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Effect of Delay Interval on Classical Eyeblink Conditioning in 5-Month-Old Human Infants[†]

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ABSTRACT: Associative learning was evaluated in human infants with simple delay classical eyeblink conditioning. A tone conditioned stimulus (CS) was paired with an airpuff unconditioned stimulus (US) at three different delay intervals (250, 650, and 1,250 ms). Independent groups of healthy, full-term 5-month-old human infants were assigned to these three paired conditions and received two identical training sessions 1 week apart. The two longer delays resulted in associative conditioning, as confirmed by comparison with unpaired control groups. However, only at the 650-ms delay were associative eyeblinks adaptively timed to avoid the airpuff. The delay function at 5 months of age appears much sharper than is observed in adults. Together with the findings of A. H. Little, L. P. Lipsitt, and C. Rovee-Collier (1984), the present study suggests a downward shift in the optimal delay interval for associative eyeblink conditioning between 1 and 6 months of age. However, this delay remains longer than what is typically reported in adults. Published 2002 Wiley Periodicals, Inc. [†]Dev Psychobiol 41: 329–340, 2002. Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/dev.10050

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Neurobehavioral studies of the development of learning and memory in human infants provide researchers with the challenge of developing behavioral proce-

dures that (a) assess cognitive abilities across a range of ages with simple perceptual and motor demands and (b) provide some indication of underlying neuro-

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logical processes. Procedures that can assess both cognitive and neural development during infancy are critical to our understanding of the progression of developmental neurobehavioral disorders and to the future prospect of being able to identify infants at risk for these disorders.

Classical eyeblink conditioning is particularly well suited for developmental studies that address both cognitive and neural aspects of learning and memory (see Ivkovich, Eckerman, Krasnegor, & Stanton, 2000a, for review). Eyeblink conditioning has been widely used in adult humans and other mammals (e.g., Woodruff-Pak & Steinmetz, 2000a, 2000b). It is a procedure with simple subject requirements [perception of a sensory stimulus (e.g., tone, light, or touch) and the ability to produce an eyeblink] that is applicable in a similar manner across a range of ages. Importantly, the procedures have been used effectively with special populations of humans including autistic children and young adults (Sears & Steinmetz, 2000), temporal lobe amnesics (Clark & Squire, 1998; McGlinchey-Berroth, Carrillo, Gabrieli, Brawn, & Disterhoft, 1997), and dyslexic children (Coffin & Boegle, 2000). The procedures enable us to distinguish associative learning (e.g., eyeblinks to a previously neutral stimulus) from the individual's ability to produce the required response reflexively (e.g., eyeblinks to an airpuff). Further, the simple perceptual and response properties can be maintained across a variety of higher order eyeblink conditioning paradigms (e.g., trace conditioning, reversal learning, latent inhibition) that can be used to evaluate more complex cognitive abilities across development (e.g., short-term memory, inhibition, attention). In addition, the sequence of neural events that mediate the behaviorally observed eyeblink responses have been clearly defined (see Lavond, Kim, & Thompson, 1993 for review), giving us a foundation for developing hypotheses about developmental changes in the brain that underlie the ontogeny of behavior. Stanton and colleagues (Stanton & Freeman, 1994, 2000; Stanton, Freeman, & Skelton, 1992) have begun to characterize the behavioral and neural developmental properties of eyeblink conditioning in rat pups. Their studies, along with our recent work with human infants (see Ivkovich, Collins, Eckerman, Krasnegor, & Stanton, 1999), suggest that developmental studies of learning and memory will benefit greatly from the integration of human and rodent studies of eyeblink conditioning.

Before we can begin to hypothesize about neurobehavioral deficits or study higher order cognitive processes with eyeblink conditioning procedures in special populations of infants and children, we need to establish the limits and optimal parameters for simple

conditioning at a given age. A few researchers have reported successful eyeblink conditioning in human infants and children (e.g., Fitzgerald & Brackbill, 1976; Little, Lipsitt, & Rovee-Collier, 1984; Ohlrich & Ross, 1968), but little parametric work has been conducted. A review of the literature, however, suggests that (barring significant laboratory procedural differences) there may be developmental changes in the parameters that support eyeblink conditioning at different ages.

Delay eyeblink conditioning is the simplest conditioning paradigm because it evaluates the ability to associate two stimuli when they overlap in time. The difficulty of the paradigm can be manipulated by altering the delay interval between the onset of a conditioned stimulus (CS; typically a tone) and the onset of an unconditioned stimulus (US; typically an airpuff). In the one existing parametric study of delay interval in infants, Little et al. (1984) found no delay conditioning in 10- to 30-day-old infants with the 500-ms interstimulus interval that is optimal for eyeblink conditioning in adults (Kimble, 1947), but moderate levels of conditioning (28–40% CRs) were observed using a delay of 1,500 ms. This finding suggests that conditioning in young infants benefits from longer delays. In another delay conditioning study using a 500-ms delay (Naito & Lipsitt, 1969), there was negligible conditioning in 1- to 2-month-old infants, further supporting and extending the Little et al. finding. A 500-ms delay interval was successful, however, with older infants around 8 months of age (range = 5–14 months; Hoffman, Cohen, & DeVido, 1985), but the US in that study was a glabellar tap (tap on the forehead between the eyes with a rubber mallet). In our own studies (Ivkovich et al., 1999; Ivkovich et al., 2000a), we demonstrated robust delay eyeblink conditioning using a 650-ms delay in infants at 4 and 5 months of age. By 4 to 6 years of age, children are able to condition with an 800-ms delay interval between tone and airpuff (Werden & Ross, 1972). Taken together, the studies suggest that from 10 days of age to 8 months of age and into childhood there may be a developmental change in the delay function such that shorter delays support conditioning at older ages. It is interesting to note that this trend may reverse across the life span as it has been shown that older adults (70–80 years) also benefit from a longer delay (1,500 vs. 500 ms; Woodruff-Pak, Jaeger, Gorman, & Wesnes, 1999), just as the very young infants did in the Little et al. study.

