

- was purified on a cobalt affinity column. Thirty-five carboxyl terminal amino acids were cleaved by chymotrypsin proteolysis. The truncated channel was purified to homogeneity by gel filtration and the detergent exchanged in a final dialysis step against 5 mM N,N-dimethyldodecylamine-N-oxide (LDAO). Crystals were grown at 20°C with the sitting drop method by mixing equal volumes of protein solution [5 to 10 mg/ml, 150 mM KCl, 50 mM tris (pH 7.5), and 2 mM dithiothreitol] with reservoir mixture [200 mM CaCl<sub>2</sub>, 100 mM Hepes (pH 7.5), and 48% PEG 400]. Through the entire preparation, the channel protein was maintained in solutions containing 150 mM KCl. For definition of K<sup>+</sup> sites, crystals were transferred into solutions where 150 mM KCl was replaced by 150 mM RbCl or 150 mM CsCl.
25. M. W. Tate *et al.*, *J. Appl. Crystallogr.* **28**, 196 (1995); D.J. Thiel *et al.*, *Rev. Sci. Instrum.* **67**, 1 (1996).
26. Z. Otwinowski, in *Data Collection and Processing*, L. Sawyer and S. Bailey, Eds. (Science and Engineering Research Council Daresbury Laboratory, Daresbury, UK, 1993), pp. 56–62.
27. Collaborative Computational Project 4 (CCP4), *Acta Crystallogr.* **D50**, 760 (1994).
28. G. M. Sheldrick, *ibid.* **46**, 467 (1990).
29. T. A. Jones, J. Y. Zou, J. Y. Cowan, M. Kjeldgaard, *ibid.* **A47**, 110 (1991).
30. A. T. Brunger, X-PLOR (Version 3.851) Manual (The Howard Hughes Medical Institute and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT).
31. S. J. Gamblin, D. W. Rodgers, T. Stehle, Proceedings of the CCP4 study weekend, Daresbury Laboratory (1996), pp. 163–169.

32. K. Y. J. Zhang and P. Main, *Acta Crystallogr.* **A46**, 377.
33. P. J. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991).
34. O. S. Smart, J. G. Neduvellil, X. Wang, B. A. Wallace, M. P. Sansom, *J. Mol. Graphics* **14**, 354 (1996).
35. We thank D. Thiel, S. Gruner, and members of the MacCHESS staff for support and assistance in data collection at A1; J. Kuriyan, S. K. Burley, S. Harrison, P. Kim, E. Gouaux, and D. Wang for helpful discussions; Y. Jiang for help in data collection; D. Gadsby and J. Kuriyan for comments on the manuscript; and T. Rahman for patience and support. R.M. is forever grateful to T. Wiesel and A. L. MacKinnon for making this project possible. R.M. is an investigator of the Howard Hughes Medical Institute.

23 February 1998; accepted 13 March 1998

# Classical Conditioning and Brain Systems: The Role of Awareness

Robert E. Clark and Larry R. Squire\*

Classical conditioning of the eye-blink response, perhaps the best studied example of associative learning in vertebrates, is relatively automatic and reflexive, and with the standard procedure (simple delay conditioning), it is intact in animals with hippocampal lesions. In delay conditioning, a tone [the conditioned stimulus (CS)] is presented just before an air puff to the eye [the unconditioned stimulus (US)]. The US is then presented, and the two stimuli coterminate. In trace conditioning, a variant of the standard paradigm, a short interval (500 to 1000 ms) is interposed between the offset of the CS and the onset of the US. Animals with hippocampal lesions fail to acquire trace conditioning. Amnesic patients with damage to the hippocampal formation and normal volunteers were tested on two versions of delay conditioning and two versions of trace conditioning and then assessed for the extent to which they became aware of the temporal relationship between the CS and the US. Amnesic patients acquired delay conditioning at a normal rate but failed to acquire trace conditioning. For normal volunteers, awareness was unrelated to successful delay conditioning but was a prerequisite for successful trace conditioning. Trace conditioning is hippocampus dependent because, as in other tasks of declarative memory, conscious knowledge must be acquired across the training session. Trace conditioning may provide a means for studying awareness in nonhuman animals, in the context of current ideas about multiple memory systems and the function of the hippocampus.

Memory is composed of several different abilities that depend on different brain systems (1). A fundamental distinction is between the capacity for conscious recollection of facts and events (declarative or explicit memory) and various nondeclarative (implicit) forms of memory that are expressed in skills, habits, and simple forms of conditioning. This distinction is dramatically evident in amnesic patients, who have bilateral damage to the hippocampal formation or related midline diencephalic brain structures. These patients have severely im-

paired declarative memory and are profoundly forgetful. Yet these same patients have a fully intact capacity for nondeclarative memory (2). Indeed, a large body of literature involving both humans and experimental animals can now be understood by recognizing that memory tasks requiring declarative memory depend on the integrity of the hippocampal formation and related structures, whereas tasks requiring nondeclarative memory can be performed normally after damage to these structures and are supported by other brain systems. Declarative memory is what is meant by the term "memory" in ordinary language. It is involved in modeling the external world, and its contents can be brought to consciousness as a verbal proposition or as a mental image. By contrast, nondeclarative memory is expressed through performance

without affording access to any conscious memory content or even awareness that memory is being used. This form of memory permits cumulative changes in perceptual and response systems and allows for the gradual development of new skills and habits.

