Awareness is essential for differential delay eyeblink conditioning with soft-tone but not loud-tone conditioned stimuli

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ABSTRACT

The role of awareness in differential delay eyeblink conditioning (DEC) remains controversial. Here, we investigated the involvement of awareness in differential DEC with a soft or a loud tone as the conditioned stimulus (CS). In the experiment, 36 participants were trained in differential DEC with a soft tone (60 dB) or a loud tone (85 dB) as the CS, paired with a corneal air-puff as the unconditioned stimulus (US). After conditioning, awareness of the relationship between the CS and the US was assessed with a 17-item true/false questionnaire. Interestingly, during differential DEC with a soft-tone CS, a higher proportion of differential conditioned responses (CRs) was evident in participants who were aware than those who were unaware. In contrast, when a loud tone was used as the CS, the proportion of differential CRs of the aware participants did not differ significantly from those who were unaware over any of the blocks of 20 trials. In unaware participants, the percentage of differential CRs with a loud-tone CS was significantly higher than that with a soft-tone CS; however in participants classified as aware, the percentage of differential CRs with a loud-tone CS did not differ significantly from that with a soft-tone CS. The present findings suggest that awareness is critical for differential DEC when the delay task is rendered more difficult.

Keywords: awareness; differential delay eyeblink conditioning; soft-tone conditioned stimulus; loud-tone conditioned stimulus

INTRODUCTION

A popular proposal in human behavior is that there are two kinds of memory, which are composed of several different abilities that depend on different brain systems^[1]. One is declarative memory that is used for conscious contingency learning. The other is non-declarative memory that does not rely on consciousness and supports skill and habit learning. In the case of associative learning, particularly Pavlovian conditioning, it is widely believed that conditioning is carried out by a reflexive, unconscious mechanism (non-declarative memory) that is quite distinct from the higher cognitive processes (declarative memory) associated with language and conscious recall of facts and events^[1]. Investigations of the distinction between declarative memory and non-declarative memory are concerned with classical conditioning.

Classical conditioning of the eyeblink response serves as an excellent model in which to investigate the mechanisms of associative learning and memory in mammals^[2–9]. In this paradigm, a conditioned stimulus (CS; e.g., a tone or light) is followed by an unconditioned stimulus (US; e.g., a corneal air-puff or periorbital shock). At first there is only a reflexive eyeblink to the US, the unconditioned response (UR). Instead, with repeated

presentations of the CS and the US, the conditioned response (CR), which is a protective eyeblink, is elicited by the CS ahead of the US. Furthermore, classical eyeblink conditioning is composed of two distinct types: trace and delay paradigms. During delay eyeblink conditioning (DEC) the CS overlaps the US and they terminate together; in contrast, during trace eyeblink conditioning (TEC), there is a temporal gap between the CS and the US and they terminate together^[9, 10]. The view is widely held that TEC is dependent on awareness^[11–14]. However, there is fervent debate about whether awareness is essential for DEC. Clark *et al.* suggested that DEC can be acquired independently, based on the evidence that differential DEC (one conditioned stimulus (CS+) is always followed by the US, and the other (CS–) is always alone) occurs in participants who show no contingency awareness^[11, 15, 16], whereas differential TEC is only acquired by participants who show awareness of the relationship between the CSs and the US^[11, 17]. Significantly, evidence from the data explanation of Clark and Squire has been cited as support for the distinction between declarative and non-declarative learning^[18], that TEC is associated with declarative knowledge and is mediated by the hippocampus and cortical circuits, and in contrast, DEC relies on non-declarative knowledge, which can be learned in a reflexive and automatic manner by sub-cortical or cerebellar circuits^[11]. Although the conclusions about DEC by Clark and Squire's group are dominant, a number of other eyeblink conditioning studies with respect to the relationship between DEC and contingency awareness are at odds with theirs. Previous studies^[12, 19–21] have shown that differential DEC only appears in participants who know the relationship between the CSs and the US, which is consistent with the latter view that DEC is awareness-dependent. Similarly, two recent reports^[13, 22] also reached the same conclusions. Thus, all these studies in favor of the latter view are opposed to the conclusions of Clark and Squire^[11].

