Ontogeny of Delay Versus Trace Eyeblink Conditioning in the Rat

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ABSTRACT: The ontogeny of delay versus trace eyeblink conditioning was examined in 19-, 23-, and 30-day-old rat pups. Pairings of a tone conditioned stimulus (CS) and periocular shock unconditioned stimulus (US; 100-ms) were presented in one of three conditioning paradigms: standard delay [380-ms CS, 280-ms interstimulus interval (ISI)], trace (380-ms CS, 500-ms trace interval), or long-delay (980-ms CS, 880-ms ISI). The results of two experiments indicated that standard delay conditioning emerged between 19 and 23 days of age whereas trace and long-delay eyeblink conditioning emerged more slowly from postnatal Days 19 to 30. Because the acquisition profile for long-delay paralleled that of trace and not standard delay, it appears that the relative deficits in the emergence of trace eyeblink conditioning during development reflect difficulty in forming associations over long ISIs rather than the short-term memory demands of the trace conditioning paradigm.

Keywords: hippocampus; cerebellum; development; learning; memory

INTRODUCTION

Classical eyeblink conditioning has become a model system for studying the behavioral and neural properties of associative learning and memory (see Gor-
mezano, Kehoe, & Marshall, 1983; Lavond, Kim, & Thompson, 1993 for reviews). Delay eyeblink conditioning is a simple associative task in which the conditioned stimulus (CS) and unconditioned stimulus (US) overlap and coterminate. The essential neural circuit underlying this form of learning is confined to the cerebellum and its interconnections with sensory and motor nuclei in the brainstem (Lavond et al., 1993). Decerebration and decortication studies (Mauk & Thompson, 1987; Norman, Buchwald, & Villablanca,
Ontogeny of Trace Eyeblink Conditioning

1977; Oakley & Russell, 1977) have demonstrated that structures above the level of the midbrain are not essential for the acquisition of delay eyeblink conditioning. However, more complex, higher order conditioning phenomena additionally involve forebrain structures associated with cognitive function such as the hippocampus, amygdala, and prefrontal cortex (Berger & Orr, 1983; Berger & Thompson, 1978; Eichenbaum, Potter, Papadoef, & Butter, 1974; Weiss, Harden, & Xiang, 1992). The availability of a variety of behavioral paradigms which engage different neural systems makes eyeblink conditioning a promising system for studying neurobehavioral changes during development. In this article, we compare the ontogeny of delay and trace eyeblink conditioning.

Trace conditioning is the simplest of the higher order phenomena to require more than the basic cerebellar–brainstem learning circuit. During trace eyeblink conditioning, the CS and US are separated by a stimulus-free period during which a memory “trace” of the CS must be maintained in order for an association with the US to occur. Several studies have demonstrated that the hippocampus is important for acquisition of trace eyeblink conditioning. Rabbits with hippocampal lesions were massively impaired in the acquisition of trace eyeblink conditioning with a trace interval of 750 ms (Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; Solomon, Vander Schaaf, Thompson, & Weisz, 1986) and 500 ms, but not 300 ms (Moyer, Deyo, & Disterhoft, 1990). More recently, Weisz, Bowneaster, Power, and Disterhoft (1999) have demonstrated that freely moving adult rats with hippocampal lesions were unable to acquire eyeblink conditioned responses (CRs) when trained with a 250-ms trace interval. In addition, neural activity that models conditioned behavior has been recorded from the hippocampus during both delay and trace eyeblink conditioning (Berger & Thompson, 1978; Solomon et al., 1986), and stimulation of the hippocampus has been shown to facilitate eyeblink conditioning (Procaccy, Kester, & Calder, 1983). The role of the hippocampus is critical during acquisition of trace conditioning but may be transitory, as Kim, Clark, and Thompson (1995) have shown that hippocampectomy impairs memory of recently, but not remotely, acquired trace eyeblink CRs. Comparisons of delay and trace eyeblink conditioning during development, therefore, may enable us to examine the ontogeny of behavioral processes, such as simple association (delay) and short-term memory (trace), and explore the functional maturation of the cerebellum and hippocampus with these paradigms.

