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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.

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

analyses were identical to those above, except the bad trial, alpha, and blink thresholds were set to 2.0 times the averaged baseline. CRs were scored almost identically ($r=0.97$) with the two different criterion, indicating our current method is accurately capturing genuine blinks.

2.5. Experiment 1: CS–US paired/unpaired eyeblink conditioning

2.5.1. Experimental design

Sixty-four rats (37 male and 27 female) underwent delay eyeblink conditioning using a mixed 2×3×7 design: two between-subjects factors, CS modality (light or tone) and conditioning ISI (280, 580, or 880 ms), and one within-subjects factor, conditioning session (1–7). An additional ten rats (6 males and 4 females) underwent explicitly unpaired eyeblink conditioning using a mixed 2 (CS modality)×7 (session) design. The CS duration for unpaired conditioning was 580 ms.

2.5.2. Behavioral training and testing

Prior to conditioning all rats underwent an initial 45 minute adaptation session in a darkened chamber. They were placed in the operant box with the commutator attached and allowed to move about. Each subject was presented with the same one hundred trials used in paired conditioning (see below), only in the absence of the CS or US—providing a baseline spontaneous blink rate, prior to the subject's exposure to any conditioning stimuli.

Paired eyeblink conditioning began the next day. Each subject was presented with the light or tone CS for 380, 680, or 980 ms, and a co-terminating 100 ms periorbital shock US. Accordingly, the conditioning ISI was 280, 580, or 880 ms. Each of the seven sessions of conditioning was composed of 10 blocks of 10 trials: 9 CS–US paired trials and 1 CS-alone trial. The intertrial interval (ITI) was 25±5 s. The six groups of rats (2 CS modalities×3 conditioning ISIs) were: light-paired 280 ($n=10$), tone-paired 280 ($n=10$), light-paired 580 ($n=11$), tone-paired 580 ($n=11$), light-paired 880 ($n=11$), and tone-paired 880 ($n=11$).

For unpaired eyeblink conditioning, rats were presented with the 580 ms CS and the 100 ms US the same number of times as occurred with paired conditioning, but the two stimuli never overlapped in time. Specifically, each of the seven sessions consisted of 100 light or tone CS trials and 90 periorbital shock US trials. The ITI ranged from 10–15 s. The two groups were: light-unpaired 580 ($n=5$) and tone-unpaired 580 ($n=5$). The unpaired eyeblink conditioning groups were included to assess non-associative blink rates to the CS with within-session US presentations.

2.6. Experiment 2: CS-alone presentations

2.6.1. Experimental design

Twenty rats (7 males and 13 females) were subjected to a mixed 2×4×7 experimental design: one between-subjects factors, CS modality (light or tone) and two within-subjects factors, trial type (CS-alone 280, CS-alone 580, CS-alone 880, and CS-spontaneous 580), and session (1–7).

2.6.2. Behavioral training and testing

Subjects were multiply presented with the same light or tone used in Experiment 1, except the US was never presented. Over 7 sessions, four trial types were pseudo-randomly presented to each rat in blocks of 20 trials. Trials were separated by an ITI of 25±5 s, and blocks were separated by approximately 30 s. The four trial types were: a 280 ms CS-alone trial (light-alone 280 or tone-alone 280), a 580 ms CS-alone trial (light-alone 580 or tone-alone 580), an 880 ms CS-alone trial (light-alone 880 or tone-alone 880), and a 580 ms trial in which EMG activity was recorded in the absence of the CS (light-spontaneous 580 or tone-spontaneous 580). The final trial type was included to assess the frequency of spontaneous blinks when alternating blocks of lights or tones were presented within the same session. Ten subjects were presented with the light CS; 10 subjects were presented with the tone CS.

3. Results

3.1. Experiment 1: CS–US paired/unpaired eyeblink conditioning

3.1.1. Adaptation

The far left of Fig. 1A–B displays spontaneous blink frequencies during adaptation, 1 day prior to the start of paired or unpaired eyeblink conditioning. To determine whether the groups differed in their spontaneous blink rates, simple one-way (group) ANOVAs were run for each CS modality. In both cases, the group main effect was statistically significant: light CS, $F(3, 33)=24.50$, $p<0.001$, and tone CS, $F(3, 33)=10.32$, $p<0.001$. The number of recorded blinks increased as a function of the trial length—i.e., the spontaneous blink frequency appears to be primarily determined by the duration of time that the EMG signal is analyzed.

3.1.2. Response frequency

Fig. 1A–B shows the percentage of associative (paired conditioning) and non-associative (unpaired conditioning) blinks generated across the seven sessions. There is a clear divergence in blink frequencies between the paired and unpaired eyeblink conditioning subjects. In support of this observation, there were no significant group differences on the first session with either the light CS (Fig. 1A) or tone CS (Fig. 1B), as indicated by a one-way (group) ANOVA. By session three, however, the three light-paired conditioning groups were producing significantly more blinks than the light-unpaired 580 rats, $F(3, 33)=6.02$, $p<0.01$. The divergence was even more rapid with the tone CS, with significance reached by session two, $F(3, 33)=5.60$, $p<0.01$. A mixed 2 (sex)×2 (CS modality)×7 (session) repeated measures ANOVA ruled out the possibility that our use of female and male rats affected frequency rates among the various groups.

Analysis of blinking in the unpaired rats, with a mixed 2 (CS modality)×7 (session) repeated measures ANOVA, revealed a significant main effect for session, $F(6, 36)=7.40$, $p<0.001$. There was a trend toward higher rates of blinking in the tone-unpaired 580 rats, relative to the light-unpaired 580 rats, across the seven sessions (Fig. 1A–B), as indicated by the near significant main effect for sensory modality, $p=0.054$.

Examination of Fig. 1A–B reveals obvious differences in the acquisition curves of light- and tone-paired rats, particularly during the early conditioning sessions with the 580 and 880 ms ISI. Rats that underwent paired eyeblink conditioning with the light CS (Fig. 1A) averaged approximately 30% CRs on the first session, regardless of the ISI at which they were trained. The frequency of blink CRs increased across training sessions, with the highest and lowest final levels of responding occurring with the light-paired 280 and light-paired 880 rats, respectively. Rats trained with the tone CS, on the other hand, displayed variable levels of conditioned responding on the first training session (Fig. 1B). The acquisition curve of the tone-paired 280 rats was similar to that for the light-paired 280 rats (Fig. 1C). The CR frequencies of the tone-paired 580 and 880 rats, however, were much higher across the first few sessions, resulting in acquisition curves that were also much flatter than the comparable curves for the light-paired 580 and 880 rats (Fig. 1D–E). Breaking the first session down into 20 trial blocks, conditioned responding was similar across all five blocks in the light-paired 280, 580, and 880 rats. The three tone-paired groups started out at comparable levels. By the second block, however, more blinks were being generated by the tone-paired 580 and 880 rats than the tone-paired 280 rats, resulting in flat frequency curves across the remaining blocks of trials (data not shown).

