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Single-cue delay eyeblink conditioning is unrelated to awareness

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We examined the importance of awareness for eyeblink conditioning by directly comparing single-cue delay eyeblink conditioning and single-cue trace eyeblink conditioning. During single-cue delay conditioning, participants who became aware of the stimulus contingencies early in the conditioning session conditioned no better than those who became aware later in the session or did not become aware. Thus, the level of awareness was unrelated to the overall level of conditioning across the session. In contrast, awareness of the stimulus contingencies early in the session predicted the success of single-cue trace conditioning. These data, together with earlier findings, show that awareness is irrelevant to single-cue delay eyeblink conditioning but is critical for single-cue trace eyeblink conditioning. The findings from the present study are related to previous findings for differential (CS^+ and CS^-) eyeblink conditioning and awareness.

Memory is composed of several different abilities that depend on different brain systems (Gabrieli, 1998; Schacter & Tulving, 1994; Squire & Zola, 1997). Declarative memory depends on the hippocampus and anatomically related structures in the medial temporal lobe and diencephalon and supports the capacity for conscious recollection of facts and events. Nondeclarative memory supports a collection of nonconscious learning abilities that are independent of the medial temporal lobe and are expressed through performance, as, for example, in skill and habit learning and simple forms of conditioning.

Human eyeblink classical conditioning provides a useful paradigm for exploring the distinction between declarative and nondeclarative forms of memory. Typically, a tone (the conditioned stimulus; CS) is presented immediately before a puff of air (the unconditioned stimulus; US) is delivered to the eye. After repeated pairings of the CS and US, individuals blink in response to the tone (the conditioned response; CR). In the standard (single-cue) procedure, a single CS is paired with the US.

Trace eyeblink conditioning, in which a short empty interval separates the CS and the US, shares several features with declarative memory. When the trace interval is sufficiently long ($\geq$ 500 msec), trace eyeblink conditioning depends on the integrity of the hippocampus (Clark & Squire, 1998; McGlinchey-Berroth, Carrillo, Gabrieli, Brawn, & Disterhoft, 1997) and requires that participants become aware that the CS predicts the US (Manns, Clark, & Squire, 2000a; Woodruff-Pak, 1999). In one study, distraction during the conditioning session reduced awareness and decreased conditioning (Manns et al., 2000a). In another study, those designated as "aware" after the session produced more conditioned responses during the first 10 conditioning trials than did those designated "unaware" (Woodruff-Pak, 1999). Finally, the degree of awareness after 10 conditioning trials predicted the overall success of conditioning across a 120-trial session (Manns et al., 2000a).

In delay eyeblink conditioning, the CS and the US overlap and coterminate. In contrast to trace conditioning, delay conditioning is intact in amnesic patients with hippocampal damage (Daum, Channon, & Canavan, 1989; Gabrieli et al., 1995). Thus, delay and trace conditioning appear to be fundamentally different from each other.

The findings in humans for delay and trace conditioning are consistent with the findings in rabbits and rats. Studies in rabbits have shown that delay eyeblink conditioning depends critically on the cerebellum and not on any forebrain structures (Thompson & Krupa, 1994). Trace eyeblink conditioning also depends critically on the cerebellum, but in contrast to delay conditioning has been found to depend in addition on the hippocampus

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(Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986) and prefrontal cortex (Kronforst-Collins & Disterhoft, 1998).

The importance of awareness for standard delay conditioning has not been explored to the extent that it has in trace conditioning. In one study, expectancy of the airpuff US was positively related to performance in individuals given trace eyeblink conditioning (i.e., higher expectancy of the US was related to higher probability of CRs), but expectancy of the US was unrelated to performance in individuals given delay eyeblink conditioning (Clark, Manns, & Squire, in press). This finding suggests that the expectation of the US has a different role in trace conditioning than it does in delay conditioning. Yet this study does not illuminate the importance of awareness itself for eyeblink conditioning, because all of the participants were aware of the stimulus contingencies. In another study of delay conditioning, participants who conditioned well appeared to be as aware (or unaware) of the stimulus contingencies as participants who conditioned poorly (Papka, Ivry, & Woodruff-Pak, 1997). In this study, the role of awareness in trace eyeblink conditioning was not assessed, and the findings for delay conditioning could not be directly compared to the findings for trace conditioning.