In the rat, both the cerebellum and hippocampus are known to continue to develop postnatally (Altman, 1982; Altman & Bayer, 1975). Acquisition of delay eyeblink conditioning also develops postnatally, emerging between Days 17 and 24 (Stanton, Freeman, & Skelton, 1992). This emergence of conditioning does not depend on motor development or changes in responsiveness to a periorcular-shock US or auditory CS and is robust across a range of parameters (Andrews, Freeman, Carter, & Stanton, 1995; Freeman, Spencer, Skelton, & Stanton, 1993; Stanton et al., 1992). Neurobiological studies with the infant rodent model, thus far, have focused on the cerebellum. The ontogeny of delay eyeblink conditioning is impaired by neonatal aspiration lesions of the cerebellar cortex, and abolished by aspirations which also include the deep nuclei (Freeman, Carter, & Stanton, 1995b). Interference with cerebellar maturation by neonatal administration of antimitotic agents also severely impairs the ontogeny of eyeblink conditioning (Freeman, Barone, & Stanton, 1995a; Stanton & Freeman, 1994). These impairments are associative in nature because neither aspiration lesions nor antimitotic agents impair sensory, motor, or motivational processes that are necessary for eyeblink conditioning (Freeman et al., 1995a, 1995b). Further developmental studies of eyeblink conditioning would extend our understanding of the ontogeny of learning processes and associated neurobehavioral changes during development.

Following are two experiments comparing the acquisition of delay and trace conditioning across development using a tone CS and periorcular-shock US. A long-delay conditioning group was included to control for the longer time between CS and US onset (interstimulus-interval; ISI) that is inherent in the trace paradigm. During long-delay conditioning, the CS duration matched the duration of the CS plus the trace interval used in the trace paradigm. As in standard delay conditioning, this CS and US overlapped and were terminated (see Figure 1). In Experiment 1, rats were trained on standard delay, trace, and long-delay eyeblink conditioning beginning on postnatal Day 23 or 30. Performance on the long-delay paradigm was expected to help interpret potential differences between standard delay and trace conditioning. If the behavioral results of long-delay conditioning were similar to standard delay conditioning, deficits in trace conditioning might be attributable to the short-term memory demands of the task. Alternatively, if long-delay were similar to trace, then deficits in trace conditioning might reflect an inability to form associations over the long ISI entailed in that paradigm. Additional unpaired control groups were run at each age to determine the rates of nonassociative eyeblink CRs produced by the different CS durations. In Experiment 2, we replicated the standard delay, trace, and long-delay conditions.
paired-conditioning groups at postnatal Days 23 and 30 and extended the study earlier in development to include groups at postnatal Day 19.

**EXPERIMENT 1**

The purpose of this experiment was to determine the acquisition profiles of standard delay, trace, and long-delay eyeblink conditioning at postnatal Days 23 and 30 with appropriate unpaired control procedures. These ages were chosen because we expected to see a developmental delay in the emergence of trace eyeblink conditioning relative to standard delay which is largely developed by postnatal Day 24 in the rat (Stanton et al., 1992). We expected this because trace eyeblink conditioning is known to be a more difficult task (Solomon et al., 1986; Thompson, Moyer, & Disteholt, 1996; Woodruff-Pak, Lavond, & Thompson, 1985) and because developmental studies of fear conditioning suggest that trace conditioning emerges later than delay conditioning (Rudy, 1992). Therefore, if hippocampal development was immature in the younger infant rats, we might expect to see greater deficits during trace conditioning by measuring adaptive rather than nonadaptive CRs.

**Methods**

**Subjects.** Subjects were 111 Long-Evans rat pups (58 male, 53 female from 25 litters) balanced for sex across groups at two ages, postnatal Days 23 and 30. No more than 1 same-sex littermate was assigned to any one condition. Animals were maintained on a 12:12 hr light:dark cycle. Litters were culled to 4 male and 4 female pups between postnatal Days 3 and 5. Pups were housed with their dams until weaning on postnatal Day 21. At weaning, pups were separated into groups of 4 same-sex littermates. During the experiment, animals were housed individually. Ad lib access to food and water was provided except during training sessions.

