Behavioral Neuroscience
2008, Vol. 122, No. 2, 471–476

0735-7044/08/$12.00 DOI: 10.1037/0735-7044.122.2.471

Magnitude and Timing of Nictitating Membrane Movements During Classical Conditioning of the Rabbit (*Oryctolagus cuniculus*)

E. James Kehoe
University of New South Wales

Elliot A. Ludvig
University of Alberta

Joanne E. Dudeney
University of New South Wales

James Neufeld and R. S. Sutton
University of Alberta

A trial-by-trial, subject-by-subject analysis was conducted to determine whether generation of the conditioned response (CR) occurs on a continuous or all-or-none basis. Three groups of rabbits were trained on different partial reinforcement schedules with the conditioned stimulus presented alone on 10%, 30%, or 50%, respectively, of all trials. Plots of each rabbit's nictitating membrane movements revealed that their magnitude rose in a continuous fashion. Response growth during acquisition followed a sigmoidal curve, and the timing of CR-sized movements was largely stable throughout the experiment. The results are discussed with respect to alternative models of CR generation.

Keywords: conditioning, timing, rabbit, partial reinforcement

We conducted this experiment to determine whether conditioned responding in the rabbit nictitating membrane (NM) preparation shows continuous or discontinuous changes during training. Averaging measures of responding across blocks of trials and across subjects assumes implicitly that conditioned response (CR) acquisition and its underlying associative process are continuous, like the sun's rising across the horizon—at first small and then growing in height and width. In this article, we evaluate this depiction of the CR on a subject-by-subject, trial-by-trial basis.

In early studies of the rabbit NM response using averaged measures, changes in the CR appeared continuous. During pairings of a conditioned stimulus (CS) with an unconditioned stimulus (US), the CR became progressively larger in magnitude, and its onset migrated to an earlier portion of the CS–US interstimulus interval (ISI; Gormezano, Kehoe, & Marshall, 1983; Smith, 1968). More fine-grained investigations, however, have put the earlier conclusions in doubt (Gallistel, Fairhurst, & Balsam, 2004; Garcia, Mauk, Weidemann, & Kehoe, 2003). In both acquisition training and generalization testing, Garcia et al. (2003) observed apparent discontinuities in the magnitude of both NM and outer eyelid movements. The aggregated distributions of movements were bimodal. The movements tended to be either negligible in magnitude, well below the criterion used for defining a CR, or well above the criterion (0.5 mm for the NM response, 0.3 mm for the outer eyelid). Similarly, when NM movements were bifurcated into non-CRs (<0.5 mm) and CRs (>0.5 mm), Gallistel et al. (2004) observed that 10 of 23 rabbits showed a one-trial transition from the non-CR range into the CR range. As for CR onset latency, changes from longer to shorter values in acquisition appear to be restricted to short ISIs. For ISIs of 500 ms and greater, the CR onset latency, at least in delay conditioning, tends to become longer as training progresses (Kehoe & Schreurs, 1986; Vogel, Brandon, & Wagner, 2003).

Continuous processes of learning and CR expression appear in many behavioral and neural models of conditioning (e.g., Hull, 1943, p. 327; Medina, Repa, Mauk, & LeDoux, 2002; Rescorla & Wagner, 1972; Sutton & Barto, 1981, 1990). Evidence for a continuous learning process at the neuronal level has been seen in the activity of Purkinje cells in the cerebellar cortex of ferrets (Jirenhed, Bengtsson, & Hesslow, 2007). Paired stimulation of afferent pathways for the CS (mossy fibers) and US (climbing fibers) produced gradual acquisition of an inhibitory response in simple spike firing. Subsequent extinction training produced a gradual loss of the inhibitory response, and reintroduction of CS–US pairings yielded rapid reacquisition. Furthermore, like eyeblink CRs, the neuronal responses were initiated during the CS–US interval and reached their peak magnitude near the time of US delivery.