Across all seven sessions, CR frequency was analyzed in light-paired and tone-paired rats as a function of ISI (Fig. 1A–B) with two mixed repeated measures ANOVAs, followed by Tukey-Kramer post-hoc analyses. For light-paired rats, the ISI×session interaction was not

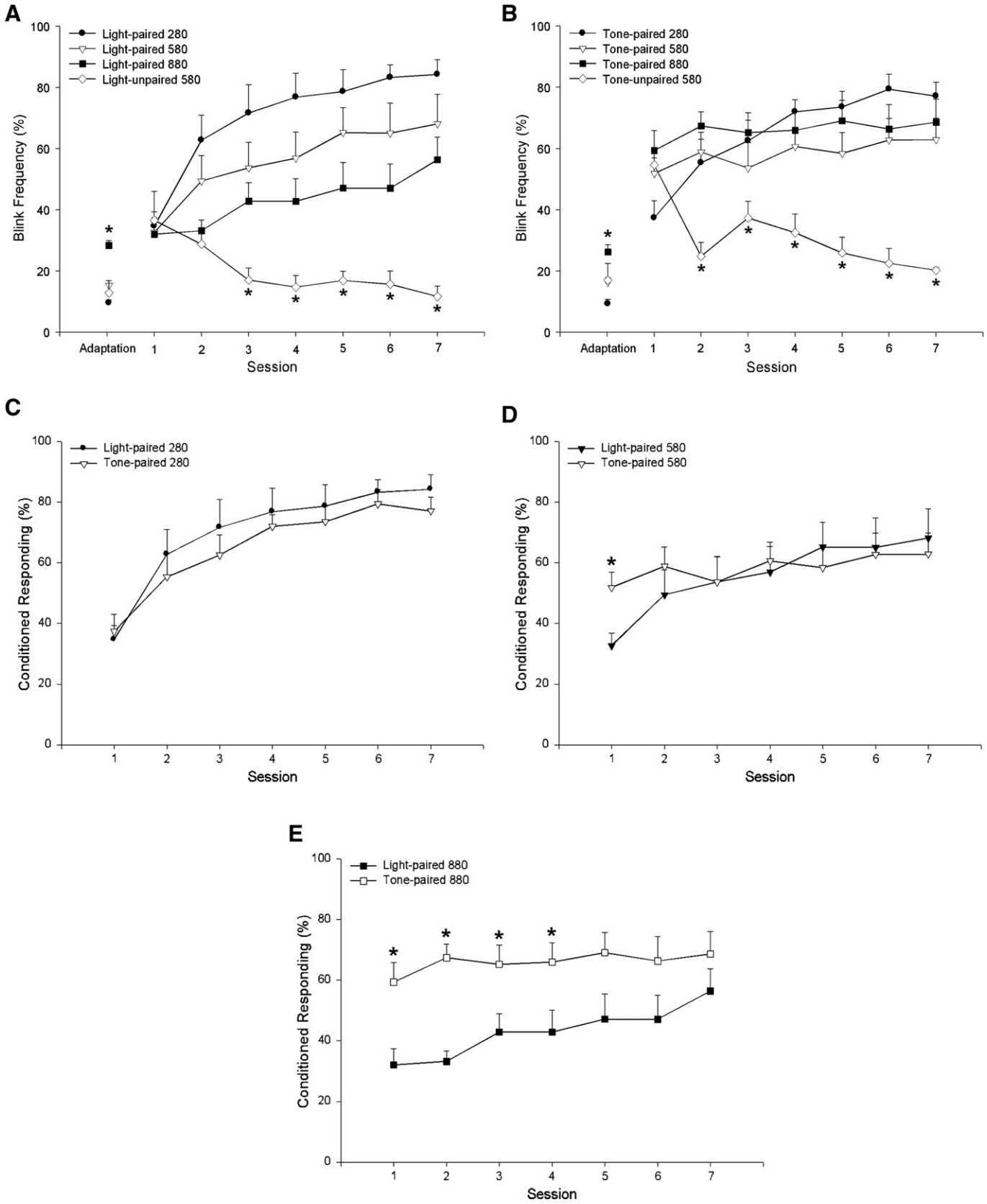

Fig. 1. Mean (±SE) percentage responding as a function of CS modality (A–B) and ISI (C–E). Conditioned response (CR) frequencies in rats trained with the light CS (A) or tone CS (B) and an interstimulus interval (ISI) of 280, 580, or 880 ms, and non-associative blink frequencies in rats that experienced explicitly unpaired eyeblink conditioning with a 580 ms light or tone CS. The paired conditioning data is re-illustrated below, with CR frequency presented for light-paired and tone-paired rats trained with the 280 ms ISI (C), the 580 ms ISI (D), and the 880 ms ISI (E). The asterisks (*) indicate significant group differences. During adaptation (A–B), light- and tone-paired 880 rats emitted significantly more spontaneous blinks than the other groups. Across the seven sessions (A–B), rats that underwent unpaired conditioning blinked significantly less than the paired eyeblink conditioning rats. The asterisks in C–E indicate significant differences in responding between the two CS modalities.

quite significant, $p=0.08$. There was, however, a significant main effect for ISI, $F(2, 174)=4.87$, $p<0.05$, with the light-paired 280 rats generating significantly more CRs than the light-paired 880 rats. As for rats presented with the tone CS, the tone-paired 280 rats had the lowest CR frequency across the first two sessions, but the highest CR frequency across the last two sessions (Fig. 1B). That is, the slope of the