**Surgery.** The day before conditioning procedures began, pups were separated from their littermates into individual cages with ad lib access to food and water, where they remained for the duration of the study. Each animal was anesthetized with Metofane and implanted with a headstage according to procedures described previously (Stanton et al., 1992). Differential electromyographic (EMG) recording electrodes were implanted in the upper eyelid muscle to monitor eyelid activity, and a ground lead was placed subcutaneously (SC) at the back of the neck. A bipolar stimulating electrode, for delivery of the US, was placed SC with its tips in a V-shape immediately caudal to the left eye. Electrode connectors were secured to the skull with dental acrylic. Following surgery, pups were returned to their individual cages and monitored during recovery from anesthesia.
Apparatus. The conditioning apparatus has been described previously (Stanton & Freeman, 1994). Briefly, animals were allowed to move about freely in a stainless-steel, wire-mesh cage (22 × 22 × 26 cm) contained within a sound-attenuated chamber (BRS/ LVE, Laurel, MD). The chamber was equipped with a fan [background noise level 65–70 dB (SPL)], dim light (15 W), and two speakers (2–12 kHz range) for presentation of the tone CS. The US was produced by a constant-current, 60-Hz square wave stimulator (World Precision Instruments, Sarasota, FL) set to deliver a 2-mA, 100-ms shock. During conditioning sessions, the animals’ headstages were connected to wire leads which passed through an opening in the chamber to a commutator suspended above the chamber. This allowed the animals maximum mobility. A custom-built Eyeblink Conditioning System (EPA, Health Effects Research Laboratory, Research Triangle Park, NC) controlled stimulus presentations and recorded EMG activity (rectified and integrated) from the eyelid (for details see Stanton & Freeman, 1994).

Design and Procedures. Animals at each age were randomly assigned to one of three conditioning groups or two unpaired control groups (see Table 1). No more than 1 same-sex littermate was assigned to each of these conditioning groups. The training protocol consisted of six conditioning sessions (three sessions/day at 5-hr intervals, over 2 days). Paired CS–US acquisition trials were presented using parameters for standard delay, trace, or long-delay conditioning (Figure 1). For the standard-delay conditioning group (D280), trials consisted of a 380-ms, 2.8-kHz, 90-dB tone CS and a 100-ms, 2-mA periocular-shock US (Stanton et al., 1992) which overlapped and coterminated to produce a delay interval of 280 ms between CS and US onset. For the trace conditioning group (T500), trials consisted of a 380-ms, 2.8-kHz, 90-dB tone CS; and a 100-ms, 2-mA periocular-shock US (Stanton et al., 1992) which overlapped and coterminated to produce a delay interval of 280 ms between CS and US onset. For the long-delay conditioning group (D880), trials consisted of a longer tone (980 ms) which overlapped and coterminated with the same 100-ms periocular shock to produce an 880-ms delay interval matching the ISI of the trace procedure (see Figure 1). Each paired session consisted of 100 trials, 10 blocks of nine paired trials and one US-alone trial. The intertrial interval averaged 30 s. For the two additional unpaired control groups the CS and US were explicitly unpaired. One unpaired procedure (UPT500) used the same short 380-ms tone that was used in the D280 and T500 conditioning groups. The other unpaired group (UPD880) experienced the long 980-ms tone comparable to the D880 paired group. Each unpaired session consisted of 200 trials, 100 CS-alone and 100 US-alone trials. Trials were presented in a pseudorandom order such that no more than three presentations of either stimulus occurred consecutively. The average intertrial interval was 15 s. Data from the UPT500 group were analyzed so as to yield control data for both the D280 and T500 groups. This approach reduced unnecessary animal usage. As a result, one statistical analysis was performed for all paired groups, and separate analyses were performed for each conditioning group against its respective control group to assess the effects of pairing.