If the cerebellar changes that underpin overt CRs are continuous, then results like those of Garcia et al. (2003) suggest that a discontinuous process occurs at some point in the downstream pathways that drive the CR. Hence, we conducted the present experiment to evaluate the continuities versus discontinuities in CR expression by examining NM movements in greater detail than has been reported previously. First, we conducted the analyses on a trial-by-trial and subject-by-subject basis to eliminate any distortion from averaging. Second, we examined transitions in the magnitude of movements under a range of partial reinforcement

E. James Kehoe and Joanne E. Dudeney, School of Psychology, University of New South Wales, Sydney, New South Wales, Australia; Elliot A. Ludvig, James Neufeld, and R. S. Sutton, Department of Computing Science, University of Alberta, Edmonton, Alberta, Canada.

Preparation of this article was supported by the Informatics Circle of Research Excellence (iCore), Alberta, Canada.

Correspondence concerning this article should be addressed to E. James Kehoe, School of Psychology, University of New South Wales, Sydney 2052, Australia. E-mail: j.kehoe@unsw.edu.au

schedules, containing different frequencies of CS-alone trials, on which the NM movements were recorded without intrusion by the unconditioned response (UR). In Garcia et al., only 20% of trials were CS alone. Thus, observing rapid changes in NM magnitude was difficult. In the present experiment, we used three frequencies for the CS-alone trials: 10%, 30%, and 50%.

Method

Subjects and Apparatus

The subjects were 24 female albino rabbits (*Oryctolagus cuniculus*), 70–80 days old, weighing around 1.5 kg on arrival. The apparatus was based on that of Gormezano (1966; cf. Kehoe & Joscelyne, 2005). The CS was a 1,000-ms, 1000-Hz, 83-dB (SPL, C scale) tone. Background noise (76 dB, SPL, C scale) was provided by white noise and a ventilating fan. The US was a 50-ms, 3-mA, 50-Hz AC current delivered via 9-mm wound clips positioned 10 mm behind the dorsal canthus of the right eye and 15 mm below the center of the eye. To avoid distorting NM movement, no eye straps were used. The CS–US interval (onset to onset) was 500 ms, and the mean intertrial interval was 60 s (range = 50–70 s).

Procedure

Each rabbit was prepared by suturing a loop of surgical silk (000) into the NM of the right eye under local anesthetic (proxymetacaine hydrochloride). The next day, the rabbits were adapted to the conditioning apparatus for 60 min. They were then assigned randomly to three groups ($n = 8$) designated as Groups 10, 30, and 50 in accordance with the percentage of trials that were CS-alone trials. (For Group 10, 1 rabbit was removed after 3 days as a result of an eye infection.)

All groups received 16 sessions of acquisition training, each of which was divided into six blocks of 10 trials. For Group 10, Trials 1–9 were all CS–US trials, and Trial 10 was a CS-alone trial. For Group 30, each block contained 7 CS–US trials and 3 CS-alone trials. For Group 50, there were 5 CS–US trials and 5 CS-alone trials in each block. For Groups 30 and 50, the mixture of CS–US and CS-alone trials was randomized, subject to the constraints that Trial 10 was always a CS-alone trial and no more than two CS-alone trials occurred consecutively.

Response Measures and Statistical Tests

Four measures of responding were recorded: (a) *magnitude*, the maximal extent of an NM movement on CS-alone trials; (b) *CR likelihood*, the proportion of all trials in each session in which the NM movement exceeded 0.5 mm during the CS but before the time of the US; (c) *CR onset latency*, the time after CS onset at which a CR was initiated; and (d) *CR peak latency*, the time at which a CR on a CS-alone trial reached its maximal extent.