A major puzzle about the distinction between conscious (hippocampus dependent) and nonconscious (hippocampus independent) forms of memory concerns classical conditioning. Classical conditioning, a phylogenetically early example of simple associative learning, has been studied extensively and would appear to be a quintessential example of nondeclarative memory (3). In perhaps the best studied classical conditioning paradigm, delay conditioning of the eye-blink response, a neutral conditioned stimulus (CS), such as a tone, is presented just before an air puff unconditioned stimulus (US). The US is then presented and the two stimuli coterminate (Fig. 1, A and B). Initially, an eye blink occurs reflexively in response to the US, but with repeated CS-US pairings a learned or conditioned response (CR) is elicited by the CS in advance of the US. The CR overlaps with the US such that the eye blink serves as an adaptive, defensive response to the air puff. Studies in the rabbit have shown that the cerebellum is essential for both the acquisition and retention of delay classical conditioning (4) and that no other forebrain structure, including the hippocampus, is required (5). Amnesic patients also exhibit intact acquisition and retention of the classically conditioned eye-blink response (6, 7). Thus, eye-blink conditioning appears to have the automatic, reflexive features that are characteristic of nondeclarative memory.

The puzzle concerns trace conditioning, a slightly different version of classical conditioning in which the CS is presented and terminated and then a short interval is imposed before the presentation of the US (8) (Fig. 1, C and D). The name comes from the fact that the CS must leave some trace in the nervous system for a CS-US associ-

R. E. Clark is in the Department of Psychiatry, University of California, San Diego, La Jolla, CA 92093, USA. L. R. Squire is at the Veterans Affairs Medical Center, San Diego, CA 92161, USA, and Departments of Psychiatry and Neurosciences, University of California, San Diego, School of Medicine, La Jolla, CA 92093, USA.

\*To whom correspondence should be addressed.

ation to be established. Trace conditioning requires the hippocampus (7, 9–11). Yet the trace interval (typically 1 s or less) is far too short to create any special difficulty for amnesic patients with hippocampal damage (12). Amnesic patients can easily hold onto information for many seconds. Accordingly, it has not been clear what aspect of trace conditioning requires the hippocampus or why trace conditioning might involve declarative memory.

We reasoned that trace conditioning might differ from delay conditioning by requiring knowledge of the CS-US relationship to build up and be remembered across many trials (13). To explore this possibility, we tested amnesic patients and control volunteers on both delay and trace conditioning, and we also assessed the knowledge that participants developed about the CS-US association.

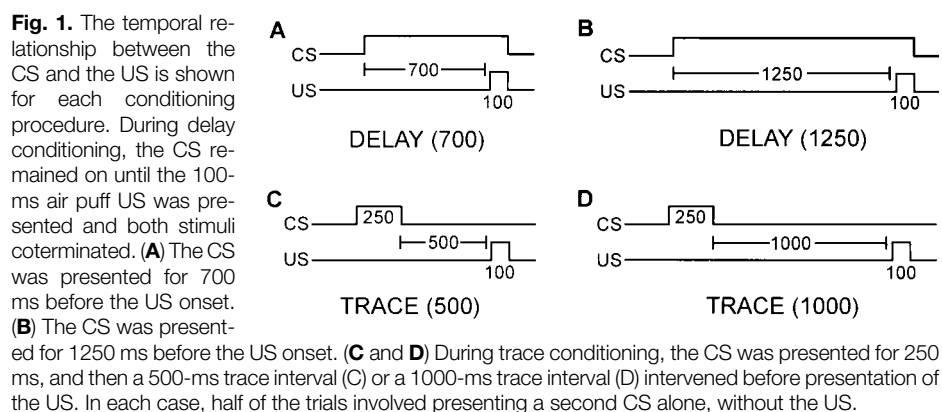
**Procedure.** We tested 4 amnesic patients (14) and 48 normal volunteers (15)

with two delay-conditioning procedures and two trace-conditioning procedures (Fig. 1). For delay conditioning, the CS was presented for 700 ms before the presentation of a 100-ms US (delay 700,  $n = 12$ ), or the CS was presented for 1250 ms before US onset (delay 1250,  $n = 10$ ). In both versions of delay conditioning, the CS and the US overlapped and coterminated. For trace conditioning, a CS was presented for 250 ms, and then a 500-ms trace interval (trace 500,  $n = 12$ ) or a 1000-ms trace interval (trace 1000,  $n = 14$ ) intervened before presentation of the US. The amnesic patients were given trace 1000 conditioning first and then 6 to 35 days later were given delay 1250 conditioning. All four conditioning paradigms used a differential conditioning procedure with two CSs in which one CS was consistently paired with the US ( $CS^+$ ) and a second CS was presented alone ( $CS^-$ ). For half of the participants, the  $CS^+$  was a tone and the  $CS^-$  was white