Data Analysis. EMG signals were sampled in 2.5-ms bins during the 1,000-ms trial epoch in the D280 group and in 3.5-ms bins during the 1,400-ms trial epoch in the T500 and D880 groups. The raw signal was rectified and integrated for analysis. Each trial epoch was divided into four time periods: (a) pre-CS period, a 280-ms baseline sampling period leading up to presentation of the tone CS; (b) startle period, first 80 ms after tone onset [On a small percentage of trials, a nonassociative short-latency startle reaction (alpha response) occurred to the CS]; (c) CS period, the 200 ms of tone presentation that immediately preceded onset of the US [EMG activity in this period constituted a conditioned response (CR)]; and (d) US period, time from onset of the US to the end of the trial [240 ms; EMG activity in this period was recorded as an unconditioned response (UR)]. The threshold for registering an EMG response was set 0.4 arbitrary units above the average baseline amplitude during the pre-CS period. These criteria for CRs and URs were as
Results

CR Percentage. Because there were no gender differences in percentage of CRs, further analyses excluded gender as a factor. The percentage of CRs across sessions is presented by age in Figure 2A for all conditioning groups. All paired groups demonstrated similar conditioning at both postnatal Days 23 and 30 with a significant increase in CR percentage across the six training sessions. However, conditioning was most robust in the D280 group. The percentage of CRs was consistently higher for D280 than for either the T500 or D880 group. These long ISI groups did not differ from one another except in Session 1. Each paired group demonstrated significant conditioning relative to its unpaired control group.

A $2 \times 3 \times 6$ (Age $\times$ Group $\times$ Session) repeated measures ANOVA on only the paired groups confirmed that there was no main effect or interaction involving age. There were significant group, $F(2, 56) = 10.82, p < .001$, and session, $F(5, 280) = 138.28, p < .001$, effects, as well as a significant Group $\times$ Session interaction, $F(10, 280) = 4.72, p < .001$. Post hoc Newman-Keuls analyses indicated that the D280 group produced significantly, $p < .01$, more CRs (77.6%) than either T500 (53.4%) or D880 (52.2%) group, and that the T500 and D880 groups were not different from one another. Further post hoc analyses indicated that this difference was evident during Ses-

FIGURE 2 Mean (±SE) percentage CRs (A) and CR amplitude (B) for rat pups trained on postnatal Days 23–24 (left) or Days 30–31 (right) using paired (filled symbols) and unpaired (open symbols) procedures as a function of training session (1–6). Three different conditioning paradigms with matching unpaired controls (UP) were used: standard delay (D280, UPD280, square), trace (T500, UPT500, circle), and long-delay (D880, UPD880, triangle). CR amplitude was measured in arbitrary EMG units.
sion 1 when the T500 group produced the least percentage of CRs. The D880 group was significantly better, \( p < .01 \), than the T500 group during Session 1, and the D280 group was significantly better than either of the other groups, \( p < .01 \). The difference between the T500 and D880 groups disappeared following Session 1, but these two groups performed more poorly than the D280 group across all sessions, \( p < .01 \).

Separate \( 2 \times 2 \times 6 \) (Age \( \times \) Pairing \( \times \) Session) repeated measures ANOVAs were performed comparing each paired group to its appropriate unpaired control group. There was a highly significant, \( p < .001 \), Pairing \( \times \) Session interaction for each group, D280: \( F(5, 195) = 24.71 \); T500: \( F(5, 205) = 48.64 \); and D880: \( F(5, 215) = 12.95 \), reflecting the greater increase in CR percentage across sessions for paired groups as compared to unpaired groups. The percentage of spontaneous CRs for all unpaired groups remained below 30%. Although the main effect of age was not significant for any of the conditioning paradigms, the D280 task did yield a significant Age \( \times \) Pairing interaction, \( F(1, 39) = 4.6 \). This interaction was not confirmed by post hoc Newman-Keuls analyses, which revealed a nonsignificant increase in CR percentage from postnatal Days 23 – 30 for the paired group and a nonsignificant decrease in the unpaired group. These nonsignificant trends may have resulted in an interaction in the ANOVA. There were no other significant interactions involving age. Post hoc analyses of the Pairing \( \times \) Session interactions for all groups revealed that asymptote was achieved by Session 2 – 6, \( p < .01 \). Post hoc Newman-Keuls revealed that for the D280 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \), as opposed to Session 2, \( p < .01 \), whereas for the D880 group the difference between paired and unpaired groups emerged on Session 2, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \). ANOVA of the T500 condition revealed an interaction of Age \( \times \) Pairing \( \times \) Session, \( F(5, 205) = 24.62 \), reflecting a significantly greater increase in CR amplitude across sessions for paired than unpaired groups. Post hoc Newman-Keuls revealed that for the D280 group the difference between paired and unpaired groups emerged on Session 2, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \). ANOVA of the T500 condition revealed an interaction of Age \( \times \) Pairing \( \times \) Session, \( F(5, 205) = 18.29 \), reflecting a significantly greater increase in CR amplitude between paired and unpaired groups earlier in training at postnatal Day 30 (by Session 3) than at postnatal Day 23 (by Session 4).