In the present study, we sought to conduct a parametric study of delay interval in 5-month-old infants using our recently developed procedures for eyeblink conditioning in human infants (Ivkovich et al., 2000a).

We present here a replication of our earlier findings demonstrating robust delay eyeblink conditioning in 5-month-old human infants over two sessions with a 650-ms delay. In addition, we extend our observations to include separate groups of infants trained with shorter (250 ms) or longer (1,250 ms) delays. The 250-ms delay is within range of the optimal intervals reported for adults (Kimble, 1947), and the 1,250-ms delay is within the range reported as effective for very young infants (Little et al., 1984). We address the possibility that the delay function changes during ontogeny and consider the neurobiological changes that may contribute to this behavioral development.

METHODS

Subjects

Infants and their parents were recruited to participate in two conditioning sessions, 6 to 8 days apart, beginning at 5 months of age (± 10 days). Participant families were recruited by mail from local county birth records and were primarily from non-Hispanic, White families (89%). In 97% of the families, both parents had graduated from high school, and in 73% of the families, both parents had completed 4 or more years of college. In response to the mailings, 10% of the families returned a reply card indicating interest in the study; 68% of these were scheduled for visits when subsequently contacted by phone.

Infants were randomly assigned to one of five conditioning groups, three paired and two unpaired groups (described later). The data presented here are from 38 infants (23 male, 15 female) who completed a minimum of 36 of 50 trials for each paired conditioning session or 67 of 100 trials for each unpaired conditioning session. Another 20 infants (14 male, 6 female) participated but were excluded from the final analysis because they did not complete the criterion number of trials ($n = 13$), they were unresponsive to the airpuff US (the mean percentage of nonresponding on US-alone trials exceeded 43% across the two sessions; $n = 3$), or there were rescheduling conflicts which precluded the completion of both sessions ($n = 5$). Chi-square analysis revealed no evidence of selective attrition based on gender, ethnic origin, or conditioning group.

Procedures

The general procedures used here have been reported in detail previously (Ivkovich et al., 1999; Ivkovich

et al., 2000a). Briefly, infants were seated in a parent's lap facing forward so that they could observe a visual display of brightly colored objects presented on a platform in front of them. To the left and right above the infant's head were two small 7-ohm speakers directed to the infant's ear level for delivery of a tone CS (1 kHz, 80 dB). The infant was outfitted with a specially crafted, soft headband supporting a flexible plastic tube through which an airpuff US ($\sim 1/20$ lb/in.²) was delivered towards the subject's right eye. Background instrumental music ("Beauty and the Beast" from Walt Disney's animated motion picture) was played at low volume so that the pure-tone CS remained distinct. A camera, placed about 1 m to the front and right side of the infant, yielded a videorecord of the infant's head and a signal box with a trial counter and lights indicating when the tone and airpuff stimuli were on. Additionally, electromyographic (EMG) records of eyeblinks were collected using three gel-pad electrodes positioned at the corner of the right eye, on the right temple, and at the back of the neck. A custom-built eyeblink conditioning system (Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC) controlled presentation of the stimuli, and amplified and integrated EMG records for subsequent analysis.

Paired Training Sessions

Paired training sessions consisted of a tone CS that overlapped and coterminated with a gentle puff of air to the right eye. The resulting delay interval was manipulated by changing the duration of the tone such that the last 100 ms always overlapped with the airpuff US. Three tone durations (350, 750, and 1,350 ms) yielded three delay conditioning groups ($n = 10$ /group): Delay 250, Delay 650, and Delay 1,250. Participants were randomly assigned to one of these groups and received two sessions of conditioning at the designated delay. The intertrial interval varied from 8 to 16 s (average = 12 s). Every sixth trial in a block of 10, as well as Trials 1 and 2 at the start of the session, was an air-alone trial to test for somatosensory responsiveness. Every 10th trial was a tone-alone trial to test for conditional responding. A maximum of 50 trials were presented for paired sessions (~ 12 –15 min), but the session was terminated earlier if the infant became overly fussy.

Unpaired Training Sessions

During unpaired training sessions, there was no associative contingency between the tone and airpuff.

Subjects experienced the same 43 tones and 45 puffs used for the paired training session, but the stimuli were presented explicitly unpaired 4 to 8 s apart (average 6 s) in a manner that matched the paired condition for stimulus density. The criteria for terminating a session were the same as those for paired sessions. Two unpaired groups in this study, one with a 750-ms tone (Unpaired 750, $n=4$) and the other with a 1,350-ms tone (Unpaired 1,350, $n=4$), matched the Delay 650 and Delay 1,250 paired conditioning groups, respectively. These groups served to control for nonassociative increases in responding to the tone. An unpaired group to match the paired group Delay 250 was not included as there was little evidence of CS-evoked responding in the paired group and the data could not be falsely interpreted as an increase in conditioning.

