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Associative and non-associative blinking in classically conditioned adult rats

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ABSTRACT

Over the last several years, a growing number of investigators have begun using the rat in classical eyeblink conditioning experiments, yet relatively few parametric studies have been done to examine the nature of conditioning in this species. We report here a parametric analysis of classical eyeblink conditioning in the adult rat using two conditioned stimulus (CS) modalities (light or tone) and three interstimulus intervals (ISI; 280, 580, or 880 ms). Rats trained at the shortest ISI generated the highest percentage of conditioned eyeblink responses (CRs) by the end of training. At the two longer ISIs, rats trained with the tone CS produced unusually high CR percentages over the first few acquisition sessions, relative to rats trained with the light CS. Experiment 2 assessed non-associative blink rates in response to presentations of the light or tone, in the absence of the US, at the same ISI durations used in paired conditioning. Significantly more blinks occurred with longer than shorter duration lights or tones. A higher blink rate was also recorded at all three durations during the early tone-alone sessions. The results suggest that early in classical eyeblink conditioning, rats trained with a tone CS may emit a high number of non-associative blinks, thereby inflating the CR frequency reported at this stage of training.

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1. Introduction

Classical eyeblink conditioning (EBC) has been used over the last half century to generate one of the largest sources of information available on the neurobiology of mammalian memory formation and storage [1–7]. Delay EBC involves multiple overlapping pairings (and often, co-terminating presentations) of a neutral conditioned stimulus (CS), such as a light or tone, with a mildly aversive unconditioned stimulus (US), such as periorbital electrical stimulation. The subject eventually learns to produce a conditioned eyeblink response (CR) to the CS, blinking near in time to the US onset.

The neural circuitry involved with eyeblink conditioning includes the cerebellum and associated brain stem structures [3,4]. CS-mediated information from the pontine nuclei and US-mediated information from the inferior olive converge in the cerebellar cortex and in the deep nuclei. Over the course of training, multiple-unit recordings in the interpositus nucleus (IP) have revealed populations of neurons that increase their discharge rates and form highly correlated amplitude-time course "models" of the eyeblink CR [8].

One of the benefits of EBC is that the CS and US can be specified and precisely controlled. For example, a vast literature has been accumulated detailing how alterations in the initial conditioning parameters in the rabbit, the predominant animal subject in EBC studies, affects acquisition of the conditioned nictitating membrane and eyelid response (reviewed in [9,10]). While a variety of CSs have been used in eyeblink conditioning, an acoustic CS has been used most frequently, typically a 1–10 kHz tone or white noise. The use of an acoustic cue is at least partly conventional, but it is also the case that rabbits appear to classically condition to auditory stimuli more readily than to visual stimuli [11]. Nevertheless, rabbits do condition to a light CS, resulting in learning that is generally comparable to that achieved with a tone CS [12–14].

The rate of learning can also be manipulated by altering the time between the onsets of the CS and the US (the interstimulus interval, or ISI). Conditioning with different CS–US intervals results in different rates and levels of CR acquisition, and correspondingly different peak latencies. Conditioning at various ISIs has also yielded acquisition rates which demonstrate that an optimal ISI for learning exists in rabbits. Indeed, a series of classic studies have demonstrated that delay EBC in the rabbit is most robust with an ISI of 200–500 ms, compared to longer or shorter ISIs [15–19].

In addition to the rabbit, a variety of species can be eyeblink conditioned, including humans, monkeys, cats, mice, and rats [20]. The latter has been increasingly used in recent years in EBC research. In a long series of studies, Mark Stanton, John Freeman and their colleagues have investigated the ontogeny of eyeblink conditioning in weanling and pre-weanling rats. Their work has established that delay EBC emerges between postnatal days 17 and 24 in the rat [21], dependent on the particular conditioning parameters. For example, 24 day old rats show conditioning that increases as a function of US intensity, whereas 17 day old rats do not [22]. Young rats are also capable of discriminating

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between two alternating interstimulus intervals [23,24]. The temporal uncertainty procedure results in double peak CRs to the same CS, one blink timed to the short ISI, one blink timed to the long ISI [23]. Alternatively, ISI discrimination, which uses two distinct CSs, results in blink CRs that are properly timed to the ISI at which each CS was conditioned [24].

To our knowledge, however, a systematic analysis of how EBC acquisition in adult rats is affected by manipulations of the CS and US, and the timing between the two, has yet to been done. To that end, we have previously examined CR acquisition at multiple US intensities in the adult rat [25]. In line with results in the rabbit [18], US intensity was found to be critically important for CR acquisition, with the rate and asymptote of conditioned responding greater in rats trained with a 2.0 than 1.0 mA periorbital shock US [25].

The current study attempts to establish the parametric variation under which two further factors, CS modality and duration, affect the rate and level of eyeblink conditioning in adult rats. In Experiment 1, all subjects underwent 1 day of adaptation, when they were exposed to the conditioning chamber, providing a baseline spontaneous blink rate for each group. Paired eyeblink conditioning commenced the next day with a light or tone CS at one of three ISIs: 280, 580, or 880 ms. A separate group of rats underwent explicitly unpaired eyeblink conditioning with a 580 ms light or tone CS. Experiment 2 examined two forms of blinking: non-associative blinks emitted in response to the light or tone CS, in the absence of the US, and spontaneous blinks that occurred independent of the two stimuli. In this experiment, two additional groups of rats were exposed to the same light or tone used in paired eyeblink conditioning for 280, 580, and 880 ms, corresponding to the conditioning ISIs in Experiment 1. In this manner, we were able to compare associative and non-associative blink frequencies to both CS modalities across the three CS–US intervals, which could, in turn, be compared to spontaneous blink rates prior to the subject's exposure to any stimuli and during sessions with intermittent light or tone presentations.