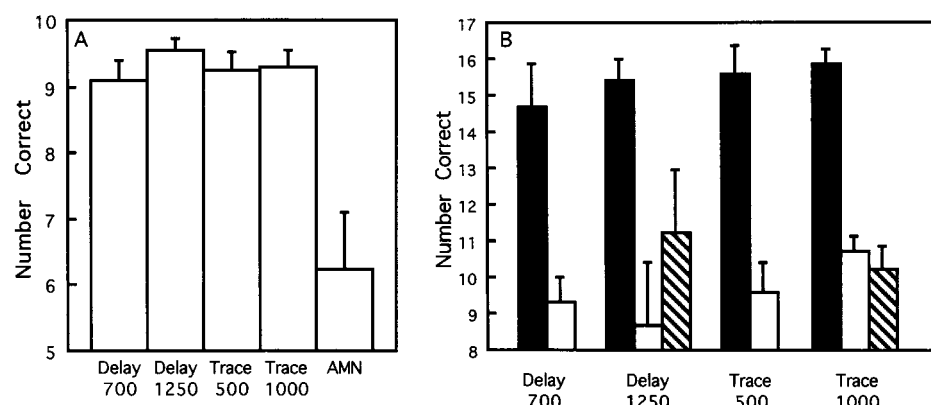
noise (static). For the other half of the participants, the  $CS^+$  and the  $CS^-$  were reversed. In all four paradigms, training consisted of 60  $CS^+$  and 60  $CS^-$  trials, which were presented while participants viewed a silent movie. Differential conditioning was measured as the percentage of CRs to the  $CS^+$  minus the percentage of CRs to the  $CS^-$  (16).

Immediately after conditioning, participants took a true or false test that asked about aspects of the conditioning session, including how well they remembered the movie (Fig. 2A) and how well they remembered the  $CS^+$ , the  $CS^-$ , and the US and their responses to the  $CS^+$  and the  $CS^-$  (17). The critical questions were 17 additional items concerning the temporal relationships between the  $CS^+$ , the  $CS^-$ , and the US. For example, true or false: I believe the air puff usually came immediately before the tone, I believe the tone usually came immediately before the static noise, I believe the static noise and air puff were always closely related in time, and I believe the tone predicted when the air puff would come. Participants responded to the test items in a fixed order and were not permitted to change earlier answers. In each of the four groups, participants who scored significantly above chance on the 17 critical questions [ $\geq 13$  correct out of the 17 test items that asked about the temporal relationships between the stimuli (18)] were designated as aware of the relationships among the stimuli, and participants who did not score significantly above chance ( $\leq 12$  test items correct) were designated as unaware (Fig. 2B). The acquisition of classical eye-blink conditioning (19) was then compared for the aware and unaware groups on each version of the task.

**Experimental results.** Knowledge of the stimulus contingencies was not related to performance on either version of delay conditioning, but it was a crucial factor in both versions of trace conditioning (Fig. 3). Specifically, normal volunteers acquired delay conditioning whether they were knowledgeable about the CS-US associations or not. For trace conditioning, only those individuals who developed knowledge of the CS-US associations successfully acquired the task. Finally, the amnesic patients, none of whom became aware of the CS-US associations (20), were unable to acquire trace conditioning, although they acquired delay conditioning at a normal rate (Fig. 3). The number of correct responses on the true or false test (out of 17 items that asked about the  $CS^+$ ,  $CS^-$ , US relationship) was not correlated with the percentage of differential responding for either version of delay conditioning (delay 700,  $r = -0.10$ ,



**Fig. 1.** The temporal relationship between the CS and the US is shown for each conditioning procedure. During delay conditioning, the CS remained on until the 100-ms air puff US was presented and both stimuli coterminated. (A) The CS was presented for 700 ms before the US onset. (B) The CS was presented for 1250 ms before the US onset. (C and D) During trace conditioning, the CS was presented for 250 ms, and then a 500-ms trace interval (C) or a 1000-ms trace interval (D) intervened before presentation of the US. In each case, half of the trials involved presenting a second CS alone, without the US.



**Fig. 2.** (A) Number of correct responses to 10 true or false questions about the content of the silent movie that participants watched during the conditioning procedure. AMN, four amnesic patients. Each of the four other groups consisted of 10 to 14 control participants given delay eye-blink conditioning or trace eye-blink conditioning (see Fig. 1). The AMN group performed no better than chance and more poorly than each of the other groups (all  $P$  values  $< 0.001$ ). (B) Number of correct responses to 17 true or false questions about the temporal relationships between the  $CS^+$ , the  $CS^-$ , and the US. The black bars are the scores of control participants who were aware of the CS-US relationship, the white bars are the scores of control participants who were unaware of the CS-US relationship, and the hatched bars are the scores for the four amnesic patients. The number of participants in each group is the same as in (A). Error bars show the SEM.

$P > 0.5$ ; delay 1250,  $r = 0.16$ ,  $P > 0.5$ ) but was significantly correlated with the percentage of differential responding for both versions of trace conditioning (trace 500,  $r = 0.74$ ,  $P < 0.01$ ; trace 1000,  $r = 0.69$ ,  $P < 0.01$ ).