Statistical tests used a Type I error of .05 (O'Brien & Kaiser, 1985), and partial eta-squared (η_p^2) measured effect size. This measure equals $SS_{effect}/(SS_{effect}+SS_{error})$. In relation to Cohen's (1988) d' measure of effect size, a η_p^2 of .20 corresponds to a d' of 1.00, which is considered a large effect size. Finally, means are accompanied by the standard error of the mean ($\pm SEM$).

Results

Magnitude and CR Likelihood

Conventional analyses using mean magnitude and mean CR likelihood revealed that Group 10 showed the fastest CR acquisition, but was followed closely by Groups 30 and 50. Across acquisition training, the mean magnitudes for Groups 10, 30, and 50 were, respectively, 1.91 mm, 1.60 mm, and 1.08 mm (±0.57 mm), $F(1, 20) = 3.60$, $p = .07$, $\eta_p^2 = .15$, and the mean CR likelihoods were 71%, 64%, and 55% CRs (±14%), $F(1, 20) = 4.64$, $p < .05$, $\eta_p^2 = .19$. At the end of training, all groups had converged on mean levels around 2.10 mm (±0.25 mm) and 83% CRs (±2%). The group mean magnitudes (filled circles) and their standard errors are plotted on a day-by-day basis in Panels B, D, and F of Figure 1.

Figure 1 also depicts the trial-by-trial, subject-by-subject magnitudes of the largest NM movement on the CS-alone trials in acquisition training. Panels A, C, and E each show the data for 2 rabbits selected randomly from each group. Thus, these 6 rabbits represent a quarter of the total sample. Inspection of these plots suggests that there was continuous growth, accompanied, however, by considerable variability, which is usually masked by averaging. The transition through the subcriterial region between 0.125 mm and 0.500 mm, in which Garcia et al. (2003) saw little activity, was relatively rapid. Only 9% (±1%) of the NM movements occurred in this region (range = 1%–23%).

To summarize the data for each rabbit, the trial-by-trial CR magnitudes were fitted with a Weibull function (Gallistel et al., 2004):

$$\text{Magnitude} = A\,(1 - 2^{-[(\text{Trial}/L)S]}),$$

where A = the asymptotic magnitude, Trial = the number of each successive CS-alone trial, L = the number of trials for the magnitude to reach half of its asymptotic magnitude, and S = the shape parameter. When S is close to 1, the curve is an inverse exponential. When S is greater than 1.5, the curve is sigmoidal, and, as S goes to infinity, the curve approximates a step function.

Panels A, C, and E show these Weibull fits superimposed on the individual data points, and Panels B, D, and F show the curves for all the rabbits in each group. As might be suspected from the variability in magnitudes, the fits explained only a modest proportion of the variance. The mean R^2 was .37 (±.03, range = .12–.58). The variability in magnitudes and hence the modest fits are consistent with observations in other species and response systems (Gallistel et al., 2004).

The fitted curves were generally sigmoidal in shape, and some were very steep. All but 2 rabbits had S parameters greater than 1.50 ($M = 13.22 \pm 5.76$, range = 0.61–129.34). The majority of S values (58%) fell between 3.00 and 10.00. To measure the speed of transition in NM magnitudes, we computed each rabbit's dynamic range, which is the number of trials between 10% and 90% of the estimated asymptotic value (A; Gallistel et al., 2004). Two rabbits—1 each in Group 30 and Group 50—failed to reach an asymptote before the end of acquisition training. Among the 21 rabbits that achieved an asymptote, the transitions were more gradual than visual inspection of the fitted curves might suggest. Specifically, when allowance was made for intervening CS–US trials, 7 rabbits required 60 total trials (CS–US and CS alone) or

