed: 19706335]
- [50]. Campolattaro MM, Halverson HE, Freeman JH. Medial auditory thalamic stimulation as a conditioned stimulus for eyeblink conditioning in rats. *Learn Mem*. 2007; 14:152–9. [PubMed: 17351138]
- [51]. Van Ham JJ, Yeo CH. Somatosensory Trigeminal Projections to the Inferior Olive, Cerebellum and other Precerebellar Nuclei in Rabbits. *Eur J Neurosci*. 1992; 4:302–17. [PubMed: 12106357]

- [52]. Jacquin MF, Woerner D, Szczepanik AM, Riecker V, Mooney RD, Rhoades RW. Structure-function relationships in rat brainstem subnucleus interpolaris. I. Vibrissa primary afferents. *J Comp Neurol*. 1986; 243:266–79. [PubMed: 3944280]
- [53]. Florence SL, Lakshman S. Topography of primary afferent projections in the trigeminal sensory nuclei of rats. *Acta Neurobiol Exp (Wars)*. 1995; 55:193–200. [PubMed: 8553912]

Highlights

Eyeblink conditioning in rabbits is slow when using traditional auditory stimuli
We found that more natural trigeminal vibrissal stimulation yields faster learning
Trigeminal stimulus-evoked conditioned blinks are a result of associative learning
Trigeminal stimulus-evoked conditioned responses are cerebellum-dependent
Trigeminal vibrissal conditioning is well-suited for studies of motor learning

Figure 1.

Reconstruction of injection sites in the IN. The identified sites were transferred to a set of standardized coronal sections of the rabbit cerebellum. A–F: Six adjacent sections through the cerebellum, separated by 0.5 mm and arranged in rostral-caudal order with “A” being most rostral. All injection sites were located directly in or proximate to the anterior interposed nucleus and the anterior interposed/dentate nuclear border. InA, anterior interposed nucleus; DN, dentate nucleus; LV, lateral vestibular nucleus; SV, superior vestibular nucleus; InP, posterior interposed nucleus; FN, fastigial nucleus; scp, superior cerebellar peduncle; icp, inferior cerebellar peduncle.

Figure 2.

Individual examples of eyeblink stack plots showing acquisition of CRs over three days of vCS training in one naïve rabbit. The first trial for each 100-trial experimental session is at the top of the stack plot and each eyeblink trace represents one trial. (A) During Day 1 of training to the vCS, this rabbit produced only a few small CRs. (B) On Day 2 of training, the rabbit began to exhibit frequent and normal-sized CRs (upward trace deflections between the CS and US markers). Towards the middle of the training session, the CRs show much larger amplitude and the peak of the response becomes time-locked with the US onset. (C) During the third day of training, the rabbit exhibited an asymptotic level of CRs.

Figure 3.

Group learning curves ($n = 4$ for each group) for the vCS and tone CS. The vCS was used for conditioning in naïve rabbits, as a second CS in tone-trained rabbits, and after three days of pseudoconditioning. (A) Training naïve rabbits with the vCS (squares) produces faster CR acquisition than training naïve rabbits with a tone CS (triangles). Rabbits reached the 90% threshold using the vCS in three days while the tone CS group required six days. (B) Training with the vCS in naïve rabbits, as a second CS, or following pseudoconditioning all produced an asymptotic level of responding by Day 3.

Figure 4.

CR incidence for rabbits during pseudoconditioning, retention, and post-pseudoconditioning training. During the three days of pseudoconditioning (P1–P3), rabbits exhibited minimal CRs to the unpaired vCS and showed no signs of learning during the retention test (Ret; 40 vCS-alone trials) performed after pseudoconditioning. Following the retention test, all rabbits showed rapid learning, reaching 90% CRs on the third day of training with paired vCS-US trials.

Figure 5.

Individual examples of behavioral effects from one rabbit following muscimol and PTX injections in the IN. The experiments start at the top and each horizontal eyeblink trace represents one trial of the 140-trial experimental session. (A) Effect of a muscimol injection (indicated by an arrow in the stack plots). After the injection, CRs (upward deflections of the trace between the CS and US markers) were abolished almost immediately and the effect lasted for the duration of the experiment. (B) PTX shortened the latency of vCS-evoked CRs and this effect also lasted for the remainder of the session. The reduced amplitude of blinks at the end of the experiment is due to PTX-induced tonic eyelid closure. (C) The control experiment in which vehicle (aCSF) had no effect on CRs.

Figure 6.

Group data ($n = 12$) for CR incidence ($\pm$ SEM) and latency ($\pm$ SEM) following injections in the IN. Dashed vertical line denotes time of injection for the experiments. (A) The effect of muscimol on CR incidence. An injection of muscimol (squares) immediately impaired CR expression and completely abolished CRs for the remainder of the experiment. The PTX (diamonds) and aCSF injections (triangles) did not significantly affect CR incidence. (B) The effect of a PTX injection on CR latency. The PTX injection (squares) significantly shortened CR latency, compared to a vehicle injection (triangles), for the duration of the experiment.