For trace 1000, the failure of the amnesic and unaware groups to demonstrate differential conditioning was due to the failure to acquire CRs to the CS<sup>+</sup>. For trace 500, unaware participants failed to demonstrate differential conditioning because they did not discriminate between the CS<sup>+</sup> and the CS<sup>-</sup>. That is, they exhibited CRs to both the CS<sup>+</sup> and the CS<sup>-</sup> (21).

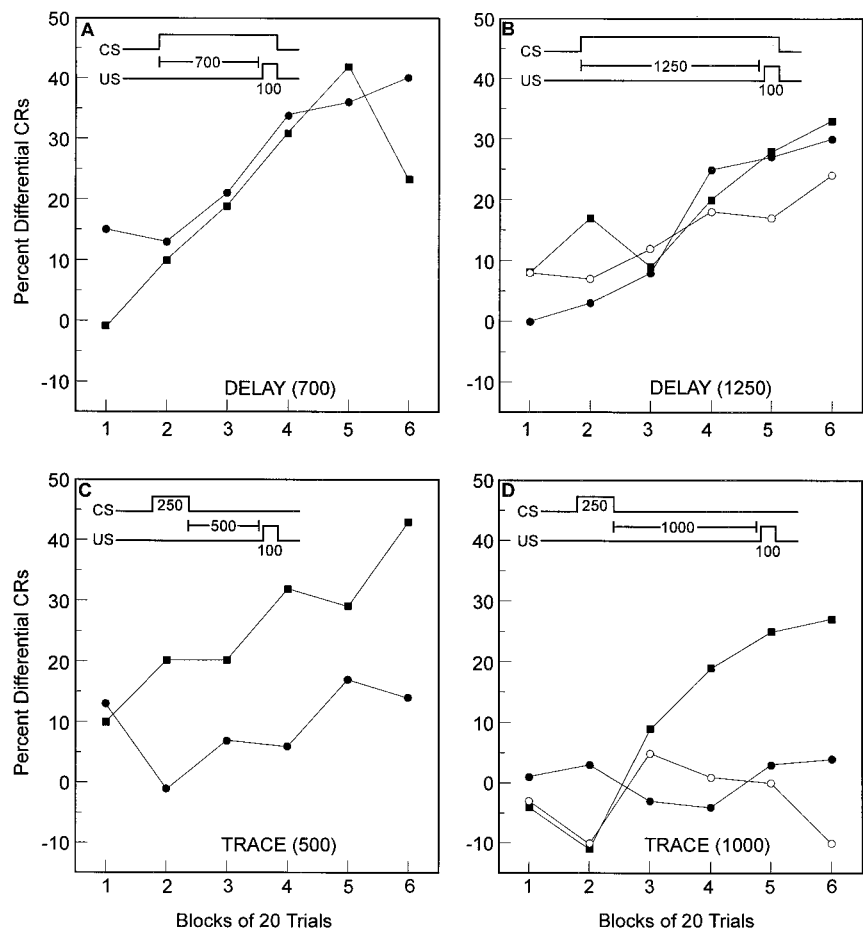
We also addressed the nature of the relationship between awareness and conditioning. Did awareness occur as a result of conditioning, or did conditioning occur because participants became aware of the CS-US associations? In both versions of delay conditioning, participants acquired differential conditioning even if they did not become aware of the CS-US associations. This finding shows that successful conditioning does not guarantee awareness. To examine this issue more directly in the case of trace conditioning, we tested two new groups of participants. With the first group (five men and three women, mean age = 70), we thoroughly explained the temporal relationships between the CS<sup>+</sup>, the CS<sup>-</sup>, and the US before trace 1000 conditioning. All participants exhibited differential conditioning. After conditioning, the group obtained a score of 16.0 correct out of 17 on the test items that asked about the temporal relationships between the stimuli. This group also exhibited significantly improved conditioning compared with the 14 control participants in the trace 1000 group (block six scores,  $P < 0.05$ ; Fig. 3D) (22). The second group (five men and three women, mean age = 63) was given trace 1000 conditioning while concurrently performing an attention-demanding task (detecting strings of three odd digits in a running sequence). This group obtained 9.0 correct out of the 17 test items that asked about the stimuli, and they did not exhibit differential conditioning (block six scores = -0.05%, significantly poorer than the 14 control participants in Fig. 3D;  $P < 0.05$ ). This finding strengthens the notion that awareness is a prerequisite for successful trace conditioning.

**Implications.** The results appear to resolve the puzzle of why trace conditioning depends on the integrity of the hippocampus. Like other tasks of declarative memory that are impaired after hippocampal lesions, trace conditioning requires the acquisition and retention of conscious knowledge across a considerable time span (in this case, the 30-min conditioning ses-

sion). Specifically, individuals must acquire and retain knowledge of the task structure if conditioning is to be successful. In earlier work, the limited ability that amnesic patients had for acquiring tasks of declarative memory correlated with their ability to verbalize the principles underlying the tasks (23). Trace conditioning may require declarative knowledge because the trace interval between the CS and the US makes it difficult to process the CS-US relationship in an automatic, reflexive way. This more complex condition likely requires the neocortex to represent the temporal relationships between the stimuli (24) and would require the hippocampus and related

structures to work conjointly with the neocortex to establish a usable representation that can persist as memory.