Response Measures

Two independent observers used frame-by-frame video coding to evaluate each trial for the occurrence and timing of a blink. A blink occurring after the airpuff, within 1 to 30 video frames (500 ms) after airpuff onset, was considered an unconditional response (UR). A blink occurring in anticipation of the airpuff was defined as a conditional response (CR). CR responses were subsequently measured in three ways. A *Total CR Including Alpha* measure, which included any blink that occurred during the entire CR period, was calculated for all delay groups (Details of this measure are not reported here because results were equivalent to the Total CR measure that follows; see Results, Percentage CRs). A *Total CR* measure, which included any blink that occurred beyond the first 17 frames (300 ms) following tone onset (the “alpha period”), was calculated for the Delay 650 and Delay 1,250 paired groups. An *Adaptive CR* measure, which included any blink within 21 frames (350 ms) prior to airpuff onset, was calculated for the Delay 650 and Delay 1,250 groups. For the Delay 250 group, the adaptive CR measure consisted of the entire 250-ms period between tone and airpuff. Adaptive CRs are well timed and reduce the impact of the upcoming airpuff (see Ivkovich, Paczkowski, & Stanton, 2000b, for further explanation of adaptive CRs). On trials in which there was no airpuff presented (tone-alone test trials), any response within the comparable CR and UR periods combined was considered a CR. Percent agreement on a conditional or unconditional response within two frames was 97%. If it was not possible to make a determination because the subject’s right eye was out of view, the trial was treated as uncodable and excluded from analyses.

The data presented here are primarily based on video codings. EMG recordings were sometimes sensitive to facial and head movements and showed large intersubject variability in signal-to-noise ratio. As a result, EMG data were unusable for about 39% of the sessions. For subjects with usable EMG records, UR amplitudes were examined to assess whether there were group differences that might produce effects on conditioning performance. Individual trial UR amplitudes, confirmed by video analysis to exclude trials with a CR, were averaged across session and conditioning group.

The primary measure of learning was the percentage of CRs across two training sessions. For paired sessions, the percentage of CRs was calculated for blocks of six paired trials for a minimum of six blocks. For unpaired training sessions, the percentage of CRs was based on six-trial blocks of corresponding tone-alone trials. Average CR and UR onset latencies for each block also were obtained from the frame-by-frame video codings (16.7 ms/frame). In a small number of instances (11 of 456 total observations), data from a particular trial block were missing for a particular subject. In these rare instances, the average of the preceding and succeeding blocks was substituted for the missing data. Data were analyzed using between/within analysis of variance (ANOVA) with a significance level of 0.05. Post hoc Newman-Keuls comparisons were performed as needed.

RESULTS

Five-month-old infants were successfully conditioned using a 650-ms delay interval, but did not demonstrate “adaptive” CRs to the tone at the 250- or 1,250-ms delays. The Delay 1,250 group did, however, show an increase in CRs that occurred at an earlier point in the CS period than that designated for “adaptive” CRs. Below are analyses of UR amplitude and CR percentage that confirm the associative nature of the increases in conditioned responding. The observance of increased CRs in both the Delay 650 and the Delay 1,250 groups led to further analyses of response-onset latency and trials-to-criterion that enabled us to characterize the differences between these groups. Analyses were first conducted to confirm learning had occurred by comparing the two groups for which some conditioning was observed (Delay 650 and Delay 1,250) relative to their unpaired control groups. A second analysis then compared the three delay-interval groups to identify the optimal delay for delay eyeblink conditioning at 5 months of age.

UR Amplitudes

UR amplitudes were analyzed to confirm that differences in conditioned responding across training groups in these studies were not secondary to differences in US efficacy or motor performance that were inadvertent (e.g., arising from sampling error). No such differences were found. Mean ($\pm SE$) UR amplitudes for the Delay 250, Delay 650, and Delay 1,250 groups were 1.99 (± 0.35), 1.63 (± 0.17), and 1.76 (± 0.20) arbitrary units, respectively, whereas these values for comparison groups Unpaired 750 and 1,350 were 1.70 (± 0.32) and 1.67 (± 0.33), respectively.

A 2 (paired vs. unpaired) \times 2 (Delay 650 vs. 1,250) \times 2 (gender) \times 2 (session) repeated measures ANOVA on UR amplitudes failed to reveal any significant statistical effects ($F_s < 1.97$, $p_s > .18$). A 3 (delay) \times 2 (gender) ANOVA on the three delay groups failed to reveal any main effects or interactions involving the delay factor ($F_s < 1$, $p_s > .71$). However, a main effect of gender was observed, $F(1, 19) = 5.3$, $p < .03$. Averaged across delay groups, females exhibited UR amplitudes of 2.15 (± 0.24) arbitrary units whereas the corresponding values for males were 1.50 (± 0.12). This outcome should be regarded with caution because we have not observed it previously (Ivkovich et al., 2000a). Moreover, gender and delay did not interact ($F < 1$). Taken as a whole, analysis of UR amplitude suggests that there were no differences in performance (unconditioned responding) that could contribute to differences in learning (CRs) across delay conditions.

Percentage CRs

Delay interval had a dramatic effect on conditioned responding. However, the nature of this effect depended on the CR sampling period ("adaptive" vs. "total"). The Total CR Including Alpha measure also was analyzed, but is not reported here because the outcome was numerically and statistically identical to that from the Total CR measure.

Adaptive CR Measure. In the adaptive CR measure (final 350 ms within the CS period for Delay 650 and Delay 1,250; entire 250 ms CS-US period for Delay 250), conditioning was observed only in the Delay 650 group. Percentage CRs increased during Session 1 and reached asymptote during Session 2 in this group (see Figure 1). This group's unpaired counterpart (Unpaired 750) did not exhibit any similar increase in CRs nor did any of the other experimental groups.