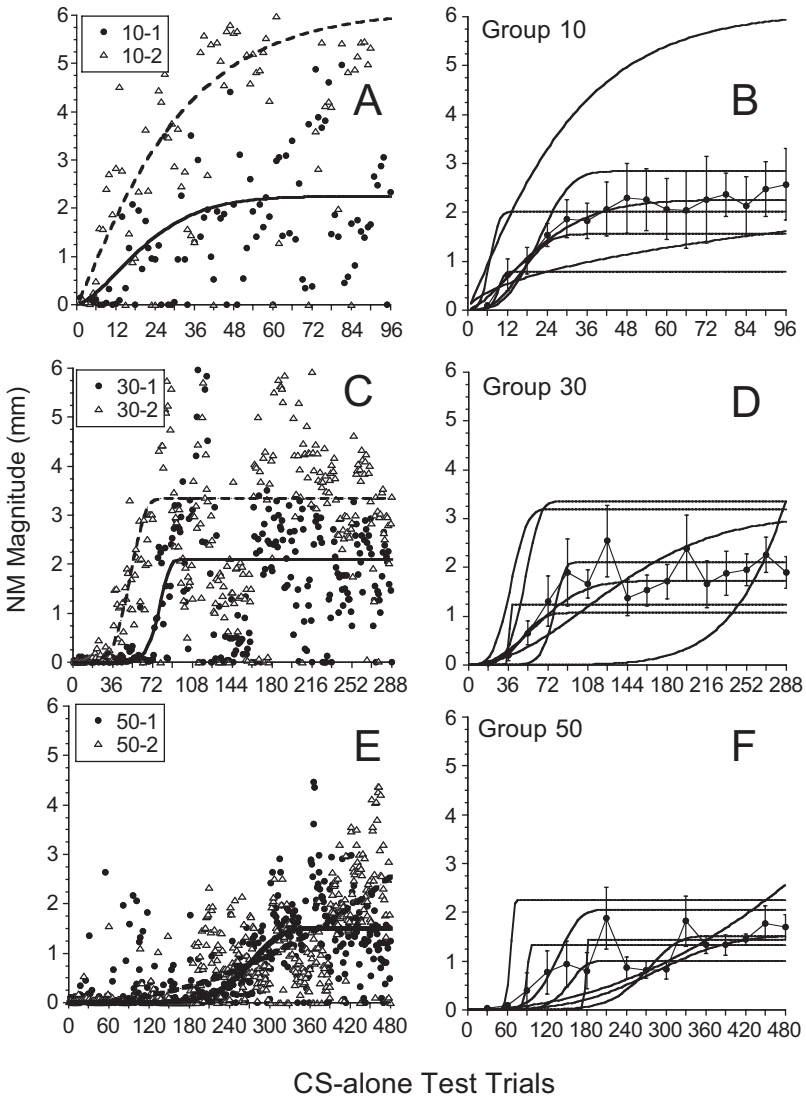

Figure 1. Magnitudes of the nictitating membrane movement on test trials of the conditioned stimulus (CS). Panels A, C, and E each show the trial-by-trial magnitudes for a pair of rabbits, which are labeled by the percentage of trials in acquisition that contained a CS-alone presentation (10, 30, or 50) and the number of the rabbit in the group (1 or 2). Panels B, D, and F show curves fitted to each rabbit's magnitudes using a Weibull function. The day-by-day group average magnitudes and standard errors are superimposed.

fewer to make the transition from 10% to 90% of asymptote, 7 required 60–180 trials, and 7 required more than 180 trials. Across all these rabbits, the average was 177 trials (±37, range = 18–550).

The only difference among groups that even approached statistical significance was in the *L* parameter, which reflects the overall rate of acquisition. When all the rabbits were included, the trend across groups approached statistical significance, $F(1, 20) = 3.40$, $p = .08$, $\eta_p^2 = .15$. Specifically, Groups 10, 30, and 50 showed mean *L* values of 42, 136, and 322 trials, respectively (largest *SEM* = 157).