Trace conditioning is dependent on the cerebellum as well as the hippocampus (25). Thus, even though trace conditioning differs from delay conditioning in its requirement for declarative memory, it resembles delay conditioning in that a nondeclarative learning circuit in the cerebellum is required for the generation of the conditioned response. Thus, in the case of trace conditioning, it appears that a representation of the CS-US relationship, dependent on the hippocampus and neocortex, can then be used by the cere-



**Fig. 3.** Performance during classical conditioning of the eye-blink response by amnesic patients (AMN) and four groups of normal volunteers. The data are presented as the percentage of differential conditioned eye-blink responses for each block of 20 trials (percentage of CRs to the CS<sup>+</sup> minus percentage of CRs to the CS<sup>-</sup>). Each 20-trial block included 10 CS<sup>+</sup> trials in which a tone (or white noise) occurred together with an air puff to the eye (US) and 10 CS<sup>-</sup> trials in which white noise (or a tone) occurred but the US did not. The temporal relationship between the CS<sup>+</sup> and the US is shown at the top of each panel. The participants in each condition were classified as aware or unaware according to their performance on a 17-item, true or false test that asked about the relationship between the CS<sup>+</sup>, the CS<sup>-</sup>, and the US. Participants scoring  $>2$  SD above the chance score of 8.5 correct (that is, 13 correct) were considered aware. Only the aware groups and not the AMN group or the unaware groups acquired differential trace conditioning (C and D). All the groups acquired delay conditioning (A and B), and, unlike trace conditioning, awareness was not a factor in the learning of differential delay conditioning. The SEMs ranged from 0.03 to 0.08. (A) ■, aware ( $n = 3$ ); ●, unaware ( $n = 9$ ). (B) ■, aware ( $n = 7$ ); ●, unaware ( $n = 3$ ); ○, AMN ( $n = 4$ ). (C) ■, aware ( $n = 5$ ); ●, unaware ( $n = 7$ ). (D) ■, aware ( $n = 7$ ); ●, unaware ( $n = 7$ ); ○, AMN ( $n = 4$ ).



bellum to support conditioning. Task awareness may develop whenever the hippocampus and neocortex are engaged during learning.

The concept of conscious knowledge is not readily applied to experimental animals. Nevertheless, an implication of the present findings is that learning and memory tasks, including trace conditioning, which are failed by animals with hippocampal lesions, are tasks about which intact animals must acquire declarative knowledge. Characteristics that have been helpful in extending the concept of declarative memory to nonhuman animals include its flexibility and the ability to use it inferentially in novel situations (26). The conjoint operation of the hippocampal system and the neocortex may be the critical element that confers awareness about knowledge that has been acquired (27, 28).

The finding that trace conditioning requires subjects to become aware of the temporal relationships among the stimuli explains why trace conditioning is declarative and hippocampus dependent, and it brings classical conditioning, the best studied of all learning paradigms, into register with current understanding of the memory systems of the brain.