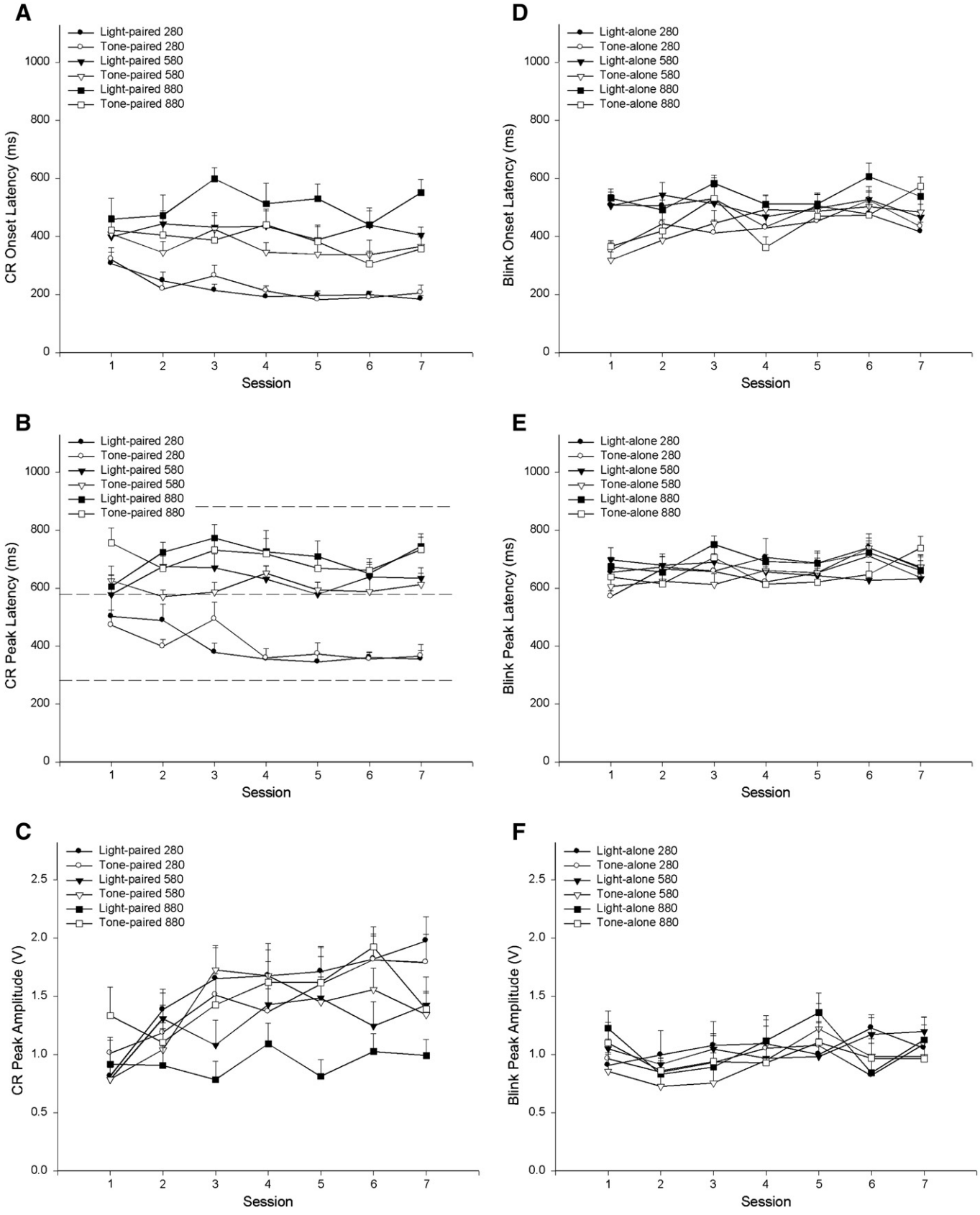

Fig. 2. Associative and non-associative blink latencies and amplitudes (mean ± SE). Conditioned response onset latencies (A), peak latencies (B), and peak amplitudes (C) are illustrated for both light- and tone-paired rats. Non-associative blink onset latencies (D), peak latencies (E), and peak amplitudes (F) are illustrated for both light- and tone-alone rats.

acquisition curve in tone-paired 280 rats is steeper and intersects the much flatter curves generated by the tone-paired 580 and 880 rats, leading to a statically significant ISI×session interaction, $F(12, 174) = 3.29$, $p<0.001$.

In Fig. 1C–E, CR frequency was broken down by the conditioning ISI and the effect of CS modality examined using three 2×7 repeated measures ANOVAs. CS modality had no significant effect on CR acquisition with the 280 ms conditioning ISI. With the 880 ms ISI the CS modality main effect was significant, $F(1, 120) = 9.29$, $p<0.001$. Only with the 580 ms ISI was a significant modality×session interaction revealed, $F(6, 120) = 2.51$, $p<0.05$. An analysis of each conditioning session, using one-way (CS modality) ANOVAs, revealed that tone-paired 580 rats produced significantly more CRs on the first conditioning session than light-paired 580 rats (Fig. 1D). Similarly, tone-paired 880 rats produced significantly more CRs than light-paired 880 rats across the first four conditioning sessions (Fig. 1E).

3.1.3. Response latency

CR onset and peak latencies on CS-alone trials are illustrated in Fig. 2A–B for all six groups across the seven conditioning sessions. For onset latencies (Fig. 2A), the mixed 2×3×7 repeated measures ANOVA revealed a significant modality×ISI interaction, $F(2, 348) = 3.27$, $p<0.05$, as well as a significant main effect for conditioning session, $F(6, 348) = 2.28$, $p<0.05$. Further examination of the interaction effect found significantly shorter onset latencies for the tone-paired than the light-paired rats. Follow-up 2×7 repeated measures ANOVAs at each ISI, found that the tone-paired 880 rats had significantly earlier onset latencies than the light-paired 880 rats, $F(1, 120) = 7.56$, $p<0.05$. The light- and tone-paired 280 rats were also the only ISI group to produce a significant conditioning session main effect, $F(6, 108) = 7.31$, $p<0.0001$, indicating a decrease in CR onset latencies across the seven sessions.

CR peak latencies, shown in Fig. 2B, varied as a function of the conditioning ISI only, as indicated by the significant repeated measures ANOVA, $F(2, 348) = 94.98$, $p<0.001$. Each of the three post-hoc comparisons for ISI were statistically significant—i.e., rats trained with the 280 ms ISI had earlier peak latencies than rats trained with the 580 and 880 ms ISI, and the 580 ms ISI rats had earlier peak latencies than the 880 ms ISI rats. And, as seen with CR onset latencies, light- and tone-paired 280 rats were the only ISI group to produce a significant conditioning session main effect, $F(6, 108) = 3.76$, $p<0.01$, indicating a decrease in CR peak latencies across the seven sessions. By the end of conditioning, peak latencies in light- and tone-paired rats at each ISI occurred near the time of US onset (dashed lines in Fig. 2B). Representative EMG traces, from tone-paired rats, are illustrated in Fig. 3. The top traces are from the first conditioning session (Fig. 3A), the bottom traces are from the last conditioning session (Fig. 3B).

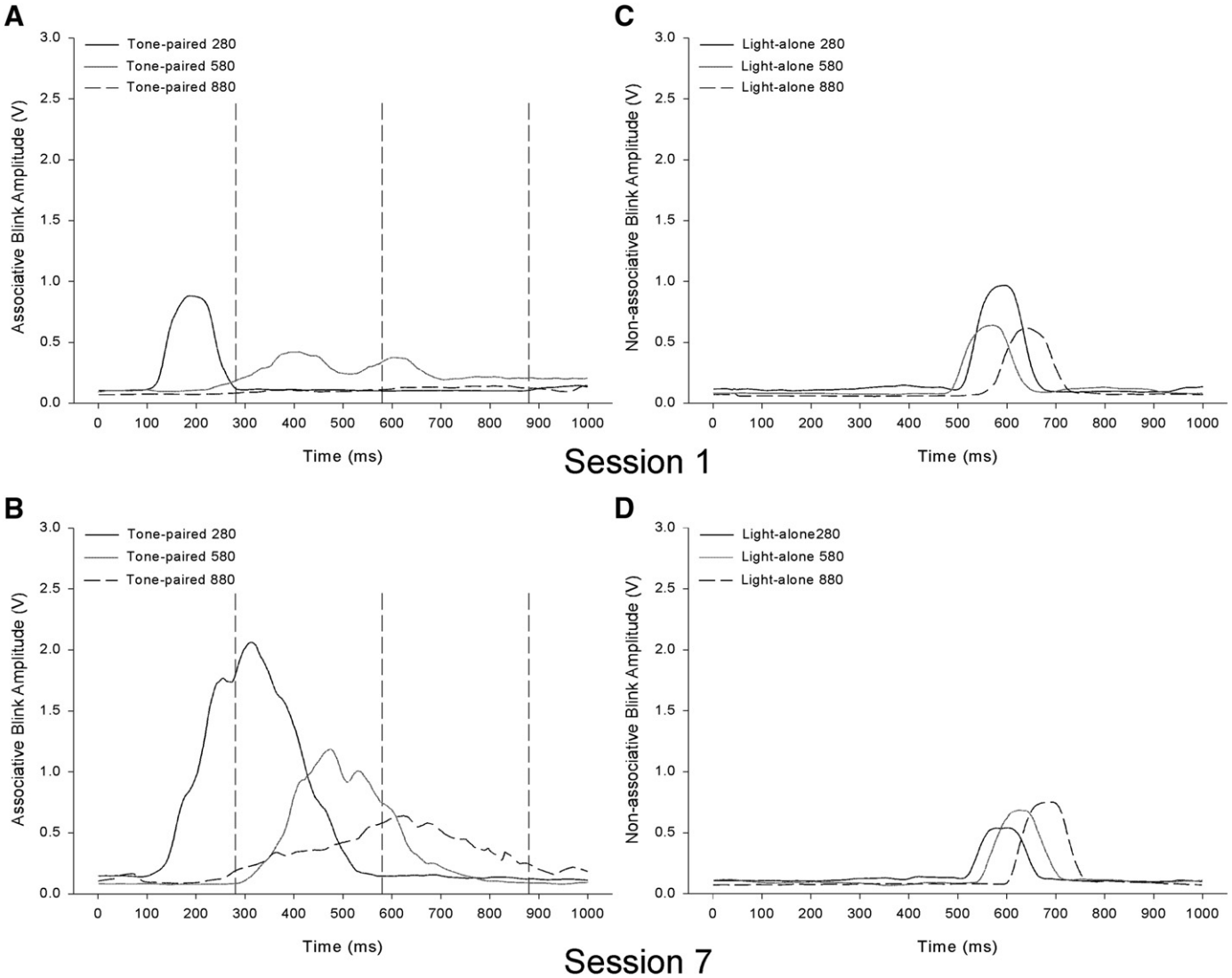

Fig. 3. Representative EMG traces from tone-paired (A–B) and light-alone (C–D) rats for session 1 (A and C) and 7 (B and D). Each averaged trace is from a single subject, based on all CS-alone trials within the session.