2. Methods

2.1. Subjects

Ninety-four experimentally naïve Long-Evans rats, 50 males and 44 females, were maintained on a 12 hour light/dark cycle with *ad lib* access to food and water. Surgical and behavioral procedures were conducted during the light phase. All procedures, including surgery and postoperative care, were in strict compliance with the Indiana University and the University of Kansas animal care guidelines, and all necessary measures were taken to minimize pain and discomfort.

2.2. Surgical procedures

All surgical procedures were performed under aseptic conditions. Beginning on postnatal day 80, rats were anesthetized using intraperitoneal (ip) injections of an anesthetic cocktail (2.0 ml/kg), consisting of physiological saline (9.0 mg/kg), ketamine (74.0 mg/kg), xylazine (3.7 mg/kg), and acepromazine (0.74 mg/kg). Ketamine boosters were administered as required to maintain anesthesia. Each subject was surgically prepared with differential electromyographic (EMG) wires and a bipolar periocular stimulator. EMG activity was recorded in the orbicularis oculi muscle surrounding the eye by passing two ultrathin (0.003 in.) Teflon-coated stainless steel wires subdermally beneath the anterior portion of the upper eyelid. Gold-coated stainless steel wires were implanted in the dorso-caudal portion of the orbicularis oculi muscle for delivery of the periorbital electrical shock US. A ground wire was connected to one of three stainless steel skull screws. The two EMG wires and a separate ground wire all terminated in gold pins inside a 3-pin plastic connector. The headstage and bipolar stimulating electrodes were fixed in dental cement. The wound was salved with antibiotic ointment (Povidone), and the animals were given at least 6 days to recover before the start of training.

2.3. Apparatus

Rats were placed in standard operant boxes (Coulburn Instruments, Allentown, PA), contained within sound-attenuating chambers. Each operant box had two stainless steel walls, two Plexiglas walls, and a grid floor composed of 0.5 cm stainless steel bars placed approximately 1.5 cm apart. The electrode leads attached to each subject's head swiveled freely on a 10-channel commutator connected to a counterbalanced pivoting arm, allowing subjects to move freely about in the conditioning chamber. All rats were presented with a light or tone CS (Experiment 1: 380, 680, or 980 ms; Experiment 2: 280, 580, and 880 ms). The light consisted of a 12 W LED assembly (Super Bright LEDs, Inc., St. Louis, MO) with an illumination intensity of 400 lux (measured about 8 cm from the source), inserted into an opening in one wall of the operant box. The tone was a 2.8-kHz, 85-dB SPL tone, delivered from an overhead speaker. The 100 ms US, used in Experiment 1, was a train of 2.0 mA, 60-Hz, constant-current square wave periocular electrical stimulation.

2.4. EMG analysis

Throughout each session, eyelid EMG activity was amplified (1000×) and band-pass filtered (300–1000 Hz) by a differential AC amplifier (model 1700, A-M Systems, Carlsborg, WA). The EMG signal was simultaneously digitized (500 Hz), rectified, smoothed (10 ms time constant), time shifted (10 ms, to compensate for smoothing), and stored for offline analysis using the Spike 2 waveform analysis system (CED Limited, Cambridge, England). On each trial, EMG activity from the orbicularis oculi muscle was sampled for 1500 ms, divided into three periods: (i) a 350-ms pre-CS period, prior to CS onset; (ii) a 280, 580, or 880 ms CS–US period, between CS onset and US onset; and (iii) an 870, 570, or 270 ms post-US period, following US onset.

The averaged EMG activity in the pre-CS period was used as a baseline for classifying behaviors and scoring trials. Trials were dropped and excluded from further analysis if EMG activity exceeded the baseline activity by ten or more standard deviations during the bad trial window, which extended from 100 ms before CS onset to 15 ms after CS onset. EMG activity that exceeded the baseline activity by ten or more standard deviations between 15 and 100 ms following CS onset was classified as an alpha response.

A blink (associative, non-associative, or spontaneous) was scored if EMG activity exceeded the baseline activity by 8 or more standard deviations beginning 100 ms after CS onset. Session-wide averages were computed for blink frequencies during the ISI (CS–US paired trials, Experiment 1) or during the CS duration (CS-alone unpaired trials, Experiment 1; CS-alone trials, Experiment 2). Blink topographies (defined below) were computed based on the 10 CS-alone trials per session (paired eyeblink conditioning, Experiment 1) or on the same CS-alone trials used to calculate frequency (Experiment 2). With no contamination by the US, the EMG response was examined from CS onset through the end of the trial. The EMG data were analyzed using *t*-tests, one-way and mixed design ANOVAs, and, when appropriate, Tukey–Kramer post hoc tests. A significant post hoc effect implies $p<0.05$.

In terms of blink topography, the onset latency refers to the point in time when the EMG signal crosses the threshold for CR detection. The peak amplitude of the EMG signal refers, behaviorally, to the point at which the eyelid is most fully extended. The time point at which the peak amplitude is reached corresponds to the peak latency.

Finally, it bears emphasizing that the eyeblink results in the current study are dependent on our criterion for scoring blinks. To ensure that blinks were accurately counted, we re-analyzed a subset of the CR data with another scoring method, based on multiples (rather than standard deviations) of the pre-CS baseline amplitude [26]. The

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