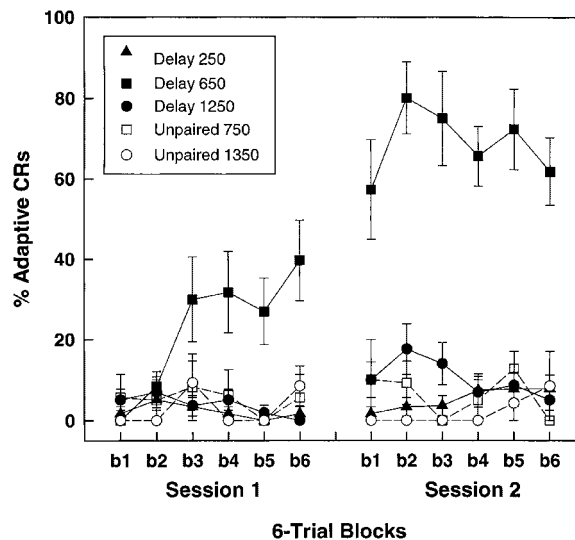


FIGURE 1 Mean ($\pm SE$) percentage responses for Adaptive CRs, as a function of trial block and training session, for 5-month-old infants in the paired (filled symbols) and unpaired groups (open symbols). Data are presented in six-trial blocks of paired trials (paired groups) or corresponding tone-alone trials (unpaired groups).

A 2 (paired vs. unpaired) \times 2 (Delay 650 vs. 1,250) \times 2 (gender) \times 2 (sessions) \times 6 (blocks) repeated measures ANOVA on percentage CRs revealed significant main effects of delay, $F(1, 20) = 11.11$, $p < .01$, pairing, $F(1, 20) = 13.65$, $p < .01$, and sessions, $F(1, 20) = 43.46$, $p < .0001$, as well as interactions of Delay \times Pairing, $F(1, 20) = 8.79$, $p < .01$, Sessions \times Pairing, $F(1, 20) = 44.72$, $p < .0001$, Sessions \times Delay, $F(1, 20) = 29.03$, $p < .0001$, and Sessions \times Delay \times Pairing, $F(1, 20) = 25.77$, $p < .001$. No other main effects or interactions were significant ($F_s < 2.13$; $p_s > .15$). Post hoc tests (Newman-Keuls) of the Session \times Delay \times Pairing interaction revealed that Group Delay 650 showed an increase in percentage CRs across sessions ($p < .01$), whereas the other three groups failed to show this increase. As a result, percentage CRs in Group Delay 650 were significantly higher than in the remaining three groups in Session 1 ($p < .01$) and Session 2 ($p < .01$). Differences among the remaining groups failed to appear in any session.

A 3 (delay) \times 2 (sessions) \times 6 (blocks) \times 2 (gender) ANOVA of percentage CRs including all three delay groups revealed significant main effects of delay, $F(2, 24) = 33.15$, $p < .0001$, sessions, $F(1, 24) = 140.81$, $p < .0001$, and blocks, $F(5, 120) = 43.46$, $p < .05$, as well as interactions of Delay \times Sessions, $F(2, 24) = 83.51$, $p < .0001$, Delay \times Blocks, $F(10, 120) = 2.21$, $p < .05$, Sessions \times Delay \times Gender, $F(2, 24) =$

3.99, $p < .05$, and Delay \times Sessions \times Blocks, $F(10, 120) = 2.53$, $p < .01$. No other main effects or interactions were significant ($F_s < 2.43$; $p_s > .13$). Post hoc tests (Newman-Keuls) of the Delay \times Sessions \times Blocks interaction revealed a pattern similar to the outcome from the previous post hoc test: There was an increase in percentage CRs across sessions and blocks ($p < .01$) in Group Delay 650, but not in the other two delay groups. Percentage CRs in Group Delay 650 were significantly higher than in the other two groups in Blocks 3 to 6 of Session 1 ($p < .01$) and all blocks in Session 2 ($p < .01$). The remaining two groups failed to differ significantly from one another on any training block except one (Session 2, Block 2: $p < .05$).

The Gender \times Sessions \times Delay interaction occurred because females in Group Delay 650 showed a modestly higher percentage of CRs in the second session than males. This gender effect should be interpreted cautiously because gender was not a consistently significant factor in the other ANOVAs in this study, nor has it been in the past (Ivkovich et al., 1999; Ivkovich et al., 2000a).

Taken together, the results from the adaptive CR measure clearly indicate that 5-month-old infants are capable of conditioning at a delay interval of 650 ms, but not at delay intervals of 250 or 1,250 ms.

Total CR Measure. In this total-CR measure, using a larger sampling period that started 300 ms after tone onset, conditioning occurred in both the Delay 650 and Delay 1,250 groups. Both groups showed similar increases in percentage CRs during Session 1 and reached asymptote during Session 2 (Figure 2). The asymptote reached by the Delay 1,250 group appeared lower than that of the Delay 650 group. There was no increase in percentage CRs across training in the unpaired counterparts of either group (Unpaired 750 or 1,350) or in the Delay 250 group.

