

The Role of Awareness in Differential Delay Eyeblink Classical Conditioning

by

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Abstract

Eyeblink classical conditioning (EBCC) is an experimental procedure widely used for understanding learning and memory in a variety of species. In EBCC, a neutral conditioned stimulus (CS; a tone) is repeatedly paired with an unconditioned stimulus (US; a corneal airpuff). Following repeated presentations, the CS alone begins to elicit an eyeblink, known as the conditioned response (CR). The CR is taken as evidence that the animal has formed a lasting association between the CS and US. However in humans, whether this form of learning depends on conscious awareness of the CS-US relationship has been largely debated. One possibility for this debate is that during differential delay EBCC, awareness may be necessary when the CSs are difficult to discriminate. The aim of the current study was to test this prediction in three groups of participants by manipulating the discriminability of tones (CSs) within a differential delay EBCC procedure and probing participant's awareness of the CS-US relationship. In Group I, 1000 Hz tone and white noise were used as CSs, whereas in Groups II and III, 1000 Hz vs 1400 Hz and 1000 Hz vs 1150 Hz tones were used as CSs respectively. Both aware and unaware participants demonstrated differential conditioning in Group I suggesting that awareness was not necessary for differential conditioning. In Group II, aware participants demonstrated early conditioning compared to unaware participants. However, in Group III no participants (aware and unaware) demonstrated conditioning suggesting that awareness was not sufficient for successful differential conditioning.

The Role of Awareness in Differential Delay Eyeblink Classical Conditioning

Introduction

Human memory has been classified into declarative and non-declarative memory systems and is considered to be mediated by distinct neural substrates (Squire & Zola-Morgan, 2015). Declarative memory refers to memories related to facts (semantic memory) and events (episodic memory) that can be consciously recollected. This type of memory system allows for rapid recollection of information and learning about the relationship between specific events in the environment as opposed to non-declarative memory which depends on repetition and practice for acquisition. Furthermore, declarative memory can be effectively manipulated to guide performance under different contexts (e.g. semantic knowledge that certain foods contain gluten and episodic knowledge that one is allergic to it, allows one to avoid foods containing gluten in the future). One implication of the ability to retrieve declarative memory under different contexts is that it depends on awareness. Successful non-declarative memory performance, on the other hand, does not require conscious awareness and is manifested independently of the declarative memory system. As such it can be expressed through motor and perceptual skills such as priming, perceptual learning, and associative learning. Simple forms of associative learning, such as classical conditioning, involve a change in the behavior of the organism in response to past exposure to two or more temporally correlated environmental stimuli (Fanselow & Wassum, 2016; Rescorla, 1988).

In classical conditioning, a neutral conditioned stimulus (CS) is presented in temporal proximity with a biologically significant unconditioned stimulus (US). Initially, the neutral stimulus alone does not elicit an associative response in the organism, while the US elicits a reflexive or autonomic unconditioned response (UR) in the organism. Following repeated presentations of the CS-US pairing, the CS alone elicits an anticipatory response called a conditioned response (CR). A CR is taken as

evidence of the formation of an association between the CS and US such that the CS serves as a cue that predicts the occurrence of the US. This form of associative learning is considered to be a fundamental form of learning by which the relationship between events in the environment is represented (Rescorla, 1988) and the basis by which more complex behavior and cognition is determined (Wasserman & Miller, 1997). Two different paradigms of classical conditioning (**Figures 1a and 1b**) have been extensively studied: delay and trace conditioning. In delay conditioning, the CS overlaps and co-terminates with the US. In trace conditioning, there is a stimulus-free period between the CS offset and US onset. Considerable evidence indicates that trace conditioning is dependent upon the declarative memory system (Clark, Manns, & Squire, 2001, 2002; Clark & Squire, 2000; Squire & Dede, 2015), and also awareness of the CS-US contingency. However, the question of whether performance in delay conditioning depends on awareness of the CS-US contingency has been largely debated (Clark et al., 2001, 2002; Clark & Squire, 2004; Lovibond & Shanks, 2002; Shanks & Lovibond, 2002; Weidemann, Broderick, Lovibond, & Mitchell, 2012; Wiens & Ohman, 2002). A better understanding of the role of awareness in this form of associative learning proves to be useful in multiple ways.

- a) Associative learning, as assessed by classical conditioning paradigms, is considered to be a fundamental form of learning and an adaptive trait in human and non-human animals (Fanselow & Wassum, 2016; Wasserman & Miller, 1997). Understanding the role of human awareness in conditioning will be informative for drawing parallels between human and non-human animal findings (Lovibond & Shanks, 2002).
- b) Findings from behavioral and neuroscientific studies of classical conditioning in human and non-human animals have been used in the development of treating clinical disorders such as phobias and anxiety (Lovibond & Shanks, 2002). For example, from a learning theory perspective the etiology and maintenance of anxiety disorders are considered to be mediated by associative learning

mechanisms in which initial panic attacks are associated with environmental cues (CS) (Bouton, Mineka, & Barlow, 2001; Mineka & Oehlberg, 2008) such that the experience of anxiety becomes a conditioned response (CR) to these environmental cues. Determining whether the formation of these associations is automatic and unconscious or depends on awareness will be valuable in developing effective treatment strategies.

- c) Moreover, determining the role of awareness during conditioning will be useful in identifying the neural mechanisms underlying classical conditioning in humans. Conditioning, when construed as an automatic, unconscious process, is mediated by the formation of excitatory and inhibitory connections between nodes of CS and US representations (e.g. LTP/LTD in the cerebellar cortex). On the other hand, when considered to be dependent on awareness, conditioning may be mediated by forming propositions about the relationship between the CS and US which involves the recruitment of a supplementary neural network (e.g. interactions between cerebral and cerebellar cortex) (Clark et al., 2002; Lovibond & Shanks, 2002).