## REFERENCES AND NOTES

1. L. R. Squire, *Annu. Rev. Neurosci.* **5**, 241 (1982); N. Cohen, in *Neuropsychology of Memory*, L. R. Squire and N. Butters, Eds. (Guilford, New York, 1984), pp. 83–103; M. Mishkin and H. L. Petri, in *ibid.*, pp. 287–296; R. J. McDonald and N. M. White, *Behav. Neurosci.* **107**, 3 (1993); D. L. Schacter and E. Tulving, *Memory Systems 1994* (MIT Press, Cambridge, MA, 1994); L. R. Squire and B. J. Knowlton, in *The Cognitive Neurosciences*, M. Gazzaniga, Ed. (MIT Press, Cambridge, MA, 1994), pp. 825–838.
2. L. R. Squire, *Psychol. Rev.* **99**, 195 (1992); D. L. Schacter, C. Y. Chiu, K. N. Ochsner, *Annu. Rev. Neurosci.* **16**, 159 (1993).
3. E. R. Kandel and R. D. Hawkins, in *Neuroscience, Memory, and Language*, R. D. Broadwell, L. L. Judd, D. C. Murphy, Eds. (Library of Congress, Washington, DC, 1995), pp. 45–58. Classical conditioning has been demonstrated in several invertebrate [T. J. Carew and C. L. Sahley, *Annu. Rev. Neurosci.* **9**, 435 (1986)] and vertebrate species [I. Gormezano, N. Schneiderman, E. Deaux, I. Fuentes, *Science* **138**, 33 (1962); I. Gormezano, W. F. Prokasy, R. F. Thompson, Eds., *Classical Conditioning* (Erlbaum, Hillsdale, NJ, 1987)].
4. D. G. Lavond, J. J. Kim, R. F. Thompson, *Annu. Rev. Psychol.* **44**, 317 (1993); R. F. Thompson and D. J. Krupa, *Annu. Rev. Neurosci.* **17**, 519 (1994).
5. L. W. Schmalz and J. J. Theios, *J. Comp. Physiol. Psychol.* **79**, 328 (1972); R. J. Norman, J. S. Buchwald, J. R. Villablanca, *Science* **196**, 551 (1977); M. D. Mauk and R. F. Thompson, *Brain Res.* **244**, 347 (1987).
6. L. Weiskrantz and E. K. Warrington, *Neuropsychologia* **17**, 187 (1979); I. Daum, S. Channon, A. G. M. Canavan, *J. Neurol. Neurosurg. Psychiatry* **52**, 47 (1989); I. Daum and H. Ackermann, *Int. J. Neurosci.* **75**, 153 (1994); J. D. E. Gabrieli et al., *Behav. Neurosci.* **109**, 819 (1995).
7. Impaired delay conditioning in one amnesic patient may have been due to the reported atrophy of the cerebellar vermis and hemispheres [D. S. Woodruff-Pak, *Behav. Neurosci.* **107**, 911 (1993)]. Two amnesic patients who had previously acquired delay conditioning subsequently exhibited little if any acquisition of trace conditioning, although during training they did achieve a string of eight CRs in nine consecutive trials.
8. I. P. Pavlov, *Conditioned Reflexes* (Oxford Univ. Press, London, 1927).
9. Trace conditioning was impaired in amnesic patients when trace intervals of 500, 750, and 1000 ms and a CS<sup>+</sup>-only (single cue) procedure were used [R. McGlinchey-Berroth, M. C. Carrillo, J. D. E. Gabrieli, C. M. Brawn, J. F. Disterhoft, *Behav. Neurosci.* **111**, 873 (1997)]. Some acquisition occurred with trace intervals of 500 or 750 ms, but no acquisition was detectable with a 1000-ms trace interval.
10. Rabbits with hippocampal lesions are impaired at trace conditioning [P. R. Solomon, E. R. Vander Schaaf, R. F. Thompson, D. J. Weisz, *Behav. Neurosci.* **100**, 729 (1986); J. R. Moyer, R. A. Deyo, J. F. Disterhoft, *ibid.* **104**, 243 (1990)].
11. Trace conditioning was impaired in rabbits when the lesion was made 1 day, but not 30 days, after conditioning [J. J. Kim, R. E. Clark, R. F. Thompson, *Behav. Neurosci.* **109**, 195 (1995)]. This temporal gradient of retrograde amnesia is characteristic of tasks that depend on the hippocampus.
12. Amnesic patients have fully intact immediate memory [A. D. Baddeley and E. K. Warrington, *J. Verb. Learn. Verb. Behav.* **9**, 176 (1970); D. A. Drachman and J. Arbit, *Arch. Neurol.* **15**, 52 (1966); C. B. Cave and L. R. Squire, *Hippocampus* **2**, 151 (1992)]. Even severely amnesic patients can retain small amounts of material in memory for a few seconds and sometimes up to a minute or more if they are not distracted [B. Milner, in *Amnesia*, C. W. M. Whitty and O. L. Zangwill, Eds. (Butterworths, London, 1966), pp. 109–133].
13. Delay conditioning in humans can occur without individuals becoming aware of the relationship between the CS and the US [E. R. Hilgard and L. G. Humphreys, *J. Gen. Psychol.* **19**, 111 (1938); K. W. Spence and J. Taylor, *J. Exp. Psychol.* **42**, 183 (1951); 29, 30]. One review concluded that “very careful, intensive, in-depth interviewing in the 1940s revealed clearly that there was little relationship between the subject’s reported awareness and his conditioning [eye blink] performance” [D. A. Grant, *Psychophysiology* **10**, 75 (1973), p. 75].
14. Four amnesic patients (three men and one woman) were tested (A.B., P.H., L.J., and E.P.). Bilateral damage to the hippocampal formation was confirmed by magnetic resonance imaging for three of the four patients [L. R. Squire, D. G. Amaral, G. A. Press, *J. Neurosci.* **10**, 3106 (1990); J. Polich and L. R. Squire, *Electroencephalogr. Clin. Neurophysiol.* **86**, 408 (1993); L. R. Squire and B. J. Knowlton, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12470 (1995)]. The remaining patient (A.B.) was suspected to have hippocampal formation damage on the basis of etiology (anoxia). The patients averaged 67.5 years of age (range, 60 to 75 years) with an average of 15.6 years of education. They averaged 105.0 on the Wechsler Adult Intelligence Scale-Revised (WAIS-R; mean subscale scores = 20.5 for information and 51.8 for vocabulary), and they averaged 100.8, 67.7, 74.3, 65.3, and 53.3 on the five indices of the Wechsler Memory Scale-Revised (attention concentration, verbal memory, nonverbal memory, general memory, and delayed memory, respectively). These scores have a mean of 100 in the normal population (SD = 15). For additional information about the four patients, see S. B. Hamann and L. R. Squire, *Behav. Neurosci.* **111**, 850 (1997); *ibid.* **109**, 1027 (1995).