Onset Latency and Peak Latency

We conducted two analyses, using different criteria for counting a movement as a CR, specifically, 0.5 mm and 0.2 mm. The 0.5-mm criterion has long been used in the analysis of rabbit NM conditioning (Garcia et al., 2003; Marshall-Goodell, Schreurs, & Gormezano, 1982). The 0.2-mm criterion was selected by examining the first five CS–US trials, in which any movements would be highly unlikely to reflect the effects of CS–US pairings. For criteria of 0.025, 0.05, 0.1, 0.2, 0.3, and 0.5 mm, the proportion of trials containing a countable movement was 8%, 8%, 3%, 1%, 1%, and 1%, respectively. Hence, the 0.2-mm criterion was selected as the smallest movement that was unlikely to include spontaneous eyelid flutters unrelated to CS–US pairings. For both criteria, we counted a movement on either a CS–US or CS-alone trial as a CR if it exceeded the criterion during a period extending from 50 ms after CS onset to 20 ms after the point of US onset. The latter point allowed for detection of movements initiated just before the point of US onset, but without including the recruitment of the UR. For

movements that did exceed the criterion, the onset latency was set for the point at which the movement first departed from the baseline by 1/16th mm (0.0625 mm; Marshall-Goodell et al., 1982). For peak latency measurements, only movements during CS-alone test trials could be counted.

For both criteria, the time course of the CR hardly varied during acquisition training. For the 0.5-mm and 0.2-mm criteria, the mean onset latency for the first CR was 326 ± 22 ms and 335 ± 26 ms, respectively. Thereafter, the onset latencies remained relatively constant. For the rest of acquisition training, the mean onset latency for the 0.5-mm criterion was 332 ± 27 ms. Similarly, for the 0.2-mm criterion, the mean onset latency was 343 ± 28 ms. For both criteria, the mean peak latency hovered around a mean of 606 ± 51 ms throughout training.

To illustrate the subject-by-subject patterns of CR timing, Figure 2 shows the CR-by-CR plots for onset latency and peak latency using the 0.2-mm criterion. The left-hand panels (A, C, E) show the CR-by-CR data for 3 rabbits, 1 from each group. By examining Figure 1, it can be seen that Rabbit 10-2 (A) showed the largest conditioned movements of any rabbit in the experiment, Rabbit 30-2 (C) showed movements near the median magnitude, and Rabbit 50-2 (E) showed movements that were smaller than most. The corresponding right-hand panels (B, D, and F) show straight lines obtained from least-square fits to the onset latencies and peak latencies for all the rabbits in Groups 10, 30, and 50, respectively. (The absence of any discernible change over training precluded Weibull curves.) The group average latencies and standard errors, divided in 16 blocks of CRs, are superimposed on Panels B, D, and F.