3.1.4. Response amplitude

Fig. 2C shows CR peak amplitudes on CS-alone trials for each of the six groups of rats, which, except for perhaps the light-paired 880 rats, all showed an increase across training. Neither CS modality nor ISI were significant, nor the interaction between them, as indicated by the mixed 2×3×7 repeated measures ANOVA. Factoring in the conditioning session resulted in significant interactions, however, for both CS modality, $F(6, 348)=2.20$, $p<0.05$, and ISI, $F(12, 348)=2.66$, $p<0.01$, indicating the effect of both variables could only be ascertained when examined across sessions. Follow-up 2×7 repeated measures ANOVAs at each ISI, found that tone-paired 880 rats generated significantly larger peak CR amplitudes than light-paired 880 rats, $F(1, 120)=9.38$, $p<0.001$.

3.2. Experiment 2: CS-alone presentations

3.2.1. Response frequency

During light-alone trials, non-associative blinks were recorded on approximately 10–30% of all trials (Fig. 4A). The blink rate was much higher in tone-alone rats, ranging from about 15–50% (Fig. 4B). In both cases, the longer the duration of the CS the higher the rate of blinking. Interestingly, the blink rate in tone-alone 580 and 880 rats declined across the seven sessions. As in Experiment 1, the non-associative blink rate was not statistically different between female and male rats exposed to the light or tone, as indicated by a mixed 2 (sex)×2 (CS modality)×7 (session) repeated measures ANOVA.

Analysis of blink frequencies, using two mixed 4×7 repeated measures ANOVAs, revealed a significant trial type main effect in both light-alone rats (Fig. 4A), $F(3, 216)=18.75$, $p<0.0001$, and tone-alone rats (Fig. 4B), $F(3, 216)=20.34$, $p<0.0001$. In both groups, post-hoc analyses found a significantly higher percentage of non-associative blinking with the two longest stimulus presentations (580 and 880 ms) than with either the shortest presentation (280 ms) or during the 580 ms spontaneous blink trial when neither the light or tone were presented.

An analysis of the three CS-alone trial types based on CS modality (Fig. 4C–E), using 2×7 repeated measures ANOVAs, revealed a significant modality×session interaction effect at each stimulus duration: light- and tone-alone 280, $F(6, 108)=2.36$, $p<0.05$; light- and tone-alone 580, $F(6, 108)=2.37$, $p<0.05$; and light- and tone-alone 880, $F(6, 108)=2.64$, $p<0.05$. In all cases, tone-alone rats emitted more non-associative blinks than the light-alone rats. One-way (CS modality) ANOVAs calculated for each individual session revealed that significantly more blinks were emitted by tone-alone than light-alone 280 rats on sessions 1 and 3 (Fig. 4C). Tone-alone 580 rats emitted more blinks than their light-alone counterparts on sessions 1 and 2 (Fig. 4D), and tone-alone 880 rats emitted more blinks than light-alone 880 rats across sessions 1–4 (Fig. 4E). Finally, there was a significantly higher spontaneous blink rate in rats that experienced within-session tones, relative to within-session lights, as indicated by a significant CS modality effect in light- and tone-spontaneous 580 rats, $F(1, 108)=6.05$, $p<0.05$.

3.2.2. Response latency

As seen in Fig. 2D, the non-associative blink onset latencies to the light and tone appear segregated during the first session, an observation supported by a significant one-way (CS modality) ANOVA, $F(1, 58)=42.06$, $p<0.001$. The mixed 2 (CS modality)×3 (CS duration)×7 (session) repeated measures ANOVA also revealed a statistically significant modality×session interaction, $F(6, 324)=3.70$, $p<0.01$. Rats presented with the light CS had significantly longer onset latencies than rats presented with the tone CS. The CS duration had no significant effect on blink onset latencies. Note, however, that the mean onset latencies in both light- and tone-alone 280 rats exceeded the duration of the CS (see Discussion).

The non-associative blink peak latencies were comparable across the seven sessions (Fig. 2E). Nevertheless, the repeated measures ANOVA revealed a significant CS modality×session interaction, $F(6, 324)=2.38$, $p<0.05$. There were no significant post-hoc comparisons. The six groups were roughly distributed by CS modality during the first session, yet by the seventh session the modality-specific segregation was gone, an effect that likely explains the significant interaction. Representative EMG traces, for light-alone rats, are illustrated in Fig. 3. The top traces are from the first session (Fig. 3C), the bottom traces are from the last session (Fig. 3D).

3.2.3. Response amplitude

Non-associative blink peak amplitudes are shown in Fig. 2F. Results of the mixed 2×3×7 repeated measures ANOVA failed to find reliable differences for CS modality or duration, nor the interaction between the two. The only a significant effect was for session, $F(6, 324)=2.86$, $p<0.01$, indicating an across-group change in peak amplitudes over the seven sessions.

3.3. Experiments 1 and 2 combined

3.3.1. Revised CR frequency

Experiment 1 found elevated CR frequencies in tone-paired rats, relative to the light-paired rats, during the early conditioning sessions. Consistent with this result, Experiment 2 found that the tone-alone rats displayed elevated non-associative blink rates during the early sessions, which then decreased in frequency across the remaining sessions. It seems the case, then, that the elevated, relatively flat acquisition curves for tone-paired 580 and 880 rats result, at least in part, from an acoustically-mediated enhancement of the non-associative blink rate. Subtracting out the non-associative blinks from the associative blinks might offer a better approximation of true learning.

For each of the six groups of rats that underwent delay eyeblink conditioning, the mean CS-alone blink frequencies (Fig. 4C–E) were deducted from the corresponding mean CR frequencies (Fig. 1C–E) across each of the seven sessions, based on the following equation:

$$\text{Revised CR} = [(\text{associative} - \text{non-associative}) / (100 - \text{non-associative})]*100.$$

All six revised CR acquisition curves, which may better represent genuine conditioned responses, display low levels of responding on the first session, negatively accelerating thereafter across the remaining six sessions (Fig. 5A–C). *T*-tests were performed on the revised light- and tone-paired response curves at each ISI. There were no significant differences in acquisition rate for rats conditioned with the 280 or 580 ms ISI. Only with the longest ISI were significantly more blinks observed in tone-paired than light-paired rats across the seven sessions, $t(12)=3.71$, $p<0.01$.

3.3.2. Associative/non-associative/spontaneous blinking rates

As discussed above, the number of recorded blinks per session seems to be at least partly dependent on the interval over which the EMG trace is analyzed. Therefore, blink frequencies are contrasted in Fig. 6, based on a single duration: 580 ms. Specifically, associative blinks (light- and tone-paired 580), non-associative blinks (light- and tone-unpaired 580; light- and tone-alone 580), and spontaneous blinks (light- and tone-spontaneous 580) are illustrated.