**CR Amplitude.** Because there were no gender differences in CR amplitude, further analyses excluded gender as a factor. Mean CR amplitudes are presented by age and conditioning group in Figure 2B and follow trends similar to CR percentage. CR amplitudes for paired groups were similar across age and increased across sessions. Amplitudes were significantly higher for the D280 group than for either the T500 or D880 groups, which did not differ. Some improvement in paired relative to unpaired conditioning was observed with age, but only for the T500 paradigm.

A \( 2 \times 3 \times 6 \) (Age \( \times \) Group \( \times \) Session) repeated measures ANOVA involving the paired groups confirmed that there were no main effects or interactions involving age. There were significant group, \( F(2, 56) = 18.09, p < .001 \), and session, \( F(5, 280) = 90.74, p < .001 \), effects as well as a significant Group \( \times \) Session interaction, \( F(10, 280) = 8.63, p < .001 \). Post hoc Newman-Keuls analyses indicated that the D280 group produced significantly higher amplitude CRs than both the T500 and D880 groups, which did not differ from each other. This difference emerged on Session 2 and remained until the end of training. Sessions 2 – 6, \( p < .01 \).

Separate \( 2 \times 2 \times 6 \) (Age \( \times \) Pairing \( \times \) Session) repeated measures ANOVAs were performed comparing paired and unpaired groups for each conditioning paradigm. As with CR percentage, there was a highly significant, \( p < .001 \), Pairing \( \times \) Session interaction for each group, D280: \( F(5, 195) = 49.82 \); T500: \( F(5, 205) = 19.85 \); and D880: \( F(5, 215) = 18.29 \), reflecting a significantly greater increase in CR amplitude across sessions for paired than unpaired groups. Post hoc Newman-Keuls revealed that for the D880 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \). ANOVA of the T500 condition revealed an interaction of Age \( \times \) Pairing \( \times \) Session, \( F(5, 205) = 2.28 \). Post hoc analyses indicated that the difference in CR amplitude between paired and unpaired groups emerged earlier in training at postnatal Day 30 (by Session 3) than at postnatal Day 23 (by Session 4).

**UR Amplitude.** UR amplitudes from the first block of the first session were compared for all animals in this experiment (see Table 2). There were no main or interaction effects involving gender in the UR analysis. There were also no age or group differences in amplitude for paired groups in a \( 2 \times 3 \times 6 \) (Age \( \times \) Group \( \times \) Session) repeated measures ANOVA. Separate \( 2 \times 3 \times 6 \) (Age \( \times \) Pairing \( \times \) Session) repeated measures ANOVAs were performed comparing paired and unpaired groups for each conditioning paradigm. As with CR percentage, there was a highly significant, \( p < .001 \), Pairing \( \times \) Session interaction for each group, D280: \( F(5, 195) = 49.82 \); T500: \( F(5, 205) = 19.85 \); and D880: \( F(5, 215) = 18.29 \), reflecting a significantly greater increase in CR amplitude across sessions for paired than unpaired groups. Post hoc Newman-Keuls revealed that for the D880 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \), whereas for the D880 group it emerged on Session 3, \( p < .01 \). ANOVA of the T500 condition revealed an interaction of Age \( \times \) Pairing \( \times \) Session, \( F(5, 205) = 2.28 \). Post hoc analyses indicated that the difference in CR amplitude between paired and unpaired groups emerged earlier in training at postnatal Day 30 (by Session 3) than at postnatal Day 23 (by Session 4).