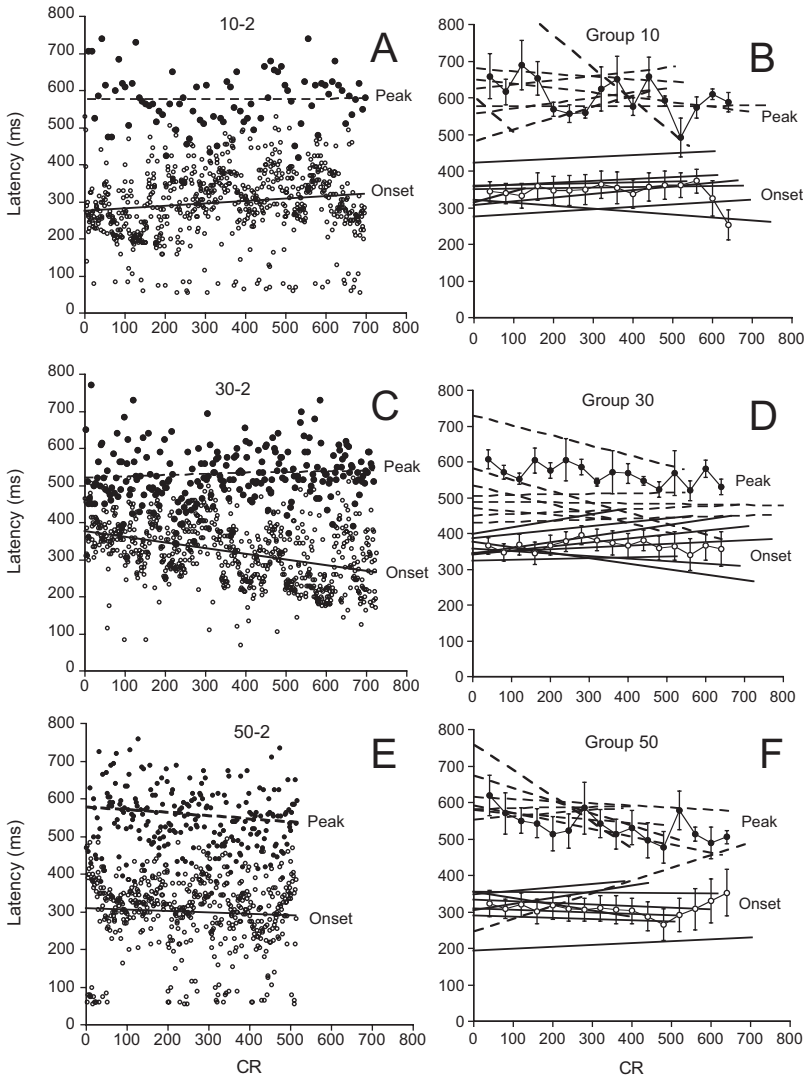

Figure 2. Onset latencies and peak latencies of the conditioned responses (CR) using a 0.2-mm magnitude as the criterion for counting a nictitating membrane movement as a CR. Panels A, C, and E each show the CR-by-CR latencies for a single rabbit; the data are labeled by the percentage of trials that contained a CS-alone presentation (10, 30, or 50) and the number of the rabbit in the group (1 or 2). The best-fitting straight lines for each type of latency are superimposed. Panels B, D, and F show the best-fitting straight lines for each rabbit's latency measures. The group average latencies and standard errors divided in 16 blocks of CRs are superimposed.

For onset latencies, the linear fits explained a tiny portion of the variance (mean $R^2 = .05 \pm .01$, range = .00–.25). Overall, slopes tended to be slightly positive ($M = 0.04 \pm 0.02$ ms per CR), $t(23) = 2.39$, $p < .05$, two-tailed, indicating a tiny increase across successive CRs. Likewise, for peak latencies, the linear fits explained only a tiny portion of the variance (mean $R^2 = .05 \pm .07$, range = .00–.24). As be seen in the figure, there were both positive and negative slopes. Overall, the peak latencies showed a slight, but not significant, decrease ($M = -0.71 \pm 0.52$ ms per CR), $t(23) = -1.38$, $p > .05$, two-tailed. Corresponding linear fits to both types of latencies using the 0.5-mm criterion yielded virtually identical results.

To test for any curvilinearity in the trends, we conducted second-order polynomial curve fits. They explained a greater portion of the variance than the linear fits for onset latency (mean $R^2 = .25 \pm .03$, range = .01–.69), but not for peak latency (mean $R^2 = .08 \pm .02$, range = .01–.27). The curvilinearity was usually slight, and there was no apparent pattern in the direction of the curves. For example, 13 subjects showed an increase and then a decrease in onset latency, and 11 subjects showed a decrease and then an increase. Similar results were obtained using the 0.5-mm criterion.

Discussion

The present experiment confirmed Garcia et al.'s (2003) observation that during acquisition, subcriterial movements were infrequent. However, fine-grained analyses revealed that transitions between negligible magnitudes and larger magnitudes were generally continuous. As indicated by the dynamic range measure, the changes in acquisition were relatively gradual, usually requiring more than the equivalent of a single session of 60 trials. Hence, the apparent continuity seen in averaged acquisition curves does not mask a set of all-or-none transitions occurring at different points in training. Having said that, averaging often produces the appearance of a negatively accelerated curve when the individual curves are usually sigmoidal (Gallistel et al., 2004).