The associative blink frequency is significantly higher than the frequency for either type of non-associative blink or spontaneous blinks. A mixed repeated measures ANOVA revealed a significant group×session interaction for both the light CS, $F(18, 192)=4.98$, $p<0.001$, and the tone CS, $F(18, 192)=2.76$, $p<0.001$. One-way ANOVAs on each session found that the light- and tone-paired 580 rats blinked significantly more than all other groups across sessions 2 through 7. On the first session, the light-spontaneous blink frequency was significantly lower than the light-paired and light-unpaired blink rates. The same held true with the

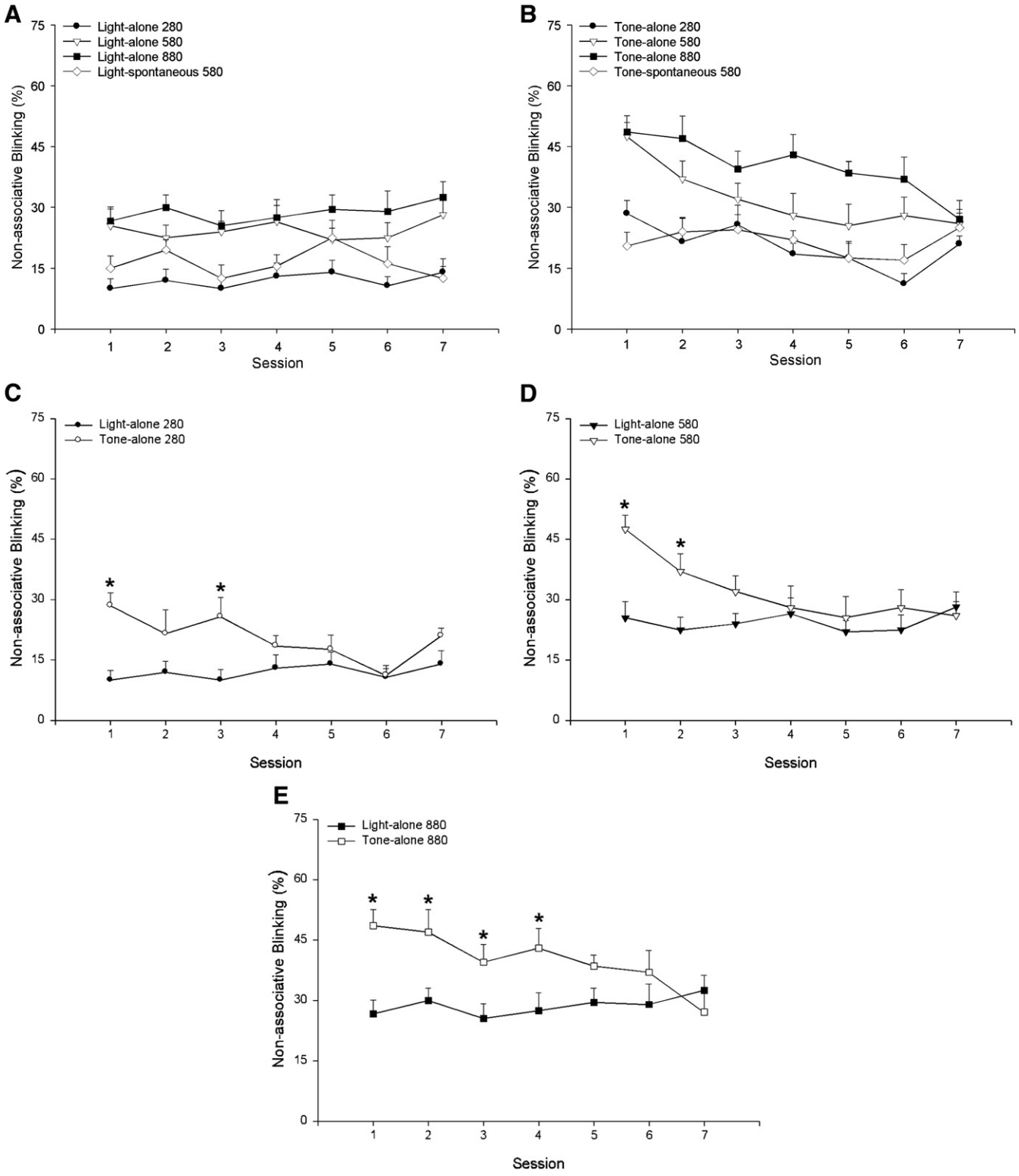

Fig. 4. Mean (±SE) percentage of non-associative blinks emitted as a function of CS modality (A–B) and duration (C–E). Spontaneous and non-associative blinks are shown for rats trained with the light CS (A) or tone CS (B). Within each session, rats were presented with the light- or tone-alone for 280, 580, and 880 ms, as well as a 580 ms interval when no cue was presented (light- or tone-spontaneous 580), capturing the spontaneous blink rate. The same data is re-illustrated below, with the non-associative blink frequency recorded during the 280 ms (C), 580 ms (D), and 880 ms (E) light or tone. The asterisks (*) in C–E indicate significant differences in responding between the two CS modalities.

tone-spontaneous blink frequency, which was also significantly lower than the tone-alone blink rate.

Examination of the non-associative blink rates (unpaired and CS-alone) shows them to be remarkably similar within each CS modality, suggesting that the number of non-associative blinks emitted per session is relatively stable, independent of whether the US is or is not presented within the same session. The spontaneous blink rate might be affected by within-session presentations of the light or tone, however. Thus, Fig. 7 illustrates response frequencies for light- and tone-paired 580 rats during the adaptation session (1 day prior to paired conditioning), as well as response frequencies for each of the groups shown in Fig. 6, averaged across all seven sessions.