A 2 (gender) \times 2 (paired vs. unpaired) \times 2 (Delay 650 vs. 1,250) \times 2 (session) \times 6 (block) repeated measures ANOVA revealed no significant main effects or interactions involving gender ($F_s < 1.26$). There were main effects of pairing, $F(1, 20) = 10.13$, $p < .01$, and sessions, $F(1, 20) = 29.97$, $p < .0001$, as well as interactions of Sessions \times Pairing, $F(1, 20) = 15.93$, $p < .001$, and Sessions \times Delay \times Pairing, $F(1, 20) = 4.86$, $p < .05$. No other main effects or interactions were significant ($F_s < 1.91$). Post hoc tests (Newman-Keuls) of the Session \times Delay \times Pairing interaction revealed that percentage CRs of both Groups Delay 650 and Delay 1,250 increased across sessions ($p < .01$) whereas this increase was not seen in the unpaired control groups. As a result, percentage CRs in Groups Delay 650 and 1,250 were

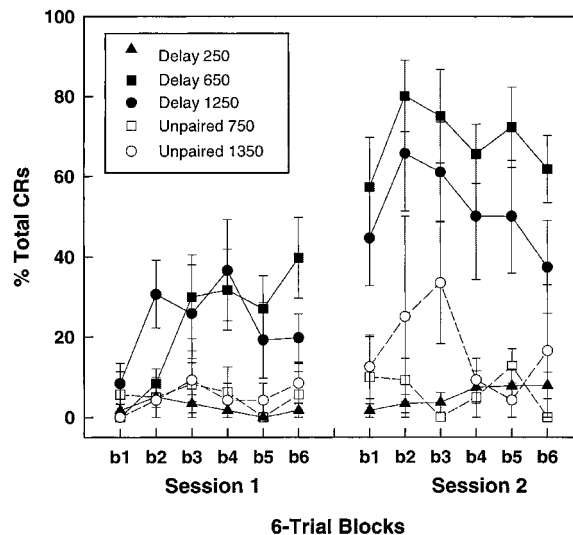


FIGURE 2 Mean ($\pm SE$) percentage of responses for Total CRs, as a function of trial block and training session, for 5-month-old infants in the paired (filled symbols) and unpaired groups (open symbols). Data are presented in six-trial blocks of paired trials (paired groups) or corresponding tone-alone trials (unpaired groups).

significantly higher than in their corresponding unpaired control groups in Session 1 ($p < 0.05$) and Session 2 ($p < .01$). In addition, Group Delay 650 showed significantly more CRs ($p < .05$) in Session 2 than Group Delay 1,250. Differences among the unpaired control groups failed to appear in any session.

A 3 (delay) \times 2 (gender) \times 2 (session) \times 6 (blocks) repeated measures ANOVA of percentage CRs from the three delay groups failed to reveal any main effects or interactions involving gender ($F_s < 1.35$). ANOVA did reveal significant main effects of delay, $F(2, 24) = 10.00$, $p < .0001$, sessions, $F(1, 24) = 79.34$, $p < .0001$, and blocks, $F(5, 120) = 4.26$, $p < .01$, as well as interactions of Delay \times Sessions, $F(2, 24) = 21.00$, $p < .0001$, Session \times Blocks, $F(2, 24) = 2.79$, $p < .05$, and Delay \times Sessions \times Blocks, $F(10, 120) = 1.90$, $p = .05$. No other main effects or interactions were significant ($F_s < 1.39$; $p_s > 0.19$). Post hoc tests (Newman-Keuls) of the Delay \times Sessions \times Blocks interaction showed an increase in percentage CRs across sessions and blocks ($p < .01$) in the Delay 650 and 1,250 groups, but not in Group Delay 250. Percentage CRs in Groups Delay 650 and 1,250 were significantly higher than in the Delay 250 group in Blocks 4 to 6 in Session 1 ($p < .05$) and all blocks in Session 2 ($p < .01$). Groups Delay 650 and 1,250 failed to differ significantly on any training block except one (Session 2, Block 6: $p < .05$).

Taken together, the results from the Total CR measure indicate that 5-month-old infants are capable of conditioning at delay intervals of 650 and 1,250 ms, but not 250 ms. These results contrast with those from the adaptive CR measure, in which conditioning was seen only at a delay interval of 650 ms. This suggests that the CRs that are acquired by infants of this age at the longest delay interval are less precisely timed to coincide with US onset relative to CRs acquired at the 650-ms delay interval. To assess this possibility, we undertook a descriptive analysis of when CRs occurred during the CR sampling period in Group Delay 1,250.

Onset Latency for Delay 1,250

The distribution of responses in the Delay 1,250 group was determined by dividing the CR sampling period into 25 50-ms bins. The number of CRs beginning in each bin of the CR period then was analyzed. The mean number of responses per bin is presented as a function of latency and session in Figure 3. Across sessions, the Delay 1,250 group produced few responses in the first 300 ms (alpha responses) or in the final 350 ms (adaptive responses) of the trial epoch. Responses in the Delay 1,250 group typically occurred early in the CR period and increased in number between Sessions 1 and 2. This increase was associative because Group Unpaired 1,350 failed to show CRs (see Figure 2).

A 2 (Session 1 vs. Session 2) × 25 (50-ms CR Bins) repeated measures ANOVA of CRs revealed signi-

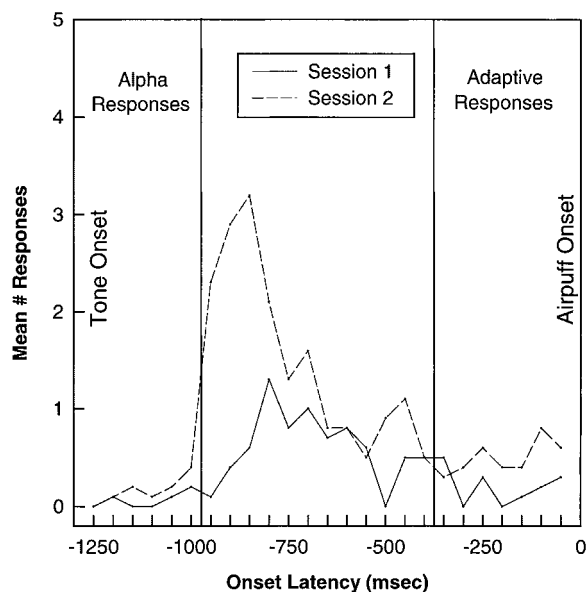


FIGURE 3 Mean number of CRs in the Delay 1,250 group as a function of onset latency and training session.