The present findings constrain the process of CR generation. The continuity in the rise of the NM magnitude largely rules out an all-or-none process, in which a full-blown CR pops into view when the level of underlying associative activation is above a threshold value. Instead, the results favor a continuous process in which the observed NM movement is proportional to the level of activation above a threshold. The sigmoidal acquisition curves and the variability in magnitudes seen even well after CRs started to occur reliably are easily explained. Long ago, Hull (1943, p. 327) postulated that associative strength grew in a continuous, negatively accelerated fashion. He also postulated trial-to-trial variability in associative activation, which is normally distributed. This process would yield a sigmoidal acquisition curve as it crossed a fixed threshold. The variability in the associative activation would yield the postasymptotic variability in the response magnitude. (Invariant associative activation growing across a variable threshold would yield the same pattern.)

This scheme for explaining the magnitude data does not easily explain the present timing data. CR onset latencies should have decreased as the magnitudes of movement rose. Even when counting small movements (>0.2 mm) as CRs, onset latencies appeared constant in acquisition, albeit with considerable variability. This constancy agrees with the recent observations by both Vogel et al. (2003) and Garcia et al. (2003). In the former case, the onset latency of the first CRs versus the last CRs revealed little change in two rabbits trained with a 500-ms ISI. In the latter case, Garcia et al. analyzed early CRs by aligning the first few blocks of trials in which CRs were displayed by each rabbit. For the NM response, they saw a small and nonsignificant decrease from 342 ms to 290 ms. For the external eyelid, they saw a larger, but still nonsignificant, decrease from 578 ms to 353 ms.

Older observations of CR onset latency have indicated a migration of CR initiation from later to earlier portions of the ISI (Gormezano et al., 1983). This apparent migration may reflect the day-by-day analyses rather than the CR-by-CR analyses conducted more recently. To test this possibility, we conducted a day-by-day analysis of the present data, and its results paralleled the older findings. On Day 1, in which 4 rabbits showed CRs using the 0.5-mm criterion, the mean onset latency was 428 ms (±27 ms). On Day 2, in which 15 rabbits showed CRs, the mean CR onset latency declined to 345 ms (±52 ms), after which the mean onset latency hovered around 363 ms. This pattern duplicated a day-by-day analysis by Garcia et al. (2003). Using the 0.2-mm criterion, however, there was no decline between Day 1 ($M = 279 \pm 42$ ms), in which 10 rabbits showed CRs, and Day 2 ($M = 300 \pm 20$ ms), in which 19 rabbits showed CRs.

Taking all these results together, the timing of CRs appears to develop across CS–US pairings in a manner that may be independent of the overall associative strength as reflected in the magnitude measure (Balsam, Drew, & Yang, 2002; Jennings, Bonardi, & Kirkpatrick, 2007; Ohyama & Mauk, 2001). This possible dissociation, at least in delay conditioning using a 500-ms ISI, is consistent with its cerebellar pathways. Plastic synaptic changes in the Purkinje cells in the cerebellar cortex modulate the plasticity and activity in deep cerebellar nuclei that drive the overt CR (Christian & Thompson, 2003; Medina et al., 2002). Temporally specific learning appears to occur first in the cerebellar cortex. This learning is expressed in behavior only after plasticity is induced in the deep nuclei (Ohyama & Mauk, 2001).

References

Balsam, P. D., Drew, M. R., & Yang, C. (2002). Timing at the start of associative learning. *Learning and Motivation, 33,* 141–155.

Christian, K. M., & Thompson, R. F. (2003). Neural substrates of eyeblink conditioning: acquisition and retention. *Learning & Memory (Cold Spring Harbor, N.Y.), 11,* 427–455.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences* (2nd ed.). Hillsdale, NJ: Erlbaum.