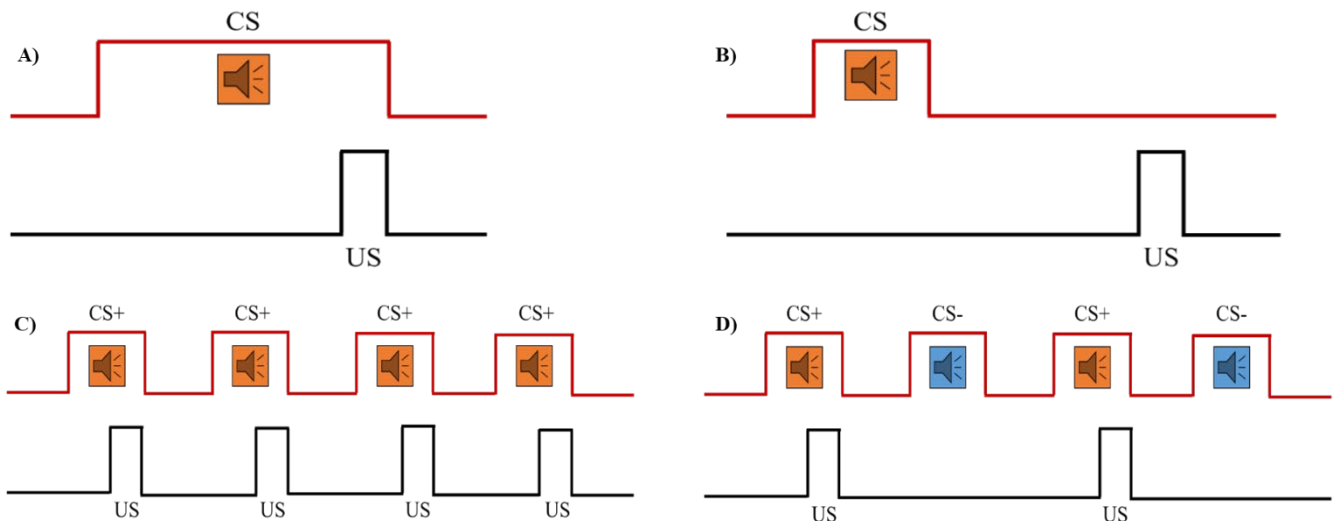


Figure 1. Typical trial structures for classical conditioning. **A)** delay and **B)** trace eyeblink conditioning procedures. A schematic of a typical trial sequence. **C)** single cue delay conditioning **D)** differential delay conditioning. In differential delay conditioning, the CS+ is always followed by and coterminates with an airpuff and the CS- is never followed by an airpuff.

Eyeblink classical conditioning (EBCC) is one of the most extensively studied forms of classical conditioning and consequently, we have a detailed understanding of the behavioral processes and its underlying neural mechanisms (Diana. S Woodruff-Pak & Steinmetz, 2000). Typically, in EBCC the CS is a tone and the US is a corneal airpuff. Following repeated presentations of the CS and the US, the CS alone begins to elicit an anticipatory eyelid closure (CR) just before the occurrence of the airpuff. Clinical applications of the EBCC procedure have been used to explore learning-related impairments in psychopathologies such as autism, bipolar disorder, fetal alcohol syndrome, amnesia, Alzheimer's disease, alcoholism and attention deficit hyperactivity disorder (Bolbecker et al., 2009; Cheng et al., 2015; Cheng et al., 2017; Fortier et al., 2008; Jacobson et al., 2011; Jacobson et al., 2008; McGlinchey-Berroth, Carrillo, Gabrieli, Brawn, & Disterhoft, 1997; McGlinchey-Berroth et al., 1995; McGlinchey-Berroth, Fortier, Cermak, & Disterhoft, 2002; McGlinchey, Fortier, Capozzi, & Disterhoft, 2005; Sears, Finn, & Steinmetz, 1994; Steinmetz, Tracy, & Green, 2001; Diana S. Woodruff-Pak, Finkbiner, & Sasse, 1990). Several characteristics of the EBCC paradigm make it well-suited for studying learning impairments in a wide range of clinical pathologies. The EBCC paradigm allows for exquisite control over stimuli presentation and also for precise measurements of the temporal properties of the conditioned response. This procedure also does not require complicated instructions, which may be misunderstood by the participant, or involve individual participant strategies. Considering these advantages, performance in EBCC has been used as a biomarker for several neurodevelopmental disorders (Reeb-Sutherland & Fox, 2015).

The majority of EBCC studies have adopted one of two paradigms: single cue or differential conditioning. Studies that have used single cue delay EBCC (**Figure 1c**) have established a dissociation between awareness and eyeblink conditioned responses (**Table 1**). When medial temporal lobe amnesics and healthy matched controls were tested on single cue delay EBCC, performance during conditioning

did not differ between the two groups indicating that single cue conditioning is not dependent on awareness and the declarative memory system (Gabrieli et al., 1995). Using a masking task to manipulate awareness, Frcka and colleagues (1983) reported that awareness was also not necessary for single cue delay conditioning. Moreover, another study showed that engaging participants in a secondary task (e.g. timed finger tapping) during the single cue delay paradigm did not affect their conditioned responses suggesting that awareness is not necessary for the single cue delay paradigm (Papka, Ivry, & WoodruffPak, 1997). However, contrary to the findings reported above, it was also observed that acquisition of CRs parallels expectancy of the US during single cue delay EBCC (Weidemann, Best, Lee, & Lovibond, 2013) indicating a close relationship between awareness and single cue delay EBCC.

While multiple single cue delay conditioning studies support the idea that awareness and delay conditioning are independent processes, trace conditioning studies report a different set of findings. In one single cue trace conditioning study, participants who were given a secondary task (e.g. digit matching) showed both impaired contingency awareness and CRs relative to participants who were not given a secondary task, indicating that performance in single cue trace conditioning is dependent upon contingency awareness (Manns, Clark, & Squire, 2000). In another study, participants were subjected to either single cue trace or delay conditioning and assessed for their contingency awareness (Manns, Clark, & Squire, 2001). Performance in participants receiving single cue delay conditioning did not differ between aware and unaware participants, suggesting a dissociation between awareness and delay CRs. However, of the participants who received single cue trace conditioning, only those who were aware of the CS-US contingency were able to demonstrate CRs, suggesting a relationship between awareness and trace CRs. Some have argued that the single cue conditioning paradigm is not well-suited to study the role of awareness in humans (Weidemann et al., 2013) because participants may become

aware of the contingency only after a few initial trials. In addition, distinguishing the associative and non-associative factors contributing to conditioning (e.g. sensitization) is more difficult in single cue compared to differential paradigms (Clark & Squire, 2000).

An alternative to the single cue paradigm is differential conditioning (**Figure 1d**). In differential delay EBCC, two tones are delivered – one tone (CS+) is always followed by and co-terminates with a corneal airpuff and the other tone (CS-) is never followed by an airpuff. The CS- in this paradigm serves as a control stimulus to which the responses to the CS+ can be compared. Greater CRs to CS+ relative to CS- indicate successful differential conditioning. In this paradigm, contingency awareness refers to the participant's ability to explicitly express that a CS+ is always followed by an airpuff and the CS- is never followed by an airpuff. If only aware but not unaware participants are able to produce differential conditioned responding, then this suggests that awareness is necessary for differential conditioning and is partially dependent upon declarative memory processes. If, on the other hand, unaware participants are also able to produce differential conditioned responding, then it suggests that awareness is not necessary for differential conditioning.

Studies addressing the role of awareness using a differential EBCC paradigm have produced inconsistent results (**Table 2**). It has been reported that both temporal lobe amnesics (mean age =67.5 years) and healthy matched controls (mean age = 66.9 years) were able to demonstrate differential delay conditioning. Importantly, neither group were able to explicitly identify the CS+ or CS-, indicating that awareness is not necessary for differential delay conditioning (Clark & Squire, 1998). This finding has also been replicated in normal healthy participants (mean age = 49.0 years) (Smith, Clark, Manns, & Squire, 2005). Further attempts to replicate these findings with younger (mean age =20.3 yrs.) participants failed in that only aware participants were able to produce differential responding, suggesting that successful differential delay conditioning is dependent on awareness (Lovibond, Liu,

Weidemann, & Mitchell, 2011). Moreover, it was reported that awareness was necessary for differential delay conditioning in both younger (20-35 years) and older (60-75 years) participants (Knuttinén, Power, Preston, & Disterhoft, 2001b). One common characteristic among these studies is that the CS+ and CS- were easily distinguishable (e.g. tone and white noise). Such studies frequently manipulate awareness by giving participants a demanding secondary task during the experiment (Carrillo, Gabrieli, & Disterhoft, 2000; Clark & Squire, 1999). The basic assumption behind using this secondary task is that these tasks occupy additional cognitive resources normally dedicated to processing the CS-US contingency and in some cases engages the neural circuitry essential for eyeblink conditioning (Papka et al., 1997). However, it has been proposed that performance in the secondary task and eyeblink conditioning can occur in parallel and that humans with a unique ability to use language can achieve an “Aha!” moment during the conditioning session (LaBar & Disterhoft, 1998) further complicating the interpretation of results.