15. The 48 controls (21 men and 27 women) averaged 66.9 years of age (range, 59 to 78 years) and 14.5 years of education and scored 20.6 and 53.2 on the information and vocabulary subscales of the WAIS-R, respectively. They were assigned to four separate groups, consisting of 10 to 14 individuals in each of four experimental conditions.
16. Participants were told that they were taking part in a study of how distraction affects learning and memory and that they would view a silent movie while being distracted by tones, static noise (white noise), and air puffs. After giving informed consent, participants were seated in a comfortable chair in a darkened room, 0.7 m from a television monitor, and shown *After the Gold Rush*, which they were asked to remember for a later test. Presentation of a silent movie during the conditioning trials is standard practice in recent studies of human eye-blink conditioning. During the movie, 120 conditioning trials occurred at an intertrial interval of 10 to 15 s. Each 20-trial block consisted of 10 CS<sup>+</sup> trials, in which a tone or white noise, presented through headphones, was paired with the air puff US, and 10 CS<sup>−</sup> trials, in which a tone or white noise occurred in the absence of the air puff US. The order of the CS<sup>+</sup> and CS<sup>−</sup> trials was random, except that neither trial type occurred more than twice consecutively. The conditioned stimuli were an 85-dB, 1-kHz tone and an 85-dB white noise. Each stimulus served equally often as CS<sup>+</sup> and CS<sup>−</sup> within each participant group. For the amnesic patients, the CS<sup>+</sup> and CS<sup>−</sup> were reversed for the second conditioning session. A pair of modified sunglasses held a nozzle for delivering the US (a 3-psi air puff to the left eye) and also held an infrared emitter detector for measuring the eye blink.
17. We used a true or false test, because recognition memory tests have been shown to reveal knowledge of stimulus contingencies during classical conditioning in instances where recall tests did not reveal any knowledge [M. E. Dawson and P. Reardon, *J. Exp. Psychol.* **98**, 308 (1973)]. Ten questions concerned the silent movie that participants watched during conditioning (Fig. 2A). Six questions asked whether the participants were aware that the CS<sup>+</sup>, the CS<sup>−</sup>, and the US had been presented. Four questions asked how, as conditioning progressed, participants had responded to the US, and eight questions asked about how participants had responded to the CS<sup>+</sup> and the CS<sup>−</sup>. All groups acquired significant knowledge of the stimuli (mean scores ranged from 5.0 to 5.9 correct out of 6,  $P < 0.05$ ) as well as knowledge about how they had responded to the air puff US (mean scores ranged from 3.0 to 3.5 correct out of 4;  $P < 0.05$ ). However, none of the groups acquired significant knowledge about how they had responded to either the CS<sup>+</sup> or the CS<sup>−</sup> (for CS<sup>+</sup>, mean scores ranged from 2.0 to 2.3 correct out of 4; for CS<sup>−</sup>, mean scores ranged from 2.3 to 2.5 out of 4; all  $P > 0.10$ ). This result was also true for those participants who were designated as aware of the relationships between the CS<sup>+</sup>, the CS<sup>−</sup>, and the US (for CS<sup>+</sup>, mean score = 2.1 out of 4; for CS<sup>−</sup>, mean score = 2.5 out of 4;  $P > 0.10$ ). These results agree with previous findings (29, 30).
18. The binomial probability of correctly answering 13 of 17 true or false questions by chance is  $P = 0.05$ .
19. For each participant in each condition, the mean eye-blink amplitude in response to the first 10 US presentations was calculated. For a response to the CS<sup>+</sup> or the CS<sup>−</sup> to be scored as a CR for that participant, the maximum eye-blink amplitude had to be at least 20% of the mean [20% criterion was used instead of some lower threshold because our pilot work indicated that with the infrared eye-blink measurement method, an eyeball movement, like that involved in scanning the television monitor, could register as high as 16% of the unconditioned response amplitude, in the absence of an actual eye blink [for a similar scoring criterion, see L. J. Sears, P. R. Fin, J. E. Steinmetz, *J. Autism Dev. Disord.* **24**, 737 (1994)]. In addition, maximum peak eye-blink amplitude had to occur less than 500 ms before the US onset [for a similar scoring criterion, see I. Daum et al., *Behav. Neurosci.* **107**, 748 (1993); R. G. Finkbiner and D. S. Woodruff-Pak, *Psychol. Aging* **6**, 109 (1991)]. This latency criterion was used to filter out nonassociative responding and voluntary responding (purposeful or volitional blinking). Voluntary eye blinks have been described as short-latency responses involving a smooth, sharp, and complete closure that is maintained until termination of the US air puff [K. W. Spence and L. E. Ross, *J. Exp. Psychol.* **58**, 376 (1959)]. Three points argue that the responses scored as CRs were not voluntary eye blinks: (i) Voluntary blinks are considered to involve full eyelid closure, and the average amplitudes of the