Gallistel, C. R., Fairhurst, S., & Balsam, P. D. (2004). The learning curve: Implications of a quantitative analysis. *Proceedings of the National Academy of Sciences of the United States of America,* 13124–13131.

Garcia, K. S., Mauk, M. D., Weidemann, G., & Kehoe, E. J. (2003). Covariation of alternative measures of responding in rabbit (*Oryctolagus cuniculus*) eyeblink conditioning during acquisition training and tone generalization. *Behavioral Neuroscience, 117,* 292–303.

Gormezano, I. (1966). Classical conditioning. In J. B. Sidowski (Ed.), *Experimental methods and instrumentation in psychology* (pp. 385–420). New York: McGraw-Hill.

Gormezano, I., Kehoe, E. J., & Marshall, B. S. (1983). Twenty years of classical conditioning research with the rabbit. In J. M. Sprague & A. N. Epstein (Eds.), *Progress in psychobiology and physiological psychology* (Vol. 10, pp. 197–275). New York: Academic Press.

Hull, C. L. (1943). *Principles of behavior.* New York: Appleton-Century-Crofts.

Jennings, D. J., Bonardi, C., & Kirkpatrick, K. (2007). Overshadowing and stimulus duration. *Journal of Experimental Psychology: Animal Behavior Processes, 33,* 464–475.

Jirenhed, D.-A., Bengtsson, F., & Hesslow, G. (2007). Acquisition, extinction, and reacquisition of a cerebellar cortical memory trace. *Journal of Neuroscience, 27,* 2493–2502.

Kehoe, E. J., & Joscelyne, A. (2005). Temporally specific extinction of conditioned responses in the rabbit (*Oryctolagus cuniculus*) nictitating membrane preparation. *Behavioral Neuroscience, 119,* 1011–1022.

Kehoe, E. J., & Schreurs, B. G. (1986). Compound-component differentiation as a function of CS-US interval and CS duration in the rabbit's nictitating membrane response. *Animal Learning & Behavior, 14,* 144–154.

Marshall-Goodell, B., Schreurs, B. G., & Gormezano, I. (1982). Ruler vs. the Apple II/FIRST system analysis of analog signals in classical conditioning. *Behavior Research Methods and Instrumentation, 14,* 519–525.

Medina, J. F., Repa, J. C., Mauk, M. D., & LeDoux, J. E. (2002). Parallels between cerebellum- and amygdala-dependent conditioning. *Nature Reviews Neuroscience, 3,* 122–131.

O'Brien, R. G., & Kaiser, M. K. (1985). MANOVA method for analyzing repeated measures designs: An extensive primer. *Psychological Bulletin, 97,* 316–333.

Ohyama, T., & Mauk, M. D. (2001). Latent acquisition of timed responses in cerebellar cortex. *Journal of Neuroscience, 21,* 682–690.

Rescorla, R. A., & Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In A. H. Black & W. F. Prokasy (Eds.), *Classical conditioning II* (pp. 64–99). New York: Appleton-Century-Crofts.

Smith, M. C. (1968). CS-US interval and US intensity in classical conditioning of the rabbit's nictitating membrane response. *Journal of Comparative and Physiological Psychology, 66,* 679–687.

Sutton, R. S., & Barto, A. G. (1981). Toward a modern theory of adaptive networks: Expectation and prediction. *Psychological Review, 88,* 135–171.

Sutton, R. S., & Barto, A. G. (1990). Time-derivative models of Pavlovian reinforcement. In M. Gabriel & J. W. Moore (Eds.), *Learning and computational neuroscience* (pp. 497–537). Cambridge, MA: MIT Press.

Vogel, E. H., Brandon, S. E., & Wagner, A. R. (2003). Stimulus representation in SOP: II. An application to inhibition of delay. *Behavioural Processes, 62,* 27–48.

Received August 6, 2007
Revision received October 29, 2007
Accepted November 13, 2007 ■