Instead of a tone and white noise, some studies have used two different pure tones as CSs and reported that awareness was necessary for differential delay EBCC (Huang et al., 2014; Ross & Nelson, 1973; Weidemann, Satkunarajah, & Lovibond, 2016). One possibility that can be inferred from these studies is that during differential delay EBCC, awareness may be necessary when the CS+ and CS- are relatively similar to each other (Clark et al., 2002). However, those studies (Huang et al., 2014; Ross & Nelson, 1973) that used two tones as CSs presented fairly distinct frequencies (e.g. 800 Hz vs 2100 Hz; 1000 Hz vs 3000 Hz tones) . The frequencies of these tones may be sufficiently distinct given that participants are able to demonstrate differential responding when the frequency difference was as low as 50 Hz (Moore, 1964). Pilot data from our lab using 1000 Hz vs 1400 Hz as CSs revealed that only participants classified as aware were able to produce differential responding, further suggesting that differential conditioning of tones of similar frequencies may rely on awareness (**Figure 2**).

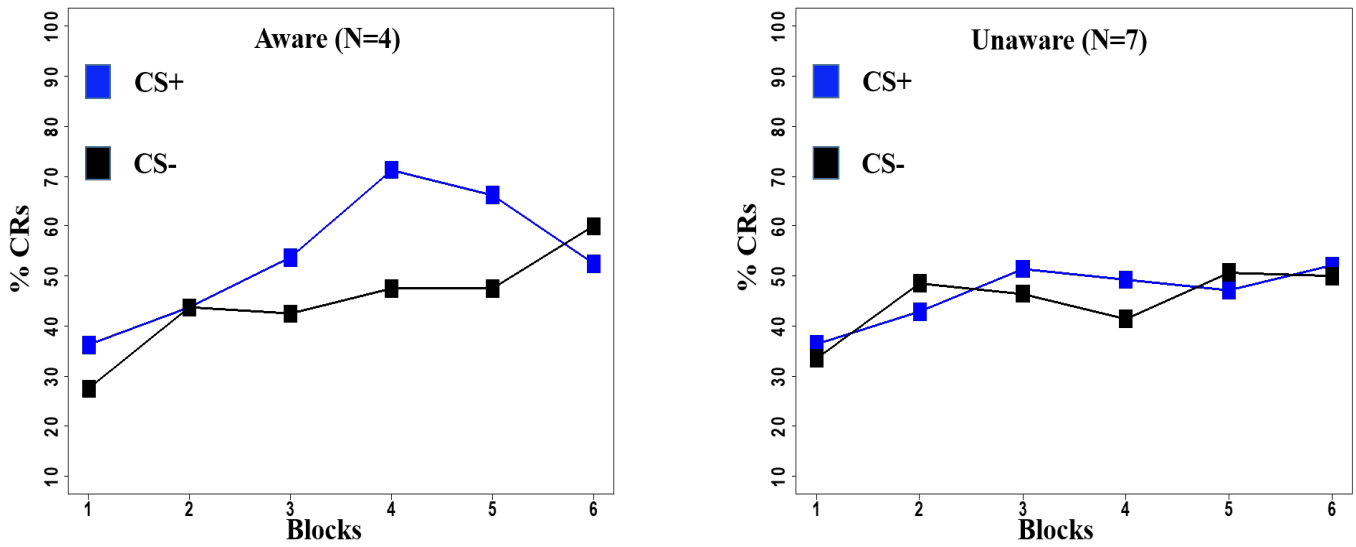


Figure 2. Results from a pilot study using differential delay EBCC procedure with 1000 Hz vs 1400 Hz as CSs indicating that awareness is necessary for differential conditioning.

The present study tested the general prediction that performance in differential delay EBCC depends on contingency awareness when two tone CSs are relatively similar. In other words, only participants who are aware of the CS-US contingency will be able to show differential eyeblink conditioning to two perceptually similar CSs.

Methods

Participants: A total of 60 (54 female) participants were recruited for the study. Participants were students (mean = 19.2 years old, standard deviation = 1.58) at Auburn University and were recruited through a research participation portal for which they received extra credit in their psychology courses. Participants were pre-screened for possible neurological or psychological disorders before participating in the study. All procedures were approved by the Institutional Review Board at Auburn University.

Apparatus: The timing and delivery of the stimuli was controlled by custom built software based on LabView (v.7.1) which was connected to a desktop PC. Auditory CSs were presented through two computer speakers located approximately two feet away. A silent movie (*'The Gold Rush'*) was played on a monitor approximately two feet away. The airpuff US was delivered through tubing connected to a solenoid valve and attached to custom modified swimming goggles. An infrared sensor attached to a fiber optic cable which continuously monitors the movement of the eyelids during the experiment was also attached to the modified goggles (Miller, Li, Weiss, Disterhoft, & Wyrwicz, 2005). These procedures and instruments have been successfully utilized in previous behavioral and functional magnetic resonance imaging (fMRI) experiments (Cheng, Disterhoft, Power, Ellis, & Desmond, 2008; Cheng, Faulkner, Disterhoft, & Desmond, 2010; Cheng et al., 2014; Cheng et al., 2017). To provide a quiet environment, conditioning took place in a sound attenuated booth (ETS. LINDGREN Acoustic Systems).

Procedure: Audiometric testing was administered to participants prior to conditioning to ensure normal hearing thresholds. The participants were instructed to pay attention to a silent movie and that they would be given a short quiz following the movie. They were also informed that the goal of the experiment was to examine the effects distracting tones and airpuffs have on their ability to remember

details related to the movie. After answering any questions, experimenters positioned participants in the sound attenuated booth and fitted them with the conditioning goggles.

Conditioned and Unconditioned Stimuli

Two hundred trials of two conditioned stimuli were presented (100 CS+ and 100 CS-). Each CS lasted 850 ms and each trial lasted 2000 ms with a variable intertrial interval (ITI) of 5-7 secs (Carrillo, Thompson, Gabrieli, & Disterhoft, 1997). Participants were randomly assigned to one of three groups. In Group I, CSs were 1000 Hz and a white noise, counterbalanced and presented in a pseudorandom order such that no more than two trials of the same CS were consecutively presented. In Group II, CSs were 1000 Hz and 1400 Hz and in Group III, CSs were 1000 Hz and 1150 Hz. CS+ trials always coterminated with US presentations and CS- trials were always presented alone. All the auditory CSs were presented at 85 dB intensity. The US was a 100 ms 5 psi (measured at the delivery site) left corneal airpuff (**Figure 3**).