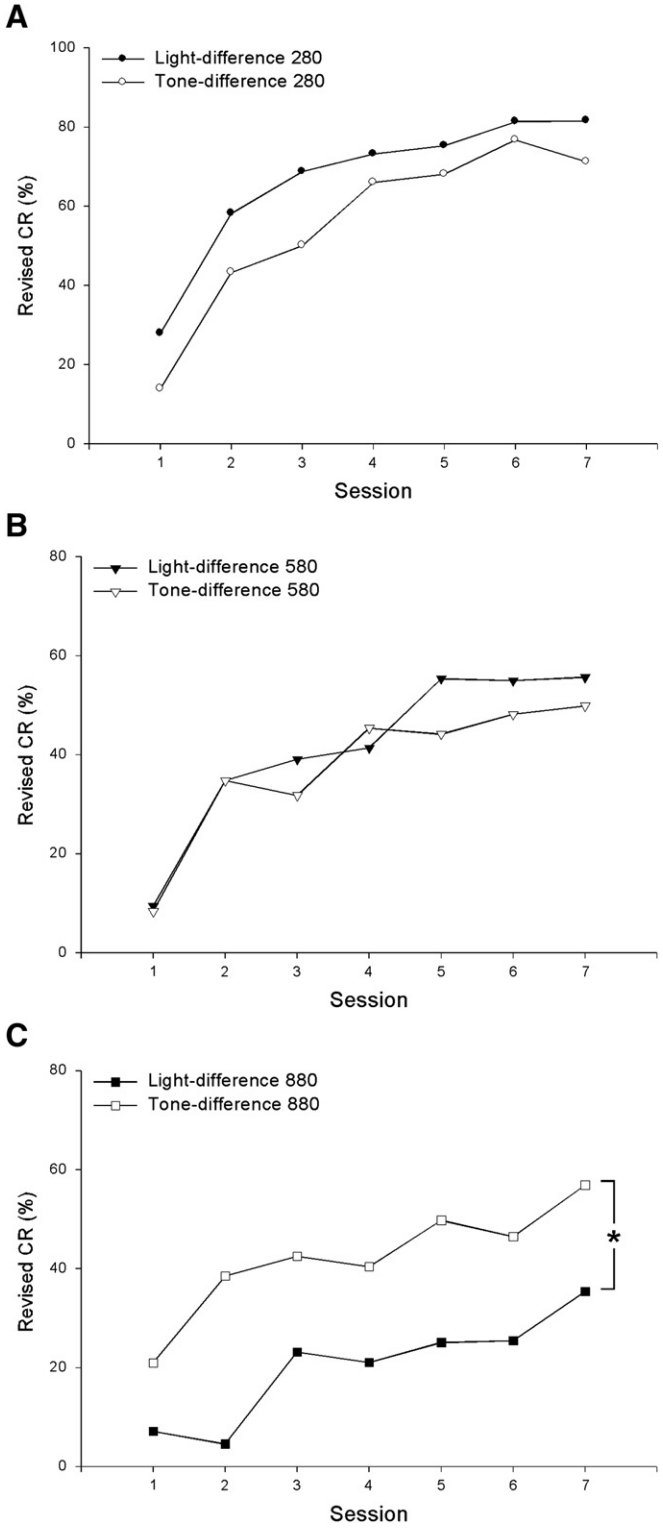

Fig. 5. Revised CR acquisition curves following the subtraction, for each session, of the mean response frequency of non-associative blinks (Fig. 4C–E) from associative blinks (Fig. 1C–E). The revised acquisition curves are statistically no different with the 280 ms (A) and 580 ms (B) ISI; only with the 880 ms ISI (C) do significant CS modality effects emerge.

During adaptation (Fig. 7, middle left), prior to the rat's exposure to any stimuli, spontaneous blinking was nearly identical for the (soon-to-be) light-paired and tone-paired rats. The spontaneous blink rate in rats exposed to intermittent tone-alone trials was significantly higher, however, than the blink rate of rats exposed to light-alone trials (Fig. 7, middle), $F(1,138)=8.29$, $p<0.01$, which was statistically equivalent to the spontaneous blink rate during adaptation. Significantly more non-associative blinks were also emitted during presentation of the tone-alone trials than the light-alone trials (Fig. 7, middle right), $F(1, 138)=11.48$, $p<0.001$. Finally, the non-associative blink rate in tone-unpaired rats was significantly higher than the blink rate in light-unpaired rats (Fig. 7, far right), $F(1, 68)=7.11$, $p<0.01$, whereas there were no significant differences in CR frequency between light- and tone-paired 580 rats (Fig. 7, far left).

4. Discussion

Results from the current study clarify how delay eyeblink conditioning in the adult rat is affected by the modality and duration of the CS. At the end of seven conditioning sessions, the highest levels of conditioned responding were observed in the light-paired and tone-paired 280 rats (Fig. 1A–B), although it cannot be ruled out that CR frequencies would continue to rise among all groups if additional training sessions were given. As the conditioning ISI was increased, CS modality effects began to emerge, becoming most pronounced with the 880 ms ISI (Fig. 1C–E). Specifically, CRs in the tone-paired 580 and 880 rats occurred at a much higher rate, had earlier onset times, and greater peak amplitudes than conditioned eyeblink responses in the light-paired 580 and 880 rats (Figs. 1 and 2). Conditioned responding across the final few sessions in tone-paired 580 and 880 rats was comparable to that for the light-paired 580 and 880 rats—only during the early acquisition sessions were unusually high levels of blinking observed in the tone-paired rats (Fig. 1D–E). In like manner, non-associative blinking in the tone-alone 580 and 880 rats was highest during the earliest sessions, decreasing in frequency across the remaining sessions (Fig. 4D–E).

With both light- and tone-alone presentations, the frequency of non-associative blinking rose as a function of CS duration (Fig. 4A–B). Nevertheless, the acoustic cue induced a much higher level of non-associative blinking than the visual cue, particularly across the first four sessions (Fig. 4C–E). The spontaneous blink rate, assessed during the same session in which alternating blocks of tones were experienced, was also significantly increased, relative to the spontaneous blink rate of rats exposed to within-session presentations of the light and naïve animals that had never been exposed to any cues (Fig. 7).

4.1. CS modality

The intensities of the light and tone CS used in the current study are comparable to those reported in other experimental work. In eyeblink conditioning, a typical light CS uses electrical energy at a rate of 6 to 10 W [12–14,27]. The energy usage of the LED assembly used in the present study was 12 W. The watt, however, is a unit of electrical power unrelated to the light output level. The actual illumination intensity of our light CS was 400 lux, measured about 8 cm from the source (a level of illumination comparable to a brightly lit office). Based on wattage, our light might be slightly brighter than other light CSs. However, minus specific information on illumination intensity, it is difficult to make direct comparisons between our own light CS and that used in other studies. That is not the case with the tone CS. Our 2.8 kHz, 85-dB tone is similar, in terms of frequency and volume, with other studies that have utilized an acoustic CS [12–14,27]. Note, however, that our light CS was inserted into an opening in one wall of the operant chamber, whereas the tone CS was presented from above. It is possible, based on the rat's location and orientation at the time of stimulus presentation that the reception of the localized light may vary more than the reception of the less localized tone.

Adult rats appear equally capable of learning to associate a light or tone CS with a mildly aversive US [24,27]. A priori, it is difficult to know whether the salience of each stimulus will be perceptually analogous for the rat. Acquisition and performance of the conditioned eyeblink response is nearly equivalent in the light- and tone-paired

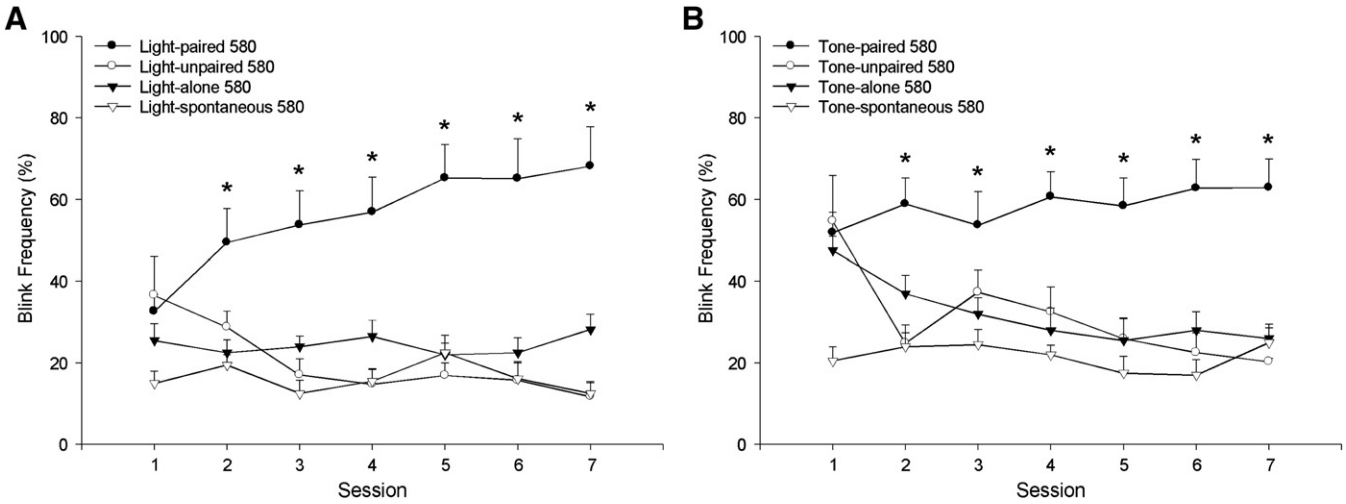

Fig. 6. Comparison of associative, non-associative, and spontaneous blink frequencies (mean±SE) at a constant 580 ms duration. Across the seven sessions, associative (paired conditioning), non-associative (unpaired conditioning, CS-alone), and spontaneous (CS-spontaneous) blink frequencies are illustrated for rats presented with the light CS (A) or the tone CS (B). The asterisks (*) indicate the light- and tone-paired rats generated a significantly higher percentage of blinks across sessions 2–7 than the other three groups of rats.