In the present study, we assessed the role of awareness in delay eyeblink conditioning. We used the same procedure as was used previously to show that awareness early in the conditioning session predicted the level of trace conditioning (Manns et al., 2000a). Participants watched a silent movie during the conditioning session and were asked about the relationship between the CS and the US early in the conditioning session, midway through the session, and at the end of the session. The question of interest was how awareness of the stimulus contingencies might be related to conditioning performance. We directly compared the results obtained here for delay eyeblink conditioning and the results from the earlier study of trace eyeblink conditioning.

METHOD

Participants

The participants (8 men, 12 women) were volunteers or employees at the San Diego Veterans Affairs Medical Center or were respondents to a newsletter. They averaged 65.2 years of age (range = 50–76) and had an average of 16.8 years of education. They obtained WAIS-III Information and Vocabulary subscale scores of 22.3 and 55.2, respectively. Older adults were tested because we have found that younger adults become aware of the stimulus contingencies rather quickly, limiting the ability to find a relationship between conditioning and awareness (for further discussion, see Clark & Squire, 2000).

Procedure

The procedure was based on one described by Manns et al. (2000a, Experiment 2). The participants were told that they were taking part in a study of how distraction affects learning and memory and that they would be distracted by tones and airpuffs. After giving informed consent, the participants were seated in a comfortable chair in a darkened room, approximately 0.7 m from a television monitor. One hundred twenty delay conditioning trials were then administered with an intertrial interval of 10–15 sec. During the conditioning session, participants watched a silent movie (*The Gold Rush*), which they were instructed to remember for a later memory test. The CS was an 85-dB, 1-kHz tone, 1,350 msec in duration, delivered through earphones. 1,250 msec after onset of the CS, the US was delivered. The US was a 100-msec, 3-psi airpuff delivered to the left eye through specially designed goggles. The goggles also included an infrared reflective sensor for recording eyeblinks (Clark & Squire, 2000). An eyeblink was considered to be a CR if it occurred between 750 msec after the onset of the CS and before the onset of the US, and if the CR was at least 20% of the amplitude of the average UR during the first 10 trials. Trials were excluded from analysis when responses had a short latency (i.e., they began more than 500 msec before the US) and were maintained until the onset of the US. Such responses have been considered to be voluntary eye closures (Spence & Ross, 1959). Finally, trials were scored as non-CR trials when no response was observed or when short-duration responses were observed that did not occur within the CR latency window just described.

Five of the first 10 participants tested in the present study produced an eyeblink response on the first trial that satisfied the criterion for being scored as a CR. These early responses could not have been true CRs and likely were nonassociative responses produced by participants who were initially sensitive to the 1,350-msec tone. To reduce the likelihood of these early nonassociative responses and to obtain a baseline with which conditioning performance could be compared, the remaining 10 participants were given 32 pseudoconditioning trials prior to the conditioning session (see Carillo, Thompson, Gabrieli, & Disterhoft, 1997; Gabrieli et al., 1995). Sixteen CS-alone and 16 US-alone trials were pseudorandomly intermixed in such a way that neither the CS nor the US occurred more than twice consecutively. As in the conditioning session, the CS was an 85-dB, 1-kHz tone, 1,350 msec in duration, and the US was a 100-msec, 3-psi airpuff delivered to the left eye. During the pseudoconditioning trials, participants watched digits appearing on a computer screen (once every 1.5 sec for a 1-sec duration) and pressed a button whenever three odd digits appeared consecutively (Mulligan & Hartman, 1996). We reasoned that pseudoconditioning trials prior to the conditioning session might reduce the probability of nonassociative eyeblinks appearing in response to the CS on the first few conditioning trials. Pseudoconditioned responses were calculated as the percentage of the 16 CS-alone trials on which participants emitted an eyeblink between 750 and 1,250 msec after the onset of the tone that had an amplitude greater than 20% of the average amplitude from the first 10 URs emitted on the US-alone trials. That is, the pseudoconditioning trials were scored in the same manner as were the conditioning trials. After the pseudoconditioning trials, the participants were given a 2-min rest before the conditioning session, during which they were given instructions about attending to the movie that they were to watch during the conditioning session.

For all 20 participants, 7 true or false questions that asked about the relationship between the CS and the US were given without forewarning after the first 10 conditioning trials, again after 60 trials, and finally after all 120 trials were completed (e.g., "I believe the airpuff came immediately before the tone"; "I believe the tone predicted when the airpuff would come"). All 7 true or false questions appear in the Appendix. At the end of the session, the participants were also given 10 true or false questions about the content of the movie.