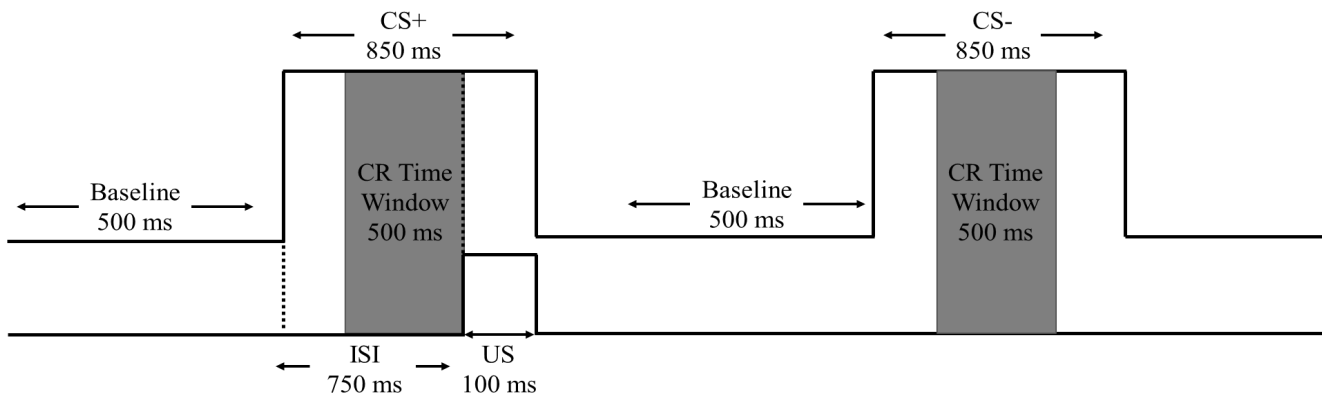


Figure 3. Timing of CS+ and CS- trial types. CSs lasted 850 ms and USs lasted 100 ms. Shaded areas indicate the time window that was sampled to classify eyeblinks as CRs. (CS: conditioned stimulus, US: unconditioned stimulus, CR: conditioned response, ISI: interstimulus interval)

Awareness

Immediately following conditioning, a 14-item questionnaire consisting of True or False statements was administered to assess participants' awareness of the CS-US contingency (**Appendix A**). This

questionnaire was the same one used in a previous single cue eyeblink conditioning study (Manns et al., 2000) but given twice to assess awareness of both CS+ and CS- contingencies. The order of presentation of CS+ and CS- items were randomized. In addition to the True/False statements, a schematic of a trial structure (**Appendix B**) was presented to further probe the participant's understanding of the temporal sequence of events. The participants were asked to report at which time point (as indicated by letters in the schematic) along the duration of CS+ and CS- presentations, the US occurred. Lastly, a True or False movie questionnaire was presented to the participant (**Appendix C**).

Analysis: An eyeblink was considered a CR if the response amplitude within a 500 ms time window before US onset was greater than four times the standard deviation of the baseline period (500 ms before CS presentation). This procedure was used to minimize the inclusion of orienting and voluntary responses (Spence & Ross, 1959). Trials were divided into five successive blocks with 20 trials per block for each CS. % CRs in each block were calculated by counting the total number of CRs in that block and dividing that by the total number of trials in that block. An increase in % CRs to CS+ relative to CS- across successive blocks was considered as successful differential delay conditioning.

Participants were categorized as aware if they scored at least 12 on the questionnaire and correctly identified when/if the US was presented on the schematic. This threshold is based on the probability of obtaining 12 correct responses out of 14 from a binomial distribution which is set to $p = 0.05$. % CRs to CS+ and CS- were compared among aware and unaware participants across the three groups. Peak latency and onset latency of the CRs were also compared across the groups to assess differences in CR topography. Peak latency refers to the time at which the maximal eyelid closure occurred during the CR and onset latency refers to the time point at which the CR began to rise. These measures reflect the temporal and adaptive properties of the CR and have also been shown to be sensitive to learning (Cheng et al., 2010; Green, Ivry, & Woodruff-Pak, 1999). Behavioral measures of learning (% CR, peak latency

of CR, and onset latency of CR) was examined in a repeated measures analysis of variance (ANOVA) of between and within subject factors (Group, Awareness, Blocks, and CS Type) to assess the effects of CS similarity and awareness using SPSS software (SPSS Version 24).

Results

A repeated measures ANOVA on % CR with Group (Group I, Group II, Group III) and Awareness (Aware, Unaware) as between subject factors and Blocks (1-5) and CS Type (CS+, CS-) as within subject factors was conducted. The omnibus F-test revealed a significant main effect of CS Type indicating overall greater responding to CS+ compared to CS- trials ($F(1,54) = 30.77, p < 0.0001$). A significant CS Type x Group interaction effect was also observed ($F(2,54) = 5.01, p = 0.01$) (**Figure 4**). Post hoc comparisons indicate that relative to Group I, Group II showed a significant increase in % CRs to CS- trials ($t(38)=2.55, p = 0.015$) and Group III showed a trend ($t(38)=1.90, p = 0.065$) (white bars in **Figure 4**). There were no significant differences in % CRs to CS+ across groups (p 's > 0.05). The main effect of Block was not significant ($F(4,216) = 0.14, p = 0.965$) but a significant interaction between CS Type and Block was observed ($F(4,216)= 2.51, p = 0.043$) (**Figure 5a**). A three way interaction between CS Type, Block, and Group was not significant ($F(8,216) = 0.969, p = 0.461$) (**Figure 5b**).

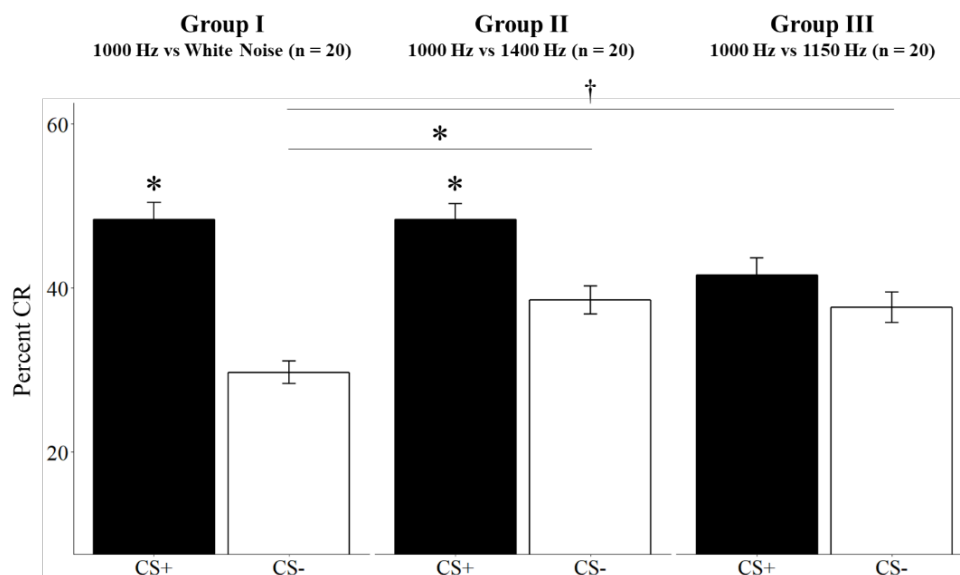


Figure 4. Overall % CRs to CS+ and CS- trials across three groups. Participants in Groups I and II showed significantly greater responses to CS+ relative to CS- while participants in Group III did not. Relative to Group I, Group II showed greater % CRs to CS- trials and Group III showed a trend.
* $p < 0.05$, † $p < 0.07$.