ficant main effects of sessions, $F(1, 216) = 12.88, p < .01$, and bins, $F(24, 216) = 4.39, p < .0001$, as well as an interaction of Sessions × Bins, $F(24, 216) = 2.82, p < .0001$. Post hoc tests (Newman-Keuls) of the Session × Bins interaction revealed an increase in responding across sessions only in the seventh to the ninth bins ($p < .01$) of the CR sampling period (350–450 ms following CS onset). During Session 1, apparent differences across the 25 bins were not statistically significant. In contrast, during Session 2, responding in the seventh to the tenth bins was significantly higher ($p < .01$) than in most other bins.

This analysis of CR timing indicates that 5-month-old infants are capable of some conditioning at long delay intervals. Unlike infants trained at a 650-ms delay interval, infants trained at a 1,250-ms delay interval do not acquire well-timed responses that coincide with US onset after two sessions. They do, however, exhibit a similar increase in amount of associative responding across sessions. Based on these findings, we then examined whether the rate of learning and maintenance of responding was similar across these groups using a trials-to-criterion measure.

Trials-To-Criterion

The number of trials-to-criterion (TTC) was determined as an additional learning measure for each session. The learning criterion was three CRs of six consecutive paired trials that was maintained for the remainder of a session. The six-trial block was advanced trial-by-trial across two training sessions until the criterion was obtained and the final trial in the sequence was noted. Subjects who did not reach criterion during a given session were assigned a score of the total number of paired trials they had experienced (maximum number of paired trials across two sessions was 76).

Delay interval had a dramatic effect on this measure of the rate of learning. As with percentage CRs, the nature of this effect depended on the CR sampling period (“adaptive” vs. “total”).

Adaptive TTC. In the adaptive-TTC measure using CRs that occurred in the final 350 ms of the CS period, only the Delay 650 group reached the criterion level of responding. The Delay 650 group reached criterion early in the second session ($M = 43.2, SE = 6.4$). Neither of the other experimental groups had reached criterion at the end of the second session. Mean ($\pm SE$) TTC for the Delay 250 and 1,250 groups were 75.6 (± 0.4), and 74.5 (± 1.1), respectively (Figure 4).

A 3 (delay) × 2 (gender) ANOVA on adaptive TTC revealed a main effect of delay, $F(2, 24) = 23.62$,

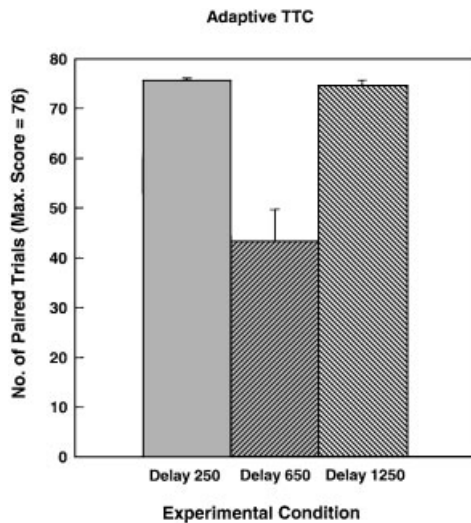


FIGURE 4 Mean ($\pm SE$) trials-to-criterion for Adaptive CRs by subjects who received two sessions of paired conditioning, as a function of conditioning group.

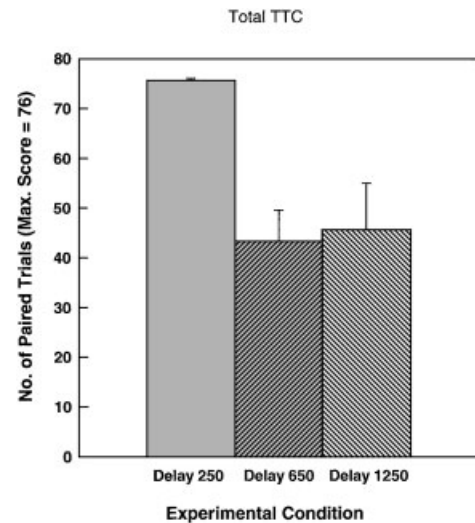


FIGURE 5 Mean ($\pm SE$) trials-to-criterion for Total CRs by subjects who received two sessions of paired conditioning, as a function of conditioning group.

$p < .0001$, but no main effect or interactions involving gender ($F_s < 1$). Post hoc tests (Newman-Keuls) of the delay main effect revealed that group Delay 650 was the only group to reach criterion ($p < .01$). The remaining two groups failed to differ significantly from each other by the end of the second session.

Total TTC. In the total-TTC measure using CRs occurring after the initial 300 ms of the CS period, both the Delay 650 and Delay 1,250 groups reached the criterion level of responding early in the second session. Mean ($\pm SE$) TTC for the Delay 650 and Delay 1,250 groups were 43.2 (± 6.4) and 45.6 (± 9.4), respectively, whereas the value for Group Delay 250 was 75.6 (± 0.4). The data are presented in Figure 5.

A 3 (delay) \times 2 (gender) ANOVA on total TTCs revealed a main effect of delay, $F(2, 24) = 6.77$, $p < .01$, but no main effect or interactions involving gender ($F_s < 1$). Post hoc tests (Newman-Keuls) of the delay main effect revealed that Group Delay 250 differed significantly from the other two groups in trials to reach criterion ($p < .01$). Groups Delay 650 and Delay 1,250 did not differ significantly from each other.