RESULTS

Figure 1A shows the percentage of CRs on each of the first 10 trials of conditioning for the 10 participants who were administered pseudoconditioning trials before the conditioning session and for the 10 who were not admin-

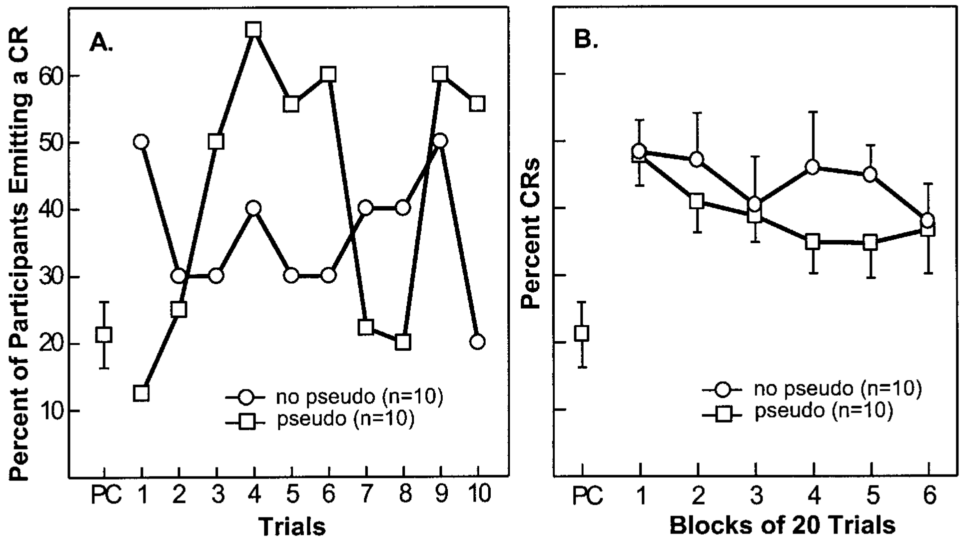

Figure 1. (A) Percentage of participants in each group who emitted conditioned responses (CRs) during the first 10 conditioning trials. One group (pseudo, squares) received 32 pseudoconditioning trials before the conditioning session. The other group (no pseudo, circles) did not receive pseudoconditioning trials. (B) Percent CRs across 6 blocks of 20 conditioning trials for participants who received 32 pseudoconditioning trials before the conditioning session (pseudo, squares) and participants who did not receive pseudoconditioning trials (no pseudo, circles). The pseudoconditioning score in both panels A and B is for the 10 participants who received pseudoconditioning. Error bars show *SEM*. PC, pseudoconditioning.

istered pseudoconditioning trials. Conditioning was evident for both groups of participants within the first 10 trials (mean % CRs ± *SEM* = 45.8 ± 7.0 and 36.0 ± 4.8 for participants who received pseudoconditioning and those who did not, respectively). In previous reports of single-cue delay conditioning (Carrillo et al., 1997, Figure 2; Gabrieli et al., 1995, Figure 1) and single-cue trace conditioning (Manns et al., 2000a, Figures 1A and 2A; McGlinchey-Berroth et al., 1997, Figure 3; Woodruff-Pak, 1999, Figure 1; Woodruff-Pak & Papka, 1996, Figure 1), CRs were also evident within the first 10 trials of conditioning.

Five of the 10 participants who did not receive pseudoconditioning trials before the conditioning session emitted an eyeblink on the 1st conditioning trial. These responses could not have been true CRs and may reflect