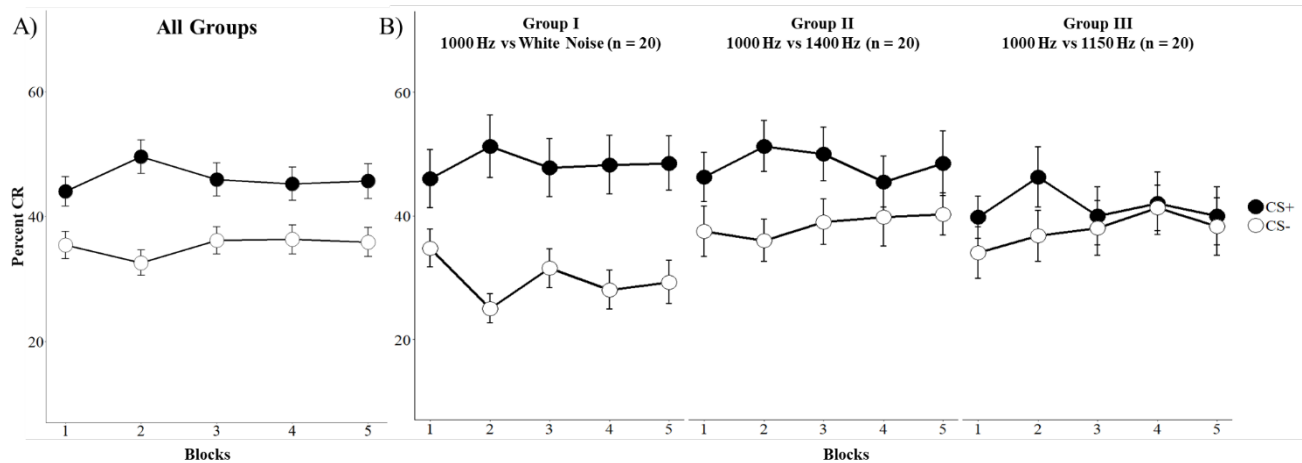


Figure 5. Overall % CRs to CS+ and CS- trials across three groups over time. **A)** A significant interaction between CS Type and Block was observed but no significant main effect of Block. **B)** Participants in Groups I and II showed significantly greater responses to CS+ relative to CS- while participants in Group III did not. These data suggest that CS similarity influenced differential conditioning.

In order to assess the role of awareness within each Group, a CS Type x Block x Awareness repeated measures ANOVA was performed for each Group.

Group I: A significant main effect of CS Type ($F(4,72) = 3.04, p = 0.022$) and a CS Type x Block interaction ($F(1,18) = 19.22, p < 0.0001$) were detected. Furthermore, the interaction between CS Type and Awareness was not significant ($F(1,18) = 0.08, p = 0.78$). These findings suggest that both aware and unaware participants were capable of producing differential CRs (**Figures 6a and 6b**).

Group II: A significant main effect of CS Type was detected ($F(1,18) = 16.46, p = 0.001$) but the interaction between CS Type and Block was not significant ($F(4,72) = 0.92, p = 0.459$). Similar to Group I, the interaction between CS Type and Awareness was also not significant ($F(1,18) = 0.238, p = 0.632$). However, the interaction between CS Type, Block, and Awareness was significant ($F(4,72) = 6.389, p < 0.0001$) (**Figures 6c and 6d**), suggesting that the time in which participants demonstrated differential conditioning depended on their awareness. Post hoc comparisons showed that aware

participants demonstrated significantly greater % CRs to CS+ relative to CS- during Block 1 ($t(10) = 5.04, p = 0.0005$) and Block 2 ($t(10) = 3.50, p = 0.006$) while unaware participants demonstrated significantly greater % CRs to CS+ relative to CS- during Block 3 ($t(8) = 3.65, p = 0.006$) and Block 5 ($t(8) = 3.47, p = 0.008$) as indicated by asterisks in Figure 6c and 6d.

Group III: No significant main effects or interactions were detected (all p 's > 0.05) (**Figure 6e and 6f**).