Taken together, analyses of the TTC measure reveals that the duration of the delay interval produces dramatic differences in the rate of conditioning. Further, as in the percentage CR measure, the CR sampling period used in longer delay intervals has a major impact on the TTC measure.

The impact of the CR sampling period on the acquisition of conditioned responding raises the possibility that infants tested at shorter delay intervals may exhibit evidence of conditioning when there are less constraints on the timing of responses. To assess this possibility for all groups, we analyzed the percentage of CRs occurring on CS-alone trials. The end of the CR period on these trials is not defined by US onset but, rather, continues for 400 ms after tone offset (the UR period on paired trials). This gives subjects in the Delay 250 group a total period of 750 ms within which to emit a CR.

Percentage CRs on CS-Alone Trials

The percentage of responses occurring on CS-alone trials was determined as an additional measure of conditioned responding. A CS-alone trial was presented once every 10 trials. Infants, therefore, experienced a maximum of five CS-alone trials per session. CRs were defined as responses occurring after tone onset and within 400 ms after tone offset. As with the previous measures of learning, the initial 300-ms alpha period was excluded from analyses for the Delay 650 and Delay 1,250 groups.

Delay interval and session had a dramatic effect on conditioned responding on CS-alone trials. Conditioning was observed across sessions in both the Delay 650 and Delay 1,250 groups. This increase was not seen in the Delay 250 group (Figure 6).

A 2 (paired vs. unpaired) \times 2 (Delay 650 vs. Delay 1,250) \times 2 (gender) \times 2 (sessions) repeated measures

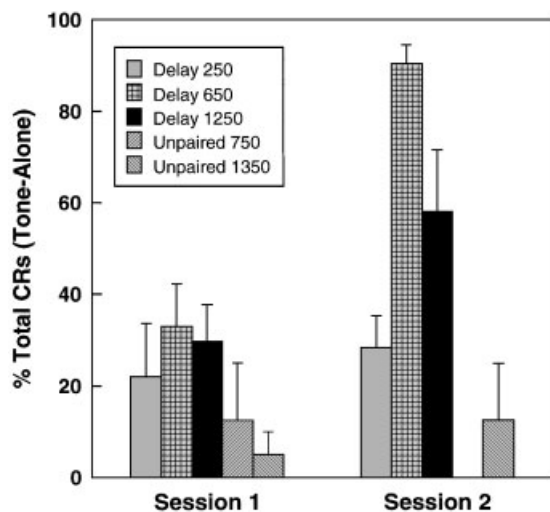


FIGURE 6 Mean (\pm SE) percentage of Total CRs occurring on Tone-alone trials. Note: There were no CRs on tone-alone trials during Session 2 for the Unpaired 750 group.

ANOVA of percentage CRs on CS-alone trials revealed no statistical effects of gender or delay ($F_s < 1.14$). There were significant main effects of pairing, $F(1, 20) = 19.64$, $p < .001$, sessions, $F(1, 20) = 7.14$, $p < .05$, and an interaction of Sessions \times Pairing, $F(1, 20) = 7.43$, $p < .05$. Newman-Keuls tests of this interaction revealed that percentage CRs increased across sessions ($p < .01$) only in the paired groups; and these groups showed more CRs than their unpaired counterparts in both Session 1 ($p < .05$) and Session 2 ($p < .01$).

A 3 (delay) \times 2 (gender) \times 2 (sessions) repeated measures ANOVA of percentage CRs on CS-alone trials from the three delay groups revealed no main effects or interactions involving gender ($F_s < 1$). There were significant main effects of delay, $F(1, 27) = 5.39$, $p < .05$, sessions, $F(1, 27) = 25.95$, $p < .0001$, and an interaction of Sessions \times Delay, $F(2, 27) = 6.03$, $p < .01$. Post hoc tests (Newman-Keuls) of this interaction revealed that percentage CRs increased across sessions in the Delay 650 and Delay 250 groups ($p < .01$), but not in Group Delay 1,250. There were no differences across the delay groups in Session 1 whereas all three groups differed significantly from one another ($p < .01$) in Session 2.

These findings from the tone-alone test trials generally confirm and extend the results from other measures of conditioning (discussed earlier). They indicate that CRs failed to appear in Group Delay 250, even when a longer sampling period was used. This suggests that the failure to observe conditioning in this group is not a result of an insufficient opportunity to emit a CR. These findings also show that the

650-ms delay interval is more optimal for conditioning than the 1,250-ms interval in 5-month-old infants, at least under these training conditions.

DISCUSSION

This study examined delay interval effects on classical eyeblink conditioning in 5-month-old infants. The primary finding was that a delay of 650 ms produces more robust conditioning than either 250- or 1,250-ms delay intervals. Although no conditioning was observed in the 250-ms delay condition, there was some conditioned responding in the 1,250-ms delay condition. However, these conditioned responses occurred early in the CS period and were poorly timed to avoid the airpuff US. Measures of TTC and percentage CRs on CS-alone test trials further supported and extended the primary finding that the optimal delay interval for conditioning at 5 months of age is 650 ms.

Consistent with our earlier reports of conditioning in 5-month-old infants using a 650-ms delay, maximal levels of conditioning were observed during the 2nd session when asymptote appears to be reached (Ivkovich et al., 1999). However, infants in the present study demonstrated clear and significant conditioning earlier in training (Session 1, blocks 3–6) than did infants in our earlier study (Session 2, block 1). Still, the multi-session training design that we have implemented continues to reveal more information than single-session designs regarding developmental processes in infant eyeblink conditioning.