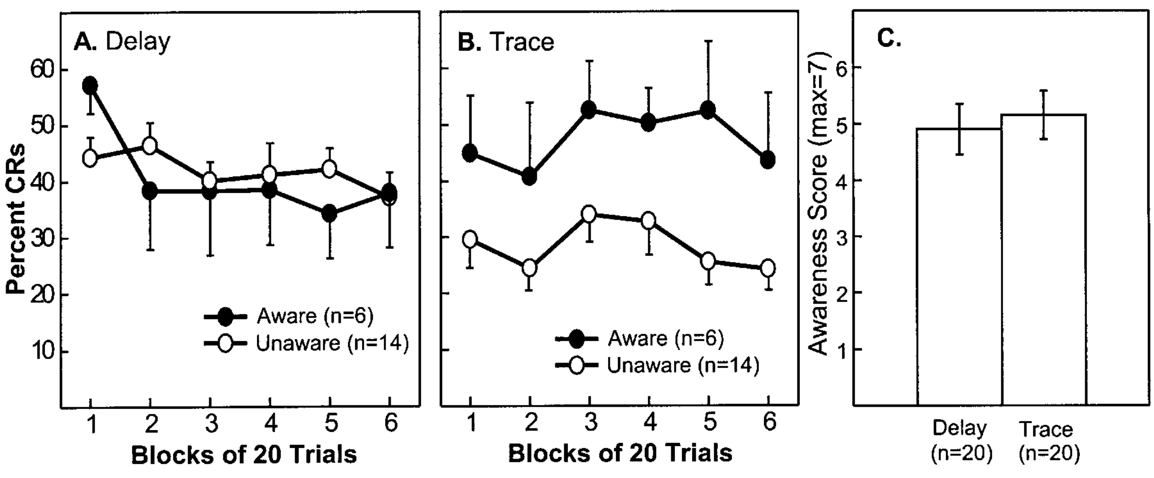

Figure 2. (A) Delay conditioning. Percent CRs across 6 blocks of 20 trials by participants who were classified as aware or unaware on the basis of their answers to the seven true or false questions given after the first 10 trials. (B) Trace conditioning. Percent CRs across 6 blocks of 20 trials by participants who were classified as aware or unaware on the basis of their answers to the seven true or false questions given after the first 10 trials. (C) Mean score obtained by each group for seven true or false questions about the relationship between the CS and the US. Error bars show *SEM*.

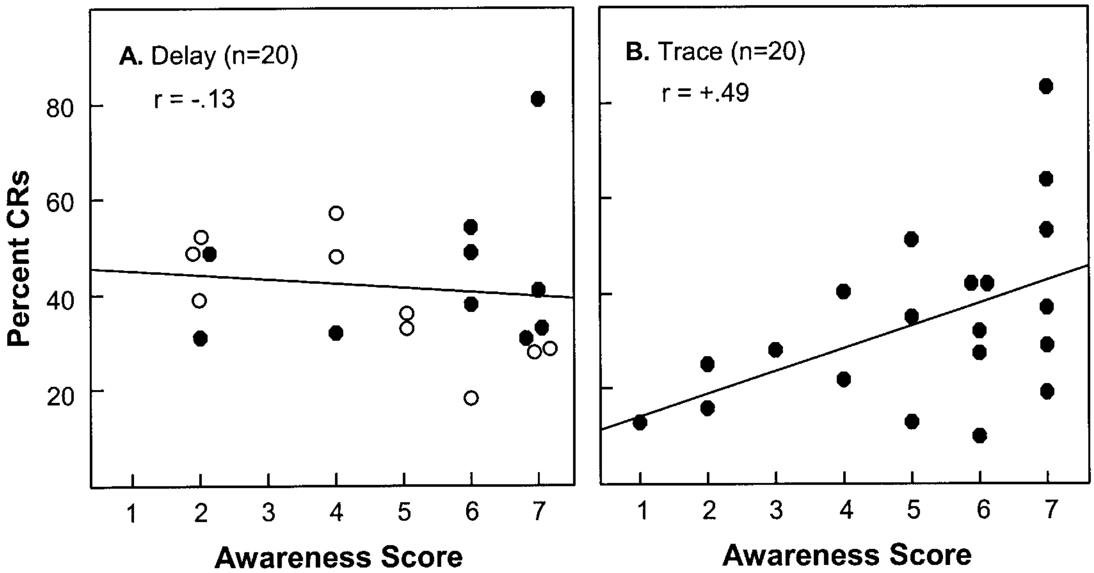

Figure 3. Relationship between awareness score obtained after the first 10 trials and the strength of conditioning (percent CRs) across all 120 conditioning trials. (A) Performance of participants who received delay conditioning ($r = -.13, p > .1$). Open circles represent participants who received pseudoconditioning before the session. Closed circles represent participants who did not receive pseudoconditioning. (B) Performance of participants who received trace conditioning ($r = .49, p < .05$).