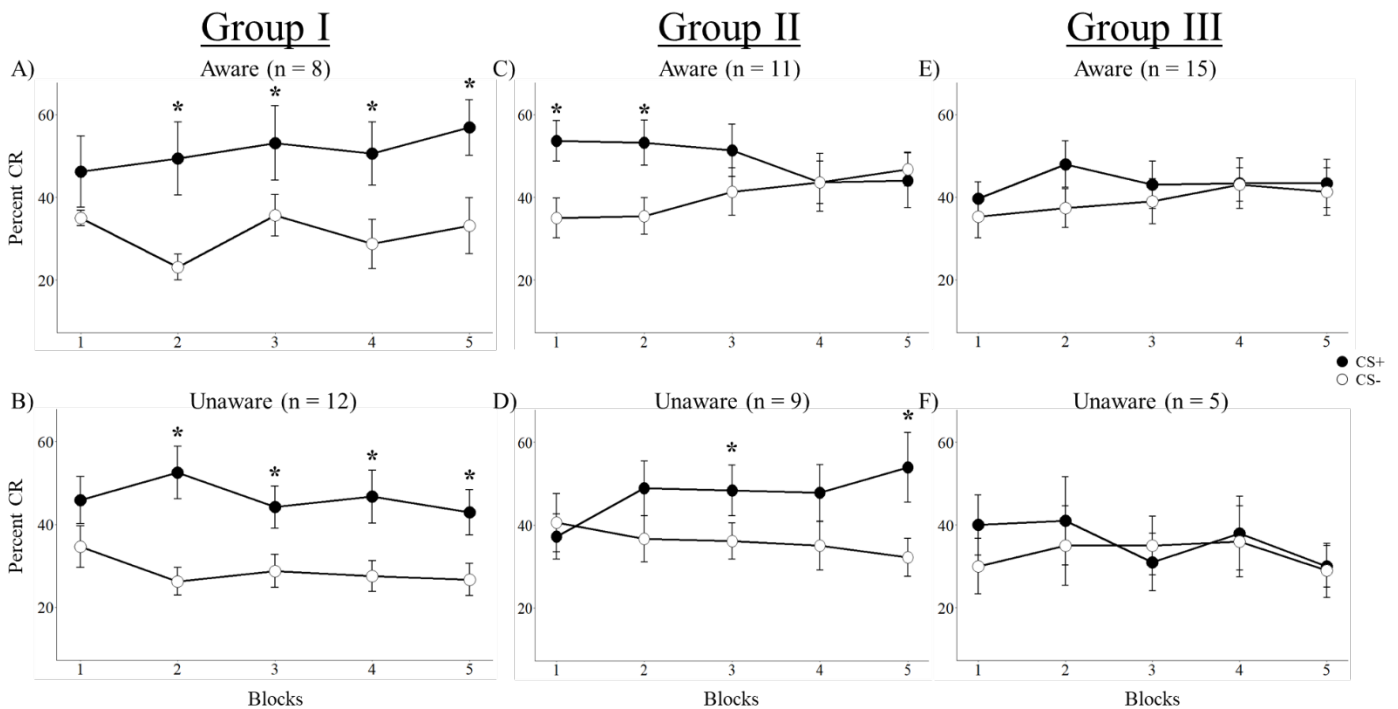


Figure 6. % CRs broken down into Groups and Awareness. **A-B)** Both Aware and Unaware Group I participants showed greater % CRs to CS+ relative to CS- when CSs were distinct (1000 Hz vs. white noise). **C-D)** Significantly greater % CRs to CS+ relative to CS- was observed early (Blocks 1 and 2) for Aware participants and later (Blocks 3 and 5) for Unaware participants when CSs were similar (1000 Hz vs. 1400 Hz). **E-F)** No significant differences were detected when CSs were very similar (1000 Hz vs. 1150 Hz).

% CRs and Awareness: Pearson correlation coefficients between participants' awareness scores and their differential CR (% CRs to CS+ minus % CRs to CS-) were calculated for each group and none reached significance (all p 's > 0.05) (**Figure 7**).

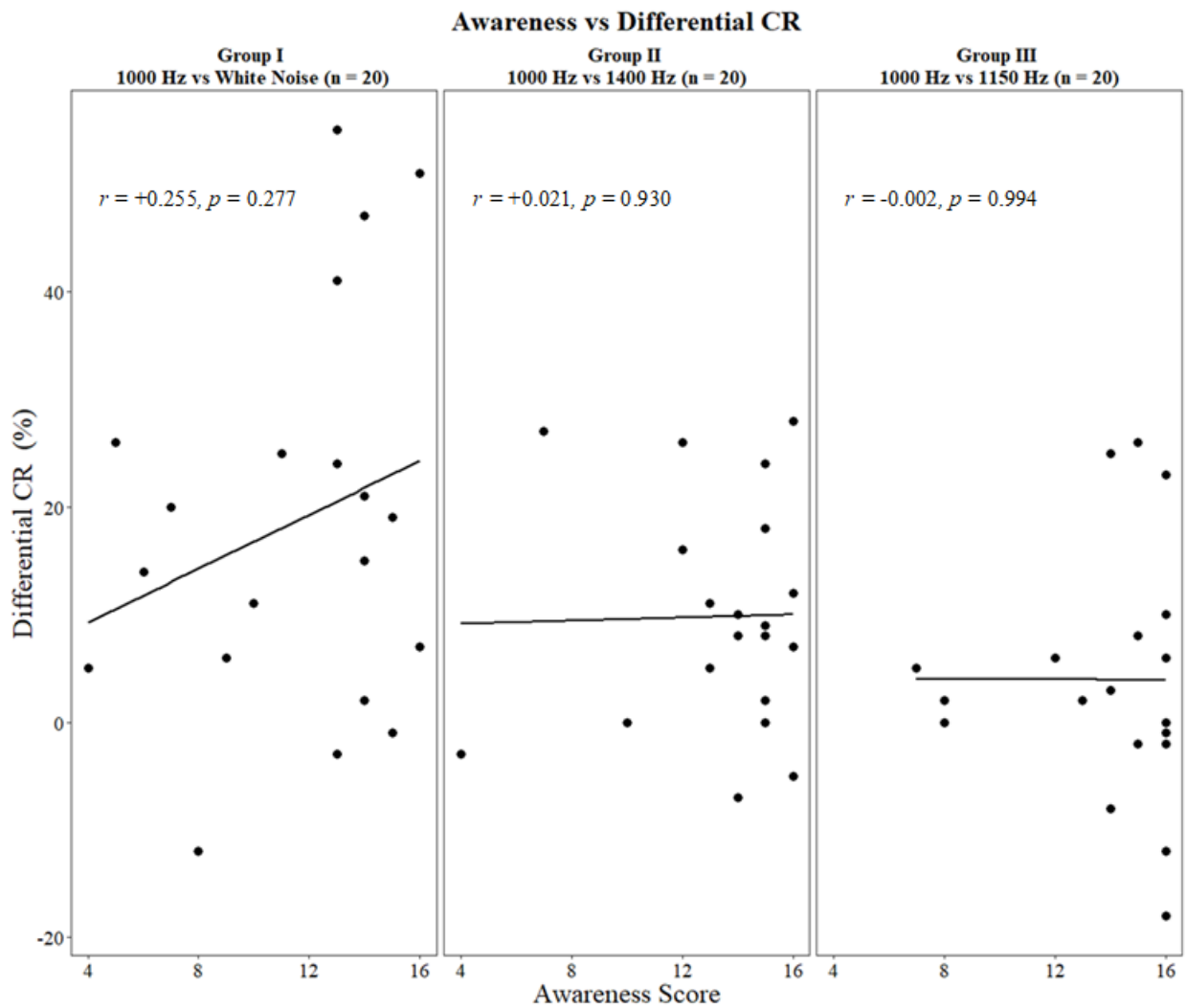


Figure 7. Relationship between awareness score and % differential CR (CS+ minus CS-) across groups. Correlation analyses indicated no significant relationship between awareness and conditioning.

Peak Latency: Peak latency of the CR (Lowgren et al., 2017) was examined with a repeated measures ANOVA with Group and Awareness as between subjects factors and CS Type and Blocks as within subject factors. None of the main effects and interaction effects were significant (all p 's > 0.05).

Onset Latency: Onset latency of the CR was defined as the time at which an eyeblink response rose to 20% of the CR amplitude (Green et al., 1999). This was examined with a repeated measures ANOVA

with Group and Awareness as between subjects factors and CS Type and Blocks as within subject factors. None of the main effects and interaction effects were significant (all p 's > 0.05).

Non-associative factors: To investigate possible non-associative reasons for group differences, several group analyses were performed. There were no group differences in hearing thresholds ($F(2,114) = 0.016, p = 0.98$), UR amplitudes ($F(2,54) = 0.32, p = 0.72$) and number of correctly answered movie questions ($F(2,54) = 0.71, p = 0.49$). The number of correctly answered questions on the relationship between CS and US was greater for aware than unaware participants ($F(1,54) = 67.4, p < 0.001$). However, the interaction between Group and Awareness was not significant ($F(2,54) = 0.74, p = 0.48$). Despite counterbalancing CS frequencies, we further tested the possibility that this manipulation could have produced differential responding (e.g. higher frequency tones elicited more CRs) and found no significant main effect of frequency on % CR ($F(3,106) = 1.961, p = 0.124$). Although there was no significant relationship between Group and the number of participants being classified as aware, a trend was observed ($\chi^2(2) = 5.02, p = 0.08$).