- CRs for each group were significantly less than a complete eye blink (range 51 to 64%). (ii) The mean latencies of the CR peaks were longer than the short-latency (<300 ms) responses of voluntary eye blinks (delay 700, 478 ms; delay 1250, 991 ms; trace 500, 527 ms; trace 1000, 996 ms). (iii) Participants were unable to answer accurately how they responded to the CS<sup>+</sup>.
20. The amnesic patients also exhibited no awareness of the CS-US relationships when training was continued beyond 120 trials and the test items were given during the intertrial intervals.
  21. Studies of single-cue trace conditioning with a 500-ms trace interval (as opposed to differential conditioning) are consistent with this finding and suggest that when only a CS<sup>+</sup> is used for training (no CS<sup>-</sup>), conditioning can proceed relatively automatically. First, amnesic patients do exhibit some learning in the single-cue condition (9). Second, normal participants exhibited conditioning with this procedure, despite a distraction task intended to reduce attention and awareness [M. C. Carillo, J. D. E. Gabrieli, J. F. Disterhoft, *Soc. Neurosci. Abstr.* **22**, 1866 (1996)].
  22. During the final four blocks of training (trials 41 to 120), differential responding progressed from 21 to 31%, closely matching the performance of the trace 1000 aware group (Fig. 3D). During the first two blocks of training (trials 1 to 20), this group also exhibited differential responding (25 to 37%). Inspection of eye-blink wave forms revealed that this finding was due to the fact that five of the eight participants exhibited several small eye blinks in response to the CS<sup>+</sup>, which became progressively larger as the US approached. This behavior extinguished as the conditioning session progressed. We suggest that responding early in the session was due to sensitization to the CS<sup>+</sup> caused by the pretraining instructions. Sensitization waned as participants habituated and became less concerned about the US.
  23. L. R. Squire, S. Zola-Morgan, K. S. Chen, *Behav. Neurosci.* **102**, 210 (1988).
  24. Lesions of the caudal area of the rabbit medial prefrontal cortex impair trace eye-blink conditioning (M. A. Kronforst-Collins and J. F. Disterhoft, *Neurobiol. Learn. Mem.*, in press).
  25. D. S. Woodruff-Pak, D. G. Lavond, R. F. Thompson, *Brain Res.* **348**, 249 (1985).
  26. H. Eichenbaum, *Annu. Rev. Psychol.* **48**, 547 (1997).
  27. D. L. Schacter, *J. Clin. Exp. Neuropsychol.* **12**, 155 (1990).
  28. Hippocampal cells in rabbits increase in activity after the US during the first four to five trials of delay classical eye-blink conditioning [T. W. Berger, B. Alger, R. F. Thompson, *Science* **192**, 483 (1976)]. This rapidly developing activity may be related to detection of the CS-US contingency and to its representation in declarative memory. The increase in activity survives lesions of the cerebellar interpositus nucleus that abolish the CR itself [G. A. Clark, D. A. McCormick, D. G. Lavond, R. F. Thompson, *Brain Res.* **291**, 125 (1984); (31)]. Another possible marker of declarative memory that occurs during classical conditioning (tone CS-shock US) is rapidly developing receptive field plasticity in the primary auditory cortex in response to the CS [J. S. Bakin and N. M. Weinberger, *Brain Res.* **536**, 271 (1990)]. These data have been interpreted as stimulus-stimulus learning (declarative), a rapidly acquired association between the CS and the US. Stimulus-response learning (procedural) is a more slowly acquired and precisely timed response specific to the US (N. M. Weinberger, *Neurobiol. Learn. Mem.*, in press). Finally, another marker of declarative memory is that the hippocampus is essential for only a limited time after learning. Three studies of eye-blink conditioning in rabbits support this feature of declarative memory. First, the hippocampus is essential for trace conditioning before and shortly after training, but not 1 month later (11). Second, after trace conditioning, the excitability of CA1 and CA3 hippocampal pyramidal neurons is increased. This increase in excitability peaks 24 hours after conditioning and decays back to baseline within 7 days [J. R. Moyer Jr., L. T. Thompson, J. F. Disterhoft, *J. Neurosci.* **16**, 5536 (1996)]. Third, multiple unit recordings from the hippocampus during delay conditioning show large learning-related activity increases in response to the CS, but this activity diminishes back to baseline within 7 days, even though animals continue to exhibit robust CRs (31).
  29. A. Freka, J. Beyts, A. B. Levey, I. Martin, *Pavlovian J. Biol. Sci.* **18**, 69 (1983).
  30. M. Papka, R. B. Ivry, D. S. Woodruff-Pak, *Psychol. Sci.* **8**, 404 (1997).
  31. L. L. Sears and J. E. Steinmetz, *Behav. Neurosci.* **104**, 681 (1990).
  32. We thank J. Moore, J. C. Frascino, and J. Zouzounis for assistance. Supported by the Medical Research Service of the Department of Veterans Affairs, National Institute of Mental Health (NIMH) grant 24600, and an NIMH postdoctoral fellowship (R.E.C.).

17 December 1997; accepted 20 February 1998

# Make a quantum leap.

SCIENCE Online can help you make a quantum leap and allow you to follow the latest discoveries in your field. Just tap into the fully searchable database of SCIENCE research abstracts and news stories for current and past issues. Jump onto the Internet and discover a whole new world of SCIENCE at the Web address:

**www.sciencemag.org**

**SCIENCE**

## Classical Conditioning and Brain Systems: The Role of Awareness

Robert E. Clark and Larry R. Squire

*Science* **280** (5360), 77-81.

DOI: 10.1126/science.280.5360.77

### ARTICLE TOOLS

<http://science.sciencemag.org/content/280/5360/77>

### REFERENCES

This article cites 22 articles, 2 of which you can access for free  
<http://science.sciencemag.org/content/280/5360/77#BIBL>

### PERMISSIONS

<http://www.sciencemag.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of Service](#)

---

*Science* (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. 2017 © The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. The title *Science* is a registered trademark of AAAS.