Confronted with the inconsistency in the role of awareness underlying differential DEC, previous studies attempted to use significant methodological differences to explain the difference. Some studies have pointed out that the questionnaire that Clark and Squire's group used to assess contingency awareness was not sensitive enough^[23]. In contrast, in order to support Clark's conclusions, Manns *et al.* considered differences in the procedures used by

other researchers, suggesting that they may have required forebrain systems involved in awareness formation, such as the measurement of eyeblinks, the complexity of the stimuli, and the different definitions of voluntary responses^[24]. Unfortunately, these studies aimed at testing whether the questionnaire used by Clark *et al.* was sensitive by endeavoring to replicate their experimental procedures have found that the questionnaire is sensitive enough to assess contingency awareness, but showed an inconsistency in the relationship between awareness and differential DEC^[13, 16]. These findings not only demonstrated that the questionnaire is sensitive and cannot explain the inconsistency, but also indicated that other potential factors affect whether awareness is essential for differential DEC.

We noted that in some previous studies, one primary difference was the intensity of the CS tone used. The intensity of the CS tone in those studies that favored the awareness-dependence of differential DEC was lower than that in other studies that proposed the awareness-independence of differential DEC (i.e. a maximum of 75 dB for awareness-dependent studies^[12, 20] *versus* a minimum of 85 dB for awareness-independent studies^[11, 16]), suggesting that the intensity of the CS tone may affect the relationship between differential DEC and awareness. Moreover, Lovibond *et al.* have proposed that the intensity of the CS tone might be a critical factor underlying whether differential DEC is awareness-dependent^[13]. Further, our recent study indicated that both electrolytic lesions and muscimol inactivation of the medial prefrontal cortex (mPFC), which is involved in many critical cognitive functions^[25–29], disrupt DEC with a soft-tone (60 dB) CS, but not a loud-tone (85 dB) CS in guinea pig^[30]. These findings led us to propose the hypothesis that awareness is critical for differential DEC with a soft-tone CS but not with a loud-tone CS. Therefore, the present study was designed to determine whether awareness is critical for differential DEC with a soft- but not with a loud-tone CS.

PARTICIPANTS AND METHODS

Participants

The participants were healthy undergraduates (36 males) with no self-reported recent hearing impairment, eye disease, or psychiatric disorder. They were divided into four groups [aware/soft group ($n = 8$; mean age, 20.25

years), unaware/soft group ($n = 11$; mean age, 21.55 years), aware/loud group ($n = 9$; mean age, 20.89 years), and unaware/loud group ($n = 8$; mean age, 21.38 years)], according to the intensity of CS tone and the scores on a 17-item true/false questionnaire. A small amount was paid to all participants. The research was approved by the Ethics Committee of the Third Military Medical University.

Apparatus

The experiment was modeled on the procedure of Clark and Squire (1998)^[11]. We also used the same silent movie [*The Gold Rush* (Chaplin, 1925)]^[11] as a masking task, and an infrared emitter/detector (FBCB30/TBBB30, Heng Sheng, Shenzhen, China) attached to spectacles for eyeblink recording. We chose two differential DEC paradigms that used a soft (60 dB) or loud (85 dB) tone CS paired with a corneal air-puff US (Fig. 1). All sound stimuli were delivered by two speakers placed 60 cm to the left and right of the participant. A sound-level meter (type 2240, Brüel & Kjær) was used to measure the intensity of the CS tone (loud: 83–87 dB, soft: 58–62 dB). The US was a 100-

ms, 5.0-psi air-puff (measured at the tip of a plastic pipe attached to modified spectacles) delivered to the left eye. The presentations of the CS and US were controlled by a self-made computer system^[30]. A data-acquisition system (RM6240BDJ, Cheng Yi, Chengdu, China) was used to digitize markers of the applied stimuli and the eyeblinks, and the data were acquired by the system software (v. 4.7). The storage and analysis of data were carried out on a dedicated Windows PC.

Behavioral Procedure

The experimental procedures were similar to those used in previous studies^[11, 16]. In the period of conditioning, participants watched the silent movie, and were asked to learn its content for a later recall test. Participants were told they would be taking part in a study about the effects of distraction on learning and memory and they would be distracted by a high-frequency tone (3 kHz), a low-frequency (1 kHz) tone, and air puffs^[11]. After participants fully understood the instructions, they put on the modified spectacles and watched the movie on the computer monitor.