Discussion

Prior studies on the role of awareness in eyeblink conditioning have yielded conflicting findings, resulting in a lack of consensus on this topic. It may be the case that awareness plays a role under limited conditions. One specific prediction is that awareness is necessary for differential delay eyeblink conditioning when the two cues are perceptually similar to each other and difficult to discriminate (Clark et al., 2002). In the current study we tested this view by manipulating the frequencies of auditory CSs in three groups of participants. Central findings from our study indicate that: 1) awareness was not necessary for differential delay EBCC when two tones were easily discriminable (Group I), 2)

awareness was also not needed for similar tones but may have facilitated early conditioning (Group II), and 3) awareness alone was not sufficient for differential delay eyeblink conditioning (Group III).

Both aware and unaware participants were able to demonstrate differential conditioning when the CSs were a 1000 Hz tone and white noise (Group I), suggesting that awareness was not necessary for differential delay EBCC when CSs were easy to discriminate. Differential delay eyeblink conditioning studies using 1000 Hz tones and white noise as CSs have produced similar findings as older and younger participants classified as unaware were still able to acquire differential conditioning (Clark & Squire, 1998, 1999; Smith, Clark, Manns, & Squire, 2005). Furthermore, medial temporal lobe amnesics were also able to show normal differential conditioning, despite not being able to explicitly report the CS-US relationship (Clark & Squire, 1998). Consistent with this view, human neuroimaging studies on fear conditioning (Carter, O'Doherty, Seymour, Koch, & Dolan, 2006; Knight, Nguyen, & Bandettini, 2006; Tabbert et al., 2011) report neural regions uniquely associated with contingency awareness, further supporting the idea that awareness and conditioning are subserved by independent processes (Squire & Dede, 2015). However, other similar studies using easily discriminable auditory CSs have produced contradictory findings and suggest that awareness is necessary for acquisition in differential delay eyeblink conditioning (Carrillo et al., 2000; Knuttinen, Power, Preston, & Disterhoft, 2001a; Lovibond et al., 2011; Weidemann & Antees, 2012). Knuttinen and colleagues (2001) performed an exhaustive series of experiments in younger and older adults using multiple ISIs and delay and trace procedures and found that awareness was a significant factor for successful differential conditioning. Furthermore, Lovibond et al. (2011) attempted to reproduce findings from Clark and Squire (1998) and Smith et al. (2005) using a shorter post-experimental questionnaire and also found that only participants who demonstrated awareness were able to show differential conditioning. The present data from Group I support the position that awareness and conditioning are independent processes, but it is important to

note several methodological differences (e.g. participant age, CS/US intensities, CR definitions, awareness measurements) between all of these studies as they may account for some of the divergent findings and should be considered when evaluating both sides of this debate.

While it has been shown that participants can show differential conditioning when CS+ and CS- frequencies differ by as little as 50 Hz (Moore, 1964), this is the first EBCC study to parametrically manipulate tone frequencies to vary CS discriminability to examine the role of awareness. A human fear conditioning study manipulated visual CS discriminability by presenting visual patterns (sine wave gratings) consisting of different spatial frequencies (Schultz & Helmstetter, 2010). When participants were presented two similarly constructed visual CSs (one predicted shock and another predicted its absence), their online US expectancy to each CS did not differ (suggesting that they were unaware) but their skin conductance responses to the CS+ was significantly greater than responses to the CS-, suggesting that autonomic fear CRs can occur without contingency awareness even when CSs are difficult to discriminate. Interestingly in Group II (1000 Hz vs. 1400 Hz), aware participants showed early differential conditioning (Blocks 1 and 2) while unaware participants showed late differential conditioning (Block 5) as indicated by a significant interaction (CS Type x Block x Awareness) and subsequent post hoc comparisons. The finding that late differential eyeblink conditioning can occur outside awareness when CSs are similar extend the results reported by Schultz and Helmstetter (2010) and also indicate that unaware participants, when provided sufficient training, eventually show differential conditioning. Unfortunately, differential conditioning was not shown by any participants at any point in time in Group III, making it impossible to examine the necessary circumstances under which conditioning can occur when CSs are very similar to each other (1000 Hz vs. 1150 Hz).

Across groups, participants showed different response patterns as a function of CS similarity. Specifically, this was characterized by a greater number of conditioned responses to CS- trials as CSs

became more similar (white bars in **Figure 4**). One possible interpretation of this response pattern is that the similarity of the CSs in Groups II and III facilitated generalization of CRs. In other words, participants mistakenly produced CRs to CS- trials that sounded like CS+ trials (Groups II and III) but not when the CS+ and CS- were easily discriminable (Group I). Spence's theory of discrimination learning (Rilling, 1977; Spence, 1937) states that responses (i.e. excitation and inhibition) acquired to CS+ and CS- during discrimination learning generalizes to stimuli that are close in dimension to these CSs such that subsequent responding during generalization testing is a result of the interaction between responses to CS+ and CS-. Formal generalization testing is needed in order to determine if this is indeed the case for our data. Interestingly, it appears that generalization did not apply in the case of awareness responses since all aware participants in Groups II and III reported hearing two different tones and correctly identified them as high and low pitch tones. Accordingly, it has been shown that repeated presentation of perceptually similar tones might enhance participants' ability to explicitly discriminate them (Roth, Amir, Alaluf, Buchsenspanner, & Kishon-Rabin, 2003; Wright & Zhang, 2009). This dissociation of CR generalization but not contingency awareness provides additional support that awareness and conditioning are mediated by two independent processes.

Despite no evidence of conditioning by participants who received very similarly sounding CSs (Group III), 75% of these participants were classified as aware. This suggests that contingency awareness does not guarantee successful differential conditioning. This finding has also been reported in both eyeblink and fear conditioning studies (Carrillo et al., 2000; Dawson & Biferno, 1973). Based on evidence related to an absence of conditioning in participants who were aware of the CS-US contingency, a "necessary-gate" hypothesis about the relationship between awareness and autonomic conditioning has been proposed (Dawson & Furedy, 1976). The main tenets of this hypothesis are that awareness of the CS-US contingency is imperative ("necessary") for but does not guarantee

conditioning and that awareness happens in an all-or-none fashion (“gate”) but also that the degree of awareness is not related to the degree of conditioning. Although results from Groups I and II argue against the “necessary” aspect of this hypothesis, behavior from Group III partially support this hypothesis in that 75% of the participants (15 out of 20) were classified as aware but failed to demonstrate differential conditioning, suggesting that awareness alone is not sufficient for conditioning. Furthermore, correlation analyses indicated that awareness scores did not predict differential responding in any of the three groups, consistent with this hypothesis’ view that there is not a significant relationship between degree of awareness and level of conditioning.

One methodological consideration among studies that investigate the role of awareness in conditioning is the method in which awareness is assessed. Two procedures are commonly used. First, a questionnaire designed to probe knowledge of the CS-US relationship is provided to participants following the conditioning session. This has disadvantages as it may not accurately reflect participants’ awareness of the contingencies during conditioning (due to forgetting) and also does not indicate when participants became aware since these measurements are taken following the conditioning session. Another procedure is to require participants to provide an online US expectancy rating on a trial by trial basis during conditioning. One disadvantage of this technique is that it directs participants’ attention to the US, which may have an unintentional effect on awareness and conditioning levels. This concurrent method of assessment also makes it difficult to distinguish between US expectancy awareness (i.e. knowing that a US is coming but not sure why) and contingency awareness (i.e. knowing that a US is coming and the reason why) (Lovibond & Shanks, 2002).