sensitivity of these participants to the 1,350-msec tone. The remaining 10 participants were given 32 pseudoconditioning (16 CS-alone and 16 US-alone) trials prior to the conditioning session to reduce the likelihood of these early nonassociative responses. Only one of the 8 participants who received pseudoconditioning (for 2 participants, data from the 1st trial was not interpretable) emitted a response on the 1st trial of the conditioning session that was scored as a CR. Thus, the pseudoconditioning trials prior to the conditioning session did reduce the likelihood of early nonassociative responses. In any case, over the entire session, the level of conditioning was similar for both groups (mean % CRs ± *SEM* = 38.9 ± 3.9 and 43.9 ± 4.9 for participants who received pseudoconditioning and those who did not, respectively; Figure 1B). Thus, it appears that pseudoconditioning prior to the conditioning session did not appreciably influence conditioning performance after the first several conditioning trials.

It is also notable that performance in the two groups did not improve from the first block of 20 trials to the last block of 20 trials. In several previous studies of single-cue eyeblink conditioning, it has been found that conditioning improved only modestly after the first 5 to 10 trials (for delay conditioning, see Carrillo et al., 1997, Figure 2; Gabrieli et al., 1995, Figure 1; for trace conditioning, see Manns et al., 2000a, Figures 1B and 2B; McGlinchey-Berroth et al., 1997, Figure 3; Woodruff-Pak & Papka, 1996, Figure 1). Thus, the present findings are not unusual.

Finally, it is worth emphasizing that associative learning did occur in our participants. First, the level of conditioning performance for all participants increased from the first 10 trials to the second 10 trials [mean % CRs ± *SEM* = 40.9 ± 4.2 and 53.7 ± 3.1; paired samples t test, $t(19) = 2.67, p < .05$]. Second, the overall level of responding across the session was greater than the level of responding observed during the pseudoconditioning trials [for 10 participants who received pseudoconditioning trials, 38.9 ± 3.9 vs. 21.3 ± 4.9, paired samples t test, $t(9) = 3.24, p = .01$; for 10 participants who did not receive pseudoconditioning trials, 43.9 ± 4.9 vs. 21.3 ± 4.9, independent samples t test, $t(18) = 3.21, p < .01$].

Figure 2A shows the percentage of CRs emitted across all 6 blocks of 20 trials by all 20 participants who were classified as aware or unaware on the basis of their answers to the seven true or false questions given after the first 10 trials. Participants classified as aware ($n = 6$) were those who answered correctly all 7 of the true or false questions given after the first 10 trials. Participants classified as unaware ($n = 14$) were those who answered fewer than 7 questions correctly [mean number of correct answers = 4.0, which is not significantly above chance, $t(13) = .29, p > .1$]. (Note that on a 7-item true or false test, a score of 7 correct is significantly above chance [binomial test, $p < .05$], but a score less than 7 is not.) Awareness did not affect conditioning. The 6 participants who were designated as aware after the first 10 conditioning trials and the 14 participants designated unaware emitted a similar percentage of CRs across all 120 conditioning trials [40.7 ± 8.4% CRs vs. 41.8 ± 3.0% CRs, respectively; $t(18) = .16, p > .1$]. In addition, the 15 participants who became aware (awareness score = 7) by the end of the 120-trial session conditioned no better than the 5 participants who remained unaware (mean awareness score = 4.4) at the end of the session [41.5 ± .4% CRs vs. 41.2 ± .4% CRs, respectively; $t(18) = .04, p > .1$]. When the data were examined separately for the participants who received pseudoconditioning trials prior to the conditioning session and for those who did not, the participants who were aware did not exhibit a noticeably higher overall level of conditioning than those who were unaware (for those who received pseudoconditioning trials, aware, 28.6 ± .5% vs. unaware, 41.5 ± 4.4%; for those who did not receive pseudoconditioning trials, aware, 46.8 ± 11.8% vs. unaware, 42.3 ± 4.1%).

Markedly different results were obtained in an earlier study in which the conditioning session was identical to the present one except that participants were given trace eyeblink conditioning (Manns et al., 2000a, Experiment 2; a 1,000-msec silent interval separated the 250-msec CS and the 100-msec US). The data from that study, which indicate that awareness did affect conditioning, are presented for comparison in Figure 2B. Specifically, the 6 participants who were designated as aware at the end of the first 10 conditioning trials emitted more CRs across the conditioning session than did the 14 unaware participants [47.4 ± 9.7% CRs vs. 28.3 ± 3.4% CRs, respectively; $t(18) = 2.36, p < .05$]. A repeated measures ANOVA for the data from delay and trace conditioning (Figures 2A, 2B) revealed a group × awareness interaction that approached significance [$F(1,36) = 3.58, p = .07$].