Fig. 1. Temporal relationship between the conditioned stimulus (CS), unconditioned stimulus (US), and analysis periods for the delay eyeblink conditioning (DEC) procedure. During DEC, the CS remained on while the 100-ms air-puff US was delivered and they terminated together. The CS was presented for 1250 ms before the US onset. In each trial, we analyzed the parameters of the conditioned eyeblink response (CR; 750–1250 ms after CS onset). These responses were based on the average amplitude at baseline (0–800 ms prior to the onset of the CS). The trace shows an example of a typical CR and UR from one participant.

For DEC, the CS was presented 1 250 ms before the US onset and they terminated together. The differential DEC procedure was used in the two conditioning paradigms. The participants were given an odd or even number according to the sequence of training. For odd-numbered participants, the CS+ (i.e. the CS paired with the US) was a 1-kHz tone and the CS– (i.e. the CS never paired with the US) was a 3-kHz tone, and for even-numbered participants, the CS+ and the CS– were reversed to avoid the effects of tone frequency on the results. While they were watching the movie, we administered 6 blocks of 120 delay conditioning trials at an inter-trial interval of 25–40 s. Each block consisted of 20 delay conditioning trials, of which half were CS+ and half were CS–, and the sequence was random.

When the conditioning session finished, we tested the participants' grasp of the movie content and asked them to carefully complete a questionnaire. This consisted of 17 items concerning the CS-US correlation (e.g., "I believe the air-puff usually came immediately after the low tone." and "I believe the low tone predicted when the air-puff would come."), as in previous studies^[11, 16].

Behavioral Data Analysis

On each CS+ or CS– trial, a 3100-ms epoch was recorded, starting 800 ms before the onset of the CS (Fig. 1). All the eyeblink data came from the left eye. The parameters of the present study were similar to those used by Smith (2005)^[16] and were analyzed with custom software. Each CS+ trial was subdivided into three discontinuous analysis periods: (1) a "baseline" period, 1–800 ms before the CS onset; (2) a "CR" period, 750–1 250 ms after the CS onset; and (3) a "UR" period, 1–500 ms after the US onset (Fig. 1). Moreover, for each CS– trial, the "baseline" period and the "CR" period were the same as in the CS+ trial.

Based on the parameters used by Smith (2005)^[16], the definition of a CR had to meet the following two conditions: first, the eyeblink must occur between 750 and 1250 ms after the CS onset (CR+ for CS+ trials and CR– for CS– trials); and second, the amplitude of the CR must exceed the baseline threshold (i.e., no less than 20% of the average amplitude of the first 10 URs in CS+ trials). During each block of 20 trials, the percentage of CR+ or CR– was defined as the ratio of the number of trials containing the CR+ or CR– to the total number of CS+ trials or CS– trials,

respectively. Based on a previous study^[11], the percentage of differential CR (differential CR%) was defined as the percentage of CR+ minus the percentage of CR–.

Following Clark and Squire's study (1998)^[11], participants were defined as being aware or unaware of the stimulus contingencies across the scores of the 17-item questionnaire. When the score was ≥ 13 correct out of 17, participants were defined as aware of the stimulus contingencies. When the score was ≤ 12 correct, participants were defined as unaware of the stimulus contingencies.

Statistical Analysis

All the data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by least significant difference (LSD) *post hoc* tests, following two-way repeated measures analyses of variance (ANOVA) and a separate one-way repeated measures ANOVA using the SPSS for Windows package (v. 18.0). $P < 0.05$ was considered to be statistically significant^[30].

RESULTS

Number of Correct Responses to Post-experimental Questionnaire

The performances of all aware and unaware individuals in answering the 17 questions of the post-experimental questionnaire concerning the temporal relationships between the CS+, the CS–, and the US are shown in Table 1.