Summary

In this study, we tested the prediction that awareness is necessary for differential delay EBCC when two CSs are perceptually similar. Our results indicate that awareness was not necessary for

differential delay EBCC when the two cues were easily discriminable (Group I). Moreover, awareness was not needed when two perceptually similar cues were used but explicit awareness may have facilitated early conditioning (Group II). Finally, awareness alone was not sufficient for differential delay EBCC (Group III). These data partially support the idea that awareness and conditioning are mediated by independent processes and possibly subserved by unique brain structures. Future studies incorporating neuroimaging (fMRI) and neuromodulation (transcranial direct current stimulation) techniques would help identify the neural substrates underlying these processes. Also, future work should include trace conditioning procedures to determine if this form of conditioning interacts with awareness differently than delay conditioning. Finally, additional approaches to modulating and measuring awareness (e.g. directly instructing participants about the contingency prior to conditioning) would aid in further characterizing this complicated psychological process. These suggestions would provide the field a more complete understanding of how conditioning and awareness are related.

Appendix A

Awareness Questionnaire (14 Items):

Can you tell me how many different tones did you hear during the experiment?

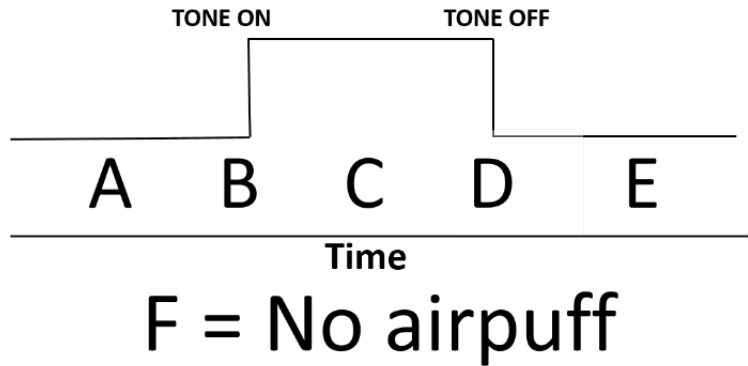
- | | | |
|--|---|---|
| 1.) I believe the airpuff usually came immediately <u>before</u> the high pitched tone | T | F |
| 2.) I believe the airpuff usually came immediately <u>before</u> the low pitched tone | T | F |
| 3.) I believe the airpuff usually came immediately <u>after</u> the high pitched tone | T | F |
| 4.) I believe the airpuff usually came immediately <u>after</u> the low pitched tone | T | F |
| 5.) I believe the high pitched tone usually came immediately <u>before</u> the airpuff | T | F |
| 6.) I believe the high pitched tone usually came immediately <u>after</u> the airpuff | T | F |
| 7.) I believe the low pitched tone usually came immediately <u>before</u> the airpuff | T | F |
| 8.) I believe the low pitched tone usually came immediately <u>after</u> the airpuff | T | F |
| 9.) I believe the low pitched tone and airpuff were <u>always</u> closely related in time | T | F |
| 10.) I believe the high pitched tone and airpuff were <u>always</u> closely related in time | T | F |
| 11.) I believe the low pitched tone and airpuff were only <u>sometimes</u> related in time | T | F |
| 12.) I believe the high pitched tone and airpuff were only <u>sometimes</u> related in time | T | F |
| 13.) I believe the low pitched tone predicted when the airpuff would come | T | F |
| 14.) I believe the high pitched tone predicted when the airpuff would come | T | F |

Appendix B

Awareness Measure Schematic:

Now I am going to show you a diagram. This diagram represents when the tone turns on, how long it stays on and when it turns off. These events are marked by the letters A, B, C, D, and E.

- 1.) For the high pitched tone, if you had to choose when the airpuff occurred in relation to the tone, which one of these letters best represent the position. The letter F indicates that the airpuff never occurred.
- 2.) For the low pitched tone, if you had to choose when the airpuff occurred in relation to the tone, which one of these letters best represent the position. The letter F indicates that the airpuff never occurred.



Appendix C

Movie Questionnaire

- | | | |
|---|----------|----------|
| 1. The main character in this movie is played by Charlie Chaplin | T | F |
| 2. The main character falls down a snowy hill and turns into a giant snowball | T | F |
| 3. In one scene a dog was eaten by a hungry man | T | F |
| 4. A bear was shot and killed | T | F |
| 5. A shoe was cooked and eaten for thanks giving dinner | T | F |
| 6. There was a drinking contest between two men | T | F |
| 7. The main character finds a new pair of shoes in the snow | T | F |
| 8. One man's coat catches on fire | T | F |
| 9. A hungry man thought he saw someone turn into a chicken | T | F |
| 10. One man dies in an avalanche | T | F |

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Table 1: Awareness in Single Cue Eyeblink Classical Conditioning

Author	Paradigm	CS	US	Awareness Necessary	Awareness Not Necessary
Papka et al., 1997	Single cue delay	1000 Hz 80 dB	5-7 psi		✓
Frcka, Beyts, Levey, & Martin, 1983	Single cue delay	N/A	2 psi and 7 psi		✓
Weidemann et al., 2013	Single cue delay	1000 Hz 85 dB	15 psi	✓	
Gabrieli at al., 1995	Single cue delay	1000 Hz 85 dB	20.6 k Pa		✓
Manns, Clark, and Squire, 2000	Single cue trace	1000 Hz 85 dB	3 psi	✓	
Manns et al., 2001	Single cue trace and delay	1000 Hz 85 dB	3 psi		✓

Table 2: Awareness in Differential Eyeblink Classical Conditioning

Author	Paradigm	CS	US	Awareness Necessary	Awareness Not Necessary
Weidemann & Antees, 2012	Differential Delay	1000 Hz and white noise	15 psi	✓	
Smith, Clark, Manns & Squire, 2005	Differential Delay	1000 Hz, 800 Hz, 2100 Hz, and white noise	3 psi, 0.75 psi		✓
Carrillo et al., 2000	Differential Delay	1000 Hz and white noise	3 psi		✓
Huang et al., 2014	Differential Delay	Soft tone: 1000 Hz, 3 kHz 60 dB Loud tone: 1000 Hz, 3 kHz, 80 dB	5 psi	✓	
Ross & Nelson, 1973	Differential Delay	800 Hz, 2100 Hz	0.75 psi	✓	
Manns, 2000	Differential Trace	1000 Hz and white noise	3 psi		✓
Clark & Squire, 1998	Differential Delay and Trace	1000 Hz and white noise	3 psi	✓ (trace)	✓ (delay)
Clark & Squire, 1999	Differential Delay and Trace	1000 Hz and white noise	3 psi	✓ (trace)	✓ (delay)
Lovibond et al., 2012	Differential Delay and Trace	1000 Hz and white noise	15 psi	✓	
Knuttinen et al., 2001	Differential Delay and Trace	1000 Hz and white noise	3 psi	✓	