Figure 2C shows the average awareness score obtained after the first 10 conditioning trials for participants administered delay conditioning in the present study and for participants administered trace eyeblink conditioning in the earlier study. The awareness scores were very similar [mean ± *SEM* = 4.9 ± .4 and 5.2 ± .4, for participants given delay and trace conditioning, respectively; $t(38) = .41, p > .1$]. Awareness scores obtained after 60 trials and after 120 trials were higher than those obtained after 10 trials and were also similar for participants given delay and trace conditioning (mean ± *SEM* = 6.3 ± .3 and 6.4 ± .2, respectively, after 60 trials; 6.4 ± .3 and 6.6 ± .2, respectively, after 120 trials).

For the 20 participants given delay conditioning, Figure 3A shows the relationship between the awareness scores obtained after 10 conditioning trials and the mean percent CRs across all 120 trials. There was no relationship between the awareness score after the first 10 conditioning trials and the strength of conditioning across the conditioning session ($r = -.13, p > .1$). In addition, the overall level of conditioning was not positively correlated with the awareness score obtained after 10 trials for either the participants who received pseudoconditioning trials or the participants who did not ($r = -.73$, $p < .05$; and $+.23$, $p > .5$, respectively). The negative correlation for the participants who received pseudoconditioning is an interesting finding but is difficult to evaluate. The slope of the relationship (Figure 3A, open circles) is shallow and depends on 2 participants who were aware but conditioned poorly.

In contrast to the findings for delay eyeblink conditioning, for the participants in the previous study who were given trace conditioning, the awareness score after the first 10 conditioning trials significantly predicted the strength of conditioning across the session (Figure 3B; $r = .49$, $p < .05$). Furthermore, the correlation for the trace group was significantly higher than the correlation for the delay group ($p < .05$; see Hays, 1994, pp. 650–651).

DISCUSSION

The present study is the first to compare directly the relationship between conditioning and awareness for both standard delay and standard trace eyeblink conditioning. During delay conditioning, participants who became aware of the stimulus contingencies early in the conditioning session conditioned no better than participants who became aware later in the session or did not become aware (Figure 2A). In contrast, participants given trace conditioning who became aware early in the session conditioned better than those who became aware later in the session or did not become aware (Figure 2B). In addition, in the case of delay conditioning, the level of awareness early in the session was unrelated to the overall level of conditioning across the session (Figure 3A). Yet, for trace conditioning, awareness early in the session predicted the overall level of conditioning across the session (Figure 3B). Furthermore, in earlier studies of trace conditioning, distraction during the conditioning session reduced awareness and decreased conditioning (Manns et al., 2000a). In another study of trace conditioning, those participants designated as "aware" after the session produced more conditioned responses during the first 10 conditioning trials than did those designated "unaware" (Woodruff-Pak, 1999).

These findings, which indicate that awareness is related to trace conditioning but not to delay conditioning, are in agreement with other studies in which more complex, differential conditioning paradigms have been used. In differential conditioning, two CSs are presented. One CS (the CS^+) is followed by the US, and the other CS (the CS^-) is presented alone. Differential conditioning is calculated as the percentage of CRs to the CS^+ minus the percentage of CRs to the CS^-. Differential trace eyeblink conditioning depends on the hippocampus, whereas differential delay eyeblink conditioning does not (Clark & Squire, 1998). In addition, for differential trace conditioning, awareness has been found to be a critical factor (Clark & Squire, 1998). For example, explaining the stimulus contingencies to participants before training facilitated differential trace conditioning, and preventing awareness of the stimulus contingencies by introducing a distracting secondary task prevented differential trace conditioning (Clark & Squire, 1999). Furthermore, when a trial-by-trial measure of awareness was used, successful differential trace conditioning and awareness appeared to develop in parallel (Manns, Clark, & Squire, 2000b). In contrast to these findings for differential trace conditioning, successful differential delay conditioning was found to be independent of awareness of the stimulus contingencies (Clark & Squire, 1998). Moreover, introducing the same distracting task that disrupted differential trace conditioning had no effect on differential delay conditioning (Clark & Squire, 1999).