Effects of Awareness on Differential DEC with a Soft-Tone CS

Compared with that in the aware/soft group, the acquisition of differential DEC with a soft-tone CS was significantly impaired in the unaware/soft group (Fig. 2A). This was confirmed by two-way repeated measures ANOVA on the differential CR%; there was no significant group-by-block

Table 1. Average scores of individuals on the 17 critical questions on the post-conditioning questionnaire

Group	Score	SEM
Aware/soft ($n = 8$)	16.50	0.27
Unaware/soft ($n = 11$)	8.09	0.37
Aware/loud ($n = 9$)	15.22	0.52
Unaware/loud ($n = 8$)	7.37	0.73

interaction [$F(5,85) = 0.777$, $P = 0.569$], but there were significant effects of group [$F(1,17) = 15.074$, $P = 0.001$] and block [$F(5,85) = 3.313$, $P = 0.009$]. Furthermore, a separate one-way ANOVA revealed that the differential CR% of the aware/soft group was significantly higher than that of the unaware/soft group on blocks 1–6 [$F(1,17) = 10.588$, $P = 0.005$; $F(1,17) = 14.409$, $P = 0.001$; $F(1,17)$

$= 5.258$, $P = 0.035$; $F(1,17) = 4.928$, $P = 0.040$; $F(1,17) = 9.099$, $P = 0.008$; and $F(1,17) = 11.791$, $P = 0.003$].

Effects of Awareness on Differential DEC with a Loud-Tone CS

We then asked whether differential DEC with a loud-tone CS depends on awareness. There was no significant group

Fig. 2. Effects of awareness and conditioned stimulus (CS) tone intensity on differential delay eyeblink conditioning. Note the significant difference in differential CR% with the soft-tone CS between the aware/soft ($n = 8$) and unaware/soft ($n = 11$) groups (A). However, there was no significant difference in differential CR% with the loud-tone CS between the aware/loud ($n = 9$) and unaware/loud ($n = 8$) groups (B). In addition, the differential CR% with a loud-tone CS in the unaware/loud group was significantly higher than that with a soft-tone CS in the unaware/soft group (C), but the differential CR% with a loud-tone CS in the aware/loud group did not differ significantly from that with a soft-tone CS in the aware/soft group (D). The error bars represent the SEM. * $P < 0.05$ vs control (unaware/soft group).

effect on the acquisition of differential DEC with a loud-tone CS between the aware/loud and unaware/loud groups (Fig. 2B). This was also confirmed by two-way repeated measures ANOVA of the differential CR%; there was no significant group-by-block interaction [$F(5,75) = 1.052$, $P = 0.394$] and no significant group effect [$F(1,15) = 0.019$, $P = 0.893$], but there was a significant block effect [$F(5,75) = 2.675$, $P = 0.028$].

Effects of CS Tone Intensity on Differential DEC in Unaware Participants

In unaware participants, the differential CR% with a loud-tone CS was significantly higher than that with a soft-tone CS (Fig. 2C). Statistical analysis using two-way repeated measures ANOVA revealed no significant group-by-block interaction [$F(5,85) = 1.397$, $P = 0.234$], but there were significant effects of group [$F(1,17) = 20.489$, $P < 0.001$] and block [$F(5,85) = 4.099$, $P = 0.002$]. Furthermore, a separate one-way ANOVA revealed that the differential CR% in the unaware/loud group was significantly higher than that of the unaware/soft group in blocks 1–3 [$F(1,17) = 5.205$, $P = 0.036$; $F(1,17) = 9.691$, $P = 0.006$; and $F(1,17) = 4.687$, $P = 0.045$], and in blocks 5 [$F(1,17) = 5.356$, $P = 0.033$] and 6 [$F(1,17) = 22.877$, $P < 0.001$].

Effects of CS Tone Intensity on Differential DEC in Aware Participants

In participants classified as aware, the CR% with a loud-tone CS did not differ significantly from that with a soft-tone CS (Fig. 2D). This was confirmed by two-way repeated measures ANOVA; there was no significant group-by-block interaction [$F(5,75) = 0.437$, $P = 0.822$] and no significant effects of group [$F(1,15) = 0.215$, $P = 0.650$] or of block [$F(5,75) = 1.770$, $P = 0.129$].