Of the delay intervals tested here, the 650-ms delay we have been using thus far (Ivkovich et al., 1999; Ivkovich et al., 2000a) yields the strongest evidence of conditioning in 5-month-olds, especially in the adaptive CR measure. Little et al. (1984) studied eyeblink conditioning in newborn to 1.5-month-old infants at delays of 500 and 1,500 ms, and found modest levels of conditioning only at the longer delay (1,500 ms). In contrast, our findings at the 1,250-ms delay suggest that conditioning in older infants does not benefit from a longer delay. However, 5-month-old infants also are not yet able to acquire the conditioned eyeblink response at the shorter 250-ms delay interval that yields robust conditioning in adults (Kimble, 1947). In this respect, our findings join those of Little et al. in suggesting that shorter delays that support conditioning in adults seem to be less effective in infants. Together with Little et al., our data suggest that there is a downward shift in the optimal delay interval for classical eyeblink conditioning between 1 month and 5 months of age.

It also is important to consider differences in the timing of conditioned responding when determining optimal delay intervals. Although we have followed the common convention of using the “adaptive” conditioned response—the response that is accurately timed to avoid the airpuff US—in our analysis of optimal delay intervals, we also found that low levels of these CRs do not mean that infants are not able to associate the CS and US because they do show CRs at other points in the CS interval. Infants trained with the 1,250-ms delay demonstrated significant levels of conditioned responding, though the responses were not adaptively timed nor was conditioning quite as robust as for the 650-ms delay. The Total CR measure, then, may reflect general associative processes whereas the Adaptive CR measure may be a measure of response timing skills (Mauk & Ruiz, 1992).

Others have suggested that there is a shift in optimal delay interval over the life span (see Rovee-Collier, Hayne, & Colombo, 2000, pp. 108–109). As with very young infants, aging adults are impaired in delay eyeblink conditioning at delays that are optimal for young adults (400–500 ms) but show significant improvement when longer delays are used (Solomon, Groccia-Ellison, Levine, Blanchard, & Pendlebury, 1990; Woodruff-Pak et al., 1999). Although the evidence for such a function relies on data across different studies in the human eyeblink conditioning literature, there is some corroborative evidence in the adult animal literature (Caldwell & Werboff, 1961; Solomon & Groccia-Ellison, 1996; Woodruff-Pak, 1988), although not in early developmental studies of eyeblink conditioning (Freeman, Spencer, Skelton, & Stanton, 1993; Ivkovich et al., 2000b). The findings of Solomon et al. (1990) suggest that the developmental changes during aging are reflective of changes in synaptic efficacy rather than sensory acuity or motor function. Similarly, we have argued that the simple sensory-response requirements of eyeblink conditioning and selection of stimuli within known perceptual parameters for infants also supports the idea of changes in synaptic efficacy during early development. However, at the earliest stages of development it may be important to additionally consider the possibility of different developmental substrates for perceptual versus motor timing (for distinction, see Nichelli, Always, & Grafman, 1996).

One brain structure that appears to play a major role in both associative (see Lavond et al., 1993; Thompson, 1986) and CR timing processes (Garcia, Steele, & Mauk, 1999) during delay eyeblink conditioning is the cerebellum. In particular, pharmacological and neurophysiological studies of the cerebellar circuitry suggest that the deep nuclei of

the cerebellum support CR generation, *per se*, whereas refinement of CR timing more critically involves the cerebellar cortex (Garcia & Mauk, 1998; Ohyama & Mauk, 2001). This is particularly interesting in light of our findings because the cerebellum is a structure that undergoes considerable postnatal development in both rats and humans (Bayer, Altman, Russo, & Zhang, 1993). In fact, the neural connectivity within the cerebellar cortex undergoes considerable change in the early postnatal period (Bayer et al., 1993), and it is the functional development of this connectivity that may explain the changes in delay functions and CR timing that we observe during infancy.

It should be noted that it is unclear whether all studies of infant eyeblink conditioning distinguish between the Total CR and Adaptive CR measure, as we do in this article. Our experience (Ivkovich et al., 2000b) suggests that this distinction becomes particularly important at longer delay intervals where responses become broader and smaller in amplitude. Levey and Martin (1968) described the shape of an eyeblink CR as an integration of both CR and UR elements, suggesting that they were examining what we refer to as the adaptive CR. This CR/UR blending is more typical of conditioning at short delays. Little et al. (1984) used an adaptive-CR measure that was even more conservative than ours, taking those responses that occurred no earlier than 170 ms prior to US onset. Others (Hoffman et al., 1985; Ohlrich & Ross, 1968) defined CRs from the end of the alpha period (our Total CR measure). Our findings indicate that whichever of these measures is used can dramatically affect both the amount of conditioning and the shape of the delay function, at least during infancy.

Knowing the limits of eyeblink conditioning delay functions at different ages during development is critical to the design of further studies that use other conditioning paradigms (e.g., trace conditioning) which incorporate additional, “higher order” processes of learning. Being able to control for timing constraints will enable us to examine the ontogeny of other memory processes that may support associative learning and that may follow different developmental trajectories (e.g., short-term memory, discrimination, inhibitory phenomena, etc.). In addition, further examination of the different CR measures discussed in this article may provide useful insights that will help us further characterize these processes. Our findings contribute to a growing line of developmental research using eyeblink conditioning. Further systematic, controlled studies of eyeblink conditioning in human infants promise to reveal interesting differences about behavioral and neural

processes underlying the development of associative learning.

NOTES

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