Other studies have indicated that complex stimulus conditions, as well as secondary tasks, can retard differential delay conditioning (Carrillo, Gabrieli, & Disterhoft, 2000; Mayer & Ross, 1969; Ross & Nelson, 1973), but it is unclear in these cases that awareness is the important factor. For example, in one study (Carrillo et al., 2000), a verbal shadowing task eliminated differential responding for aware and unaware individuals alike. In another study (Ross & Nelson, 1973), unaware participants in some groups exhibited substantial differential responding, and other task factors in addition to awareness were considered to be important for conditioning. However, in a more recent study, differential delay conditioning did correlate with awareness in both young and aged participants (Knuttinen, Power, Preston, & Disterhoft, in press).

There are several differences between the study that revealed a role for awareness in differential delay conditioning (Knuttinen et al., in press) and our earlier studies (Clark & Squire, 1998, 1999) that did not. Knuttinen et al. (in press) presented 30 pseudoconditioning trials prior to the conditioning session and used a less intense CS. A potentially more important factor is that different criteria were used in the two studies to identify and score the CRs. One reason that the criteria could be important is that it has been recognized that true CRs can be difficult to distinguish from voluntary eye closures (Coleman & Webster, 1988). Voluntary eye closures have been described as short-latency responses involving complete eye closures that are maintained until the onset of the US (Spence & Ross, 1959; Spence & Taylor, 1951). We have tried to identify and exclude putative voluntary eye closures by excluding responses that begin earlier than 500 msec before the US and that persist until the US (see Method; also see Clark & Squire, 1998, 1999). Knuttinen et al. (in press) did not discuss voluntary responses, and one does not know that such responses were a factor in their study. We note only that the average CRs illustrated in their Figure 3 begin more than 700 msec before the US and persist until the US. By our criteria, individual responses with these characteristics would have been excluded from analysis. If voluntary eye closures were frequent and were sometimes scored as CRs, then the performance of aware individuals (who would be capable of voluntary eye closures) would likely be better than the performance of unaware individuals (who would not exhibit voluntary eye closures).

We also rescored the present data for single-cue delay eyeblink conditioning, using the same scoring window as was described in the Knuttinen et al. (in press) study (responses occurring between 100 msec after the onset of the CS and the onset of the US). The results were similar to those obtained with our original scoring window. There was no difference in the level of conditioning between aware and unaware participants (mean % CRs ± $SEM = 62.5 \pm 6.5$ and 67.8 ± 3.6, respectively). We also examined the frequency of responses that were likely to be voluntary eyeblinks (early onset, large amplitude, long duration, no effect of the airpuff US). Aware participants did emit on the average more of these responses than did unaware participants [mean number of responses per participant ± $SEM = 3.8 \pm 2.2$ and $.5 \pm .3$, respectively; $t(18) = 2.25, p < .05$]. However, this small percentage of voluntary responses among the total number of trials would not have produced a difference between aware and unaware participants, even if they had been scored as conditioned responses. Nevertheless, this finding suggests that it will be useful to monitor the frequency of voluntary eye closures in studies of eyeblink conditioning and distinguish them from valid conditioned responses.

In any case, there is now substantial support for the importance of awareness in single-cue trace eyeblink conditioning, and the present study provides strong evidence that awareness is unrelated to single-cue delay eyeblink conditioning. Taken together, these results indicate that trace conditioning requires an additional level of processing that is not required for delay conditioning. The results also help explain why the hippocampus is important for trace eyeblink conditioning but not for delay eyeblink conditioning. We propose that though awareness per se may not directly contribute to successful trace conditioning, awareness serves as an indicator that the hippocampus and related structures (as well as the neocortex) are effectively engaged so as to make trace conditioning possible (Manns et al., 2000b). For delay conditioning, awareness (and the integrity of the hippocampus) appears to be superfluous.

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APPENDIX
Questions Asked About the Relationship Between CS and US

1. I believe the airpuff usually came immediately *before* the tone.	T	F
2. I believe the airpuff usually came immediately *after* the tone.	T	F
3. I believe the tone usually came immediately *before* the airpuff.	T	F
4. I believe the tone usually came immediately *after* the airpuff.	T	F
5. I believe the tone and airpuff were always closely related in time.	T	F
6. I believe the tone and airpuff were only sometimes related in time.	T	F
7. I believe the tone predicted when the airpuff would come.	T	F

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