DISCUSSION

The original goal of the present study was to investigate our hypothesis that awareness is essential for differential DEC when a soft tone but not a loud tone is used as the CS. The following findings appear to support our hypothesis: (1) in the soft-tone CS trials, the acquisition of differential CRs was significantly associated with contingency awareness; (2) by contrast, in the loud-tone CS trials, the acquisition of differential CRs was unrelated to contingency awareness; and (3) in unaware participants, the percentage of differential CR with a loud-tone CS was significantly

higher than that with a soft-tone CS; but (4) in participants classified as aware, the percentage of differential CR with a loud-tone CS did not differ significantly from that with a soft-tone CS. Thus, these experimental findings firmly support the hypothesis and demonstrate that contingency awareness plays an important role specifically in the acquisition of differential delay CRs when a soft tone but not a loud tone is used as the CS. In other words, the intensity of the CS is a crucial factor that affects the relationship between differential DEC and contingency awareness. In fact, the present results do not appear to contradict either of the two distinctly different views, but provide a way to interpret the long-standing argument that the present results on differential DEC with a loud-tone CS coincide with the view of Clark *et al.* and Smith *et al.* that contingent awareness is not critical for differential DEC^[11, 15, 16], whereas the present results on differential DEC with a soft-tone CS are entirely consistent with the view of other studies that differential DEC only appears in participants who have contingency awareness^[12, 19, 20].

Why is contingency awareness essential for differential DEC with a soft-tone CS but not a loud-tone CS? Although the present data have not directly answered this question, evidence from our recent study^[30] on DEC in experimental animals with mPFC lesions may be cited in this regard. This study showed that lesions of the mPFC, forebrain structures involved in awareness formation^[31–33], induce deficits in acquisition of the CR with a soft-tone CS, but not a loud-tone CS^[30]. While the concept of conscious knowledge is not readily applicable to experimental animals, it is believed that the critical element that confers awareness about acquired knowledge comes from the conjoint operation of the neocortex and the hippocampal system^[11]. Accordingly, it can be reasoned that the animals with mPFC lesions in our previous study failed to acquire contingency awareness.

It has been demonstrated that during the tone stimulation of eyeblink conditioning, only a small percentage of auditory-driven mossy fibers show sustained responses that persist until the CS tone offset even when a loud tone is used^[34–36], therefore, the soft-tone CS may drive much less sustained mossy-fiber activity, which is essential for the integration and processing of CS and US information during conditioning. Fortunately, sustained responses may also be driven by input from other sources

such as the mPFC^[37] and hippocampus^[38, 39], in this case, with input from these forebrain areas, mossy-fiber activity driven by the soft-tone CS may also effectively support eyeblink conditioning. In contrast, it would be insufficient to support the CR if there were no signal input from the mPFC or hippocampus due to their inactivation or lesioning.

Our finding that differential DEC with a loud-tone CS did not depend on awareness differs from the results of two recent studies^[13, 22] with the same loud-tone CS, in which differential DEC was reported to be awareness-dependent. Although it is not clear what underlying differences of procedure might be responsible for this inconsistency, some possible candidates should be taken into account. One possible factor is the way awareness is assessed. Lovibond *et al.*^[13] used two questionnaires, a long one, based on Clark and Squire (1998)^[11], and a short one, based on the recommendations of Dawson and Reardon (1973)^[40]. In addition, Weidemann *et al.*^[22] used a button-press measure of expectancy of the US rather than the long questionnaire, whereas the participants in our study were only given the long questionnaire based on Clark and Squire (1998)^[11]. The button-press measure may reinforce the contingency awareness of CS-US during experiments, hence affecting the results. Another possible factor is that the pressure of the air-puff in these two studies was higher than that in our study (15 psi^[13, 22] vs 5 psi). Indeed, some recent studies on animals have shown that TEC is affected more by lesions of the mPFC when a relatively non-aversive airpuff, rather than an aversive periorbital shock, is used as the US^[41-43], implying that the intensity of the US may be an important factor affecting the results in differential DEC studies. This hypothesis needs to be further tested. Anyway, assessing awareness using the questionnaire of Clark and Squire (1998)^[11], our results suggested that differential DEC is not dependent on awareness when a loud tone is used as the CS, paired with a 5-psi, 100-ms air-puff US.

In conclusion, we showed that awareness is critical for differential DEC when the delay task is rendered more difficult. Moreover, the results also provide a way to interpret the long-standing argument on whether differential DEC is awareness-dependent.

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