280 rats, however, suggesting the salience and associability of each stimulus is similar. We did not vary CS intensity in this study, however. It is possible that blinking rates to the light or tone CS would change if, for example, a brighter light or lower intensity tone were used as CSs.

The blink CRs generated by the tone-paired 880 rats had, on average, higher amplitudes and shorter onset latencies than the CRs generated by the light-paired 880 rats (Fig. 2A–C). The tone-paired 880 rats, as well as the tone-paired 580 rats, also produced unusually high CR percentages during the first few sessions, a result that appears to be at least partly attributable to an increase in non-associative blinking. The revised CR acquisition curves in Fig. 5 are proposed to approximate the true rates of associative learning among the various groups, once the non-associative blinks are subtracted out. In doing so, the acquisition curves to the light and tone CS are much more comparable across all three ISIs.

The CS modality-mediated differences in conditioned responding might arise because: (i) there is increasingly rapid learning as the CS–US interval is lengthened in the tone-paired rats; (ii) non-associative blinks to the tone are conflated with genuine conditioned responses; or (iii), the tone increases the spontaneous blink rate in a way that the light does not. The first possibility is unlikely based on numerous reports that find optimal rabbit EBC, defined as faster acquisition and a higher maximal asymptote, with ISIs in the range of 200–500 ms [15,17,18]. In adult rats, a recent ISI discrimination study found slower acquisition with an 880 ms ISI, relative to a 280 ms ISI, although the final percentage of conditioning responding was similar for both ISIs after 15 sessions [24]. Nevertheless, the tone-paired 580 and 880 learning curves in the current study display near maximal CR production in the very first session, with little further gain across the remaining sessions. It seems reasonable to suggest that the high level of responding during the first session is the result of tone-mediated non-associative blinks being counted as CRs, rather than the rat learning to near asymptote within the first 100 trials.

The results from Experiment 2 support the notion that the 85 dB tone increases non-associative blinking during its presentation. Investigations of the acoustic startle response in rats have found a 90 dB burst of white noise sufficient for evoking a whole-body startle response [28,29], which typically includes a reflex blink. It is possible, therefore, that a proportion of the tone-mediated non-associative blinks are the result of a mild startle blink response. However, Experiment 2 also found an increase in the within-session spontaneous blink rate in rats exposed to the tone, a result hard to reconcile if the non-associative blinks are solely startle responses. A more likely explanation then is that the tone induces a heightened level of responsiveness in the rat, which in turn leads to an overall increase in blinking [30].

The acquisition of EBC varies as a direct function of the tone CS intensity over the range of 65–86 dB [31]. While the non-associative blink rate found in the present study could presumably be reduced by utilizing a less intense tone, there would likely be a concomitant decrease in the rate of learning. Considering that eyeblink conditioning with longer, non-optimal ISIs results in only moderate levels of conditioned responding, careful consideration must be given to the trade-off between learning and non-associative blinking when utilizing a tone CS. Indeed, the current results lend strong support to the notion that a light might be a preferable CS for eyeblink conditioning adult rats, particularly if ISIs of 580 ms or longer are used.

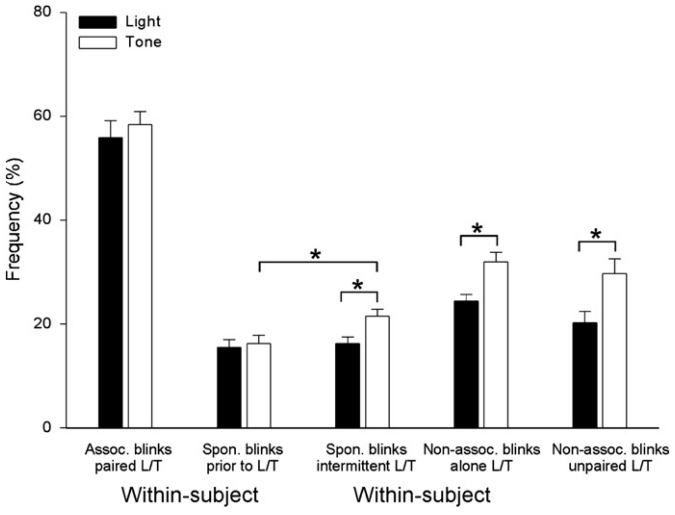

Fig. 7. Averaged associative, non-associative, and spontaneous blink frequencies (mean ± SE) at a constant 580 ms duration. The far left displays the frequency of associative blinks (CRs) in rats trained with the light or tone CS. The middle left displays the spontaneous blink rate during the adaptation session, 1 day prior to the start of paired eyeblink conditioning. The middle displays the spontaneous blink rate in rats intermittently exposed to lights or tones within the same session. Exposure to the tone resulted in a significant increase in the spontaneous blink rate, relative to light-exposed and naïve rats. The middle right displays the non-associative blink rate during presentation of the light or tone alone, with a higher percentage of blinking to the tone than the light. Finally, the far right displays non-associative blink rates for rats that underwent unpaired eyeblink conditioning, with a higher percentage of responding to the tone than the light.

Multiple-unit and single-unit recordings in the dorsolateral IP have revealed populations of neurons that discharge to the CS and US, with other units exhibiting firing patterns that are tightly coupled to the execution of the behavioral response in the rat [32,33] and rabbit [8,14]. The majority of studies that have recorded conditioning-related IP units have utilized a tone CS, however, leaving other sensory modalities relatively unexplored.

We have previously subjected rabbits to eyeblink conditioning with a light-tone compound CS or with alternating light and tone CS presentations, with both groups then tested with the light-alone, tone-alone, or light-tone compound [14]. IP units were recorded after asymptotic levels of conditioned responding were reached. Approximately half the sampled units responded in some fashion on the test trials, while the other half did not respond at all. In rabbits trained with the alternating light and tone CS presentations, which best corresponds to the present study, nearly 90% of the responsive units responded exclusively, and in approximately equal numbers, to the light, to the tone, or to the compound, although the latter had never been presented to the subject until testing. The results indicate that uni-modal units exist in the IP, as well as units that respond to the compound stimulus discretely and separately from the light and tone. Indeed, it might be case that the mammalian brain has evolved to process multisensory signals, and utilization of a single sensory stimulus, as is typical of most conditioning studies, might not fully engage available multisensory learning mechanisms [34].

4.2. CS–US interstimulus interval

Based on a large body of research [15,17,18,26], we hypothesized there would be slower rates of acquisition and lower final percentages of CRs as the conditioning ISI was lengthened. This is exactly what was observed in rats trained with the light CS, at least through seven sessions of conditioning. The acquisition curves for rats trained with the tone CS, on the other hand, did not show as straightforward a relationship to the ISI length.

Light-paired 280 rats displayed faster rates of acquisition and a higher final level of conditioned responding than light-paired 580 or 880 rats. Tone-paired 280 rats displayed higher levels of conditioned responding by the end of training, relative to tone-paired 580 and 880 rats, yet they also demonstrated the slowest rate of acquisition over the first few sessions. As discussed above, we posit that the unusually high percentage of blink CRs in the tone-paired 580 and 880 rats during the first few acquisition sessions is the result of a generalized training effect to the tone that increased the number of non-associative blinks emitted during the tone CS [30].

The light- and tone-paired 280 rats were also the only groups to display significant cross-session alterations in CR latency. That is, across the seven sessions of conditioning the CR onset and peak latencies were shortened, with the latter occurring near US onset. The eyeblink CRs produced by rats conditioned with the 580 and 880 ms ISI underwent more modest alterations across the seven sessions. As the ISI was increased, the distribution of CR onset latencies were more dispersed and shifted towards the US (Fig. 2A), a pattern similar to that observed in rabbits [10,35].

In terms of CR peak latency, the data suggests that rats might not be as capable of accurately timing the US onset as rabbits. CR temporal peaks centered around the time when the US is normally presented have been consistently observed in rabbits, with ISIs between 125 and 1000 ms [16,18,36,37]. In contrast, while peak latencies in our own light- and tone-paired ISI groups were comparable and approached the interstimulus interval, the peak latencies did not fall around the US onset (Fig. 2B). By the end of training, the mean peak latencies with the 280 and 580 ms ISIs occurred after US onset, while the peak latencies with the 880 ms ISI occurred before US. Perhaps more training sessions would yield better timed peak amplitudes. It is also possible that the poor alignment between CR peak latencies and the conditioning ISI resulted from the intrusion of non-associative blinks. However, non-associative blinking decreased across the seven sessions, the point at which CR timing should be most accurate. We provisionally suggest, therefore, that rats might not be capable of temporally encoding the ISI as accurately as rabbits.

It does appear, however, that an ISI of 200–500 ms is optimal for learning in both the rat and rabbit. That is, as in previous studies with the rabbit [15,17,18], the 280 ms ISI in the current study resulted in the fastest acquisition, at least in light-paired rats, and the highest levels of conditioned responding. As mentioned above, however, adult rats acquired approximately the same level of asymptotic responding in an ISI discrimination study with 280 and 880 ms ISIs, albeit the latter took more training sessions [24]. Thus, rate of acquisition may be more sensitive to the conditioning ISI than the final asymptotic level of conditioned responding.

4.3. Spontaneous and non-associative blinks

The percentage of spontaneous blinking during adaptation, prior to the rats' exposure to any stimuli, varied (from 9–29%) as a function of the EMG analysis window (Fig. 1A–B). Nevertheless, with a 580 ms trial duration, naïve rats blinked on approximately 15% of all trials (Fig. 7, middle left), a much higher rate than observed in rabbits with a 500 ms trial duration, which yielded a spontaneous blink rate of less than 3% [9]. The low spontaneous blink rate and toleration of restraint are two primary reasons that eyeblink conditioning research has utilized rabbits as subjects over the years.

In rats exposed to within-session light presentations the averaged spontaneous blink rate is statistically equivalent to the blink rate during adaptation, whereas a significant increase in blinking, rising to about 22%, is seen in rats exposed to the tone (Fig. 7, middle). Non-associative blinking during the presentation of the light or tone is also significantly higher than the spontaneous blink rate of the same subject within the same session, albeit rats still blink significantly more to the tone than the light (Fig. 7, middle right). Thus, it appears that the presentation of either a visual or auditory cue is sufficient to elicit an increase in blinking, relative to the baseline blink rate in the absence of any cues. Interestingly, the non-associative blink rate during light and tone-alone presentations is similar to the non-associative blink rate to the CS during explicitly unpaired CS–US eyeblink conditioning in rats (Fig. 6). And, in fact, both non-associative blink rates (Fig. 7; averaging ~30%) were comparable to those observed in other unpaired eyeblink conditioning studies with a tone CS [38,39].

As shown in Fig. 2D, the mean onset latencies in light- and tone-alone 280 rats exceeded the total duration of the stimulus presentation. In other words, the initiation of many non-associative blinks occurred after CS offset, although as seen in Fig. 4C at least a subset of blinks were recorded when the EMG analysis was restricted to the 280 ms stimulus interval. The discrepancy lies in the way the blink frequency was analyzed versus blink latency. Frequency was calculated during the CS only, just as the CR frequency was calculated based on CS–US paired trials during the ISI. Latency, on the other hand, is calculated from CS onset to the end of the trial, again, just as was done with the CS-alone trials during paired conditioning. As a result, only a portion of the non-associative blinks were being counted with the frequency analysis. Indeed, examining the first session only (Fig. 4A–B), the light- and tone-alone 280 rats blinked on 10% and 29% of the trials, respectively. Analysis of the whole trial from CS onset, however, reveals that the light-alone rats blinked on 35% of the first session trials, and tone-alone rats blinked on 53% of the trials. In comparison, the light-alone 580 and 880 rats blinked on 42% and 32% of the first session trials, whereas tone-alone 580 and 880 rats blinked on 55% and 53% of the trials, respectively. These rates are again higher than the frequency rates presented in Fig. 4, although the differences are not as large.

It appears that, within each CS modality, all rats blink at approximately the same rate, independent of the CS duration. This suggests that non-associative blinking might be tied to the onset of the light or tone, and that, when emitted, the resulting blink is fairly uniform in terms of

latency and amplitude (Fig. 2D–F). The biggest differences in non-associative blinking to the two stimuli relate to the frequency and onset latencies. If all blinks are counted, light-alone rats blinked on 37% of all first session trials, whereas the tone-alone rats blinked on 54%. The blinks emitted by the tone-alone rats also had shorter onset latencies. Both points fit with our ongoing hypothesis that the tone, but not the light, induces a heightened level of responsiveness in the rat, resulting in more blinks with earlier onsets.

What, then, is the minimum duration that the light or tone must be presented in order to evoke a non-associative blink? Based on data from a recent study we suggest that the minimum is less than 50 ms. The study examined the effects of hippocampal lesions on short- and long-trace eyeblink conditioning in rats [40]. The CS was a 50 or 500 ms tone CS and the trace duration was 500 or 50 ms in duration, resulting in a 550 ms ISI for both groups. The CR frequency in the 500 ms CS/50 ms trace (control) group was approximately 54% on the first day of conditioning, similar to our own results in the tone-paired 580 rats. The CR frequency in the 50 ms CS/500 ms trace (control) group was approximately 32% on the first day of conditioning. In the current study, only modest learning (i.e., conditioned responding) is proposed to occur on the first session of delay EBC once non-associative blinks are deducted (see Fig. 5B, 580 ms ISI). Presumably, then, a proportion of the first session blinks observed during the more difficult trace EBC are also non-associative. The difference in CR frequency between the short- and long-trace eyeblink conditioning groups might reflect not only differential learning rates, but higher levels of non-associative blinking with the longer (500 ms) than shorter (50 ms) tone CS. Nevertheless, if correct, the 50 ms tone is sufficient to evoke non-associative blinks.

Presentation of either CS increases the rate of non-associative blinking, relative to the spontaneous blink rate, which can itself be increased by mere exposure of the subject to the tone, but not the light. Once initiated, the timing and amplitude of the non-associative blinks appear to be relatively uniform, with the tone producing more blinks, and during the first session, earlier onsets. Further study is warranted to more precisely define the kinetics of each type of blink in the rat, as well as the underlying neural circuitry [cf. 41].

4.4. Summary

The present study provides new information regarding delay EBC in the adult rat. The acquisition and expression of blink CRs proved most efficacious for both the light and tone CS with the 280 ms ISI. Rats trained with the tone CS at the two longest ISIs exhibited elevated CR frequencies during the early acquisition sessions, most likely due to an increase in non-associative blinking. The light CS might be a better cue, therefore, for assessing eyeblink conditioning in the adult rat, particularly with CS–US intervals on the order of 580 ms or longer.

The current results also detail the frequencies at which adult rats spontaneously blink, prior to their exposure to any stimuli and during within-session exposures to the light or tone. Together with the non-associative blink rates for both CSs, with or without US presentations in the same session, the data provides a baseline for assessing CR frequency rates. Such information is relevant not just for paired and unpaired eyeblink conditioning, but also for studies that employ ISI discriminations or discrimination learning (CS+/CS−) in the adult rat.

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