### Table 2. Mean (±SE) UR Amplitudes for the First Block of Conditioning Presented by Age and Group for Experiment 1

<table>
<thead>
<tr>
<th>Conditioning Group</th>
<th>Age</th>
<th>D280</th>
<th>T500</th>
<th>D880</th>
<th>UPT280 &amp; UPT500</th>
<th>UPT880</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnatal Day 23</td>
<td>4.4 ± 1.4</td>
<td>6.2 ± 1.9</td>
<td>4.3 ± 1.4</td>
<td>5.7 ± 1.6</td>
<td>5.8 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Postnatal Day 30</td>
<td>5.6 ± 1.9</td>
<td>4.8 ± 1.5</td>
<td>5.2 ± 1.5</td>
<td>3.4 ± 1.0</td>
<td>3.5 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

Note: Amplitude, in arbitrary EMG units, was determined using paired CS–US trials for paired groups (T500, D280, D880) or US-alone trials for unpaired groups (UPT500, UPT280, UPT880).
Table 3. Distribution of Subjects Across Three Ages and Three Conditioning Groups in Experiment 2

<table>
<thead>
<tr>
<th>Age</th>
<th>D280</th>
<th>T500</th>
<th>D880</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnatal Day 19</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>(6 m, 5 f)</td>
<td>(6 m, 5 f)</td>
<td>(5 m, 6 f)</td>
<td>(4 m, 4 f)</td>
</tr>
<tr>
<td>Postnatal Day 23</td>
<td>11</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>(4 m, 7 f)</td>
<td>(6 m, 7 f)</td>
<td>(5 m, 5 f)</td>
<td></td>
</tr>
<tr>
<td>Postnatal Day 30</td>
<td>15</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>(6 m, 9 f)</td>
<td>(8 m, 3 f)</td>
<td>(4 m, 5 f)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

CR percentage and amplitude trends were very similar. There were no main effects of age. Acquisition was faster and stronger for the D280 group than for the T500 or D880 groups, suggesting that the difficulty in acquiring trace conditioning in these experiments was due to the long ISI rather than the temporal gap between stimuli created by the trace interval. Experiment 2 was designed to replicate and extend the developmental comparison downward to include 19-day-old rat pups in order to better determine whether these conditioning paradigms have different ontogenetic profiles across a broader period of development.

EXPERIMENT 2

The purpose of this experiment was to assess the ontogeny of delay versus trace eyeblink conditioning across a broader developmental range. Because conditioning performance did not change markedly in Experiment 1 between postnatal Days 23 and 30 for any of the paradigms and conditioning levels were fairly robust, we chose to look at a younger age to determine if conditioning developed differently across these three task conditions. Experiment 2 was a replication of the paired groups from the previous experiment with the addition of a younger age group (Day 19). Conditioning took place across 2 days beginning at one of three ages: postnatal Day 19, Day 23, or Day 30. Previous studies suggested that rat pups would demonstrate poorer acquisition of delay eyeblink conditioning at the youngest age, but that conditioning would be robust by postnatal Days 23–30 (Stanton et al., 1992). We hypothesized that there might be a developmental dissociation in the emergence of trace versus standard delay conditioning between Days 19 and 23. Performance of the long-delay conditioning group would help determine whether developmental deficits in trace relative to standard delay were attributable to the trace interval or ISI.

Methods

Subjects and Apparatus. Subjects were 99 Long-Evans rat pups (48 male, 51 female from 35 litters) balanced for sex across groups at three ages, postnatal Day 19, Day 23, and Day 30 (see Table 3). Animals were maintained as in Experiment 1. Pups were housed with their dams until weaning on postnatal Day 21 or the beginning of conditioning procedures. The apparatus was as described in Experiment 1.

Design and Procedures. In a $3 \times 3 \times 6$ (Age × Group × Session) design, animals at each age were randomly assigned to one of three paired CS–US conditioning groups (see Table 3). As previously, the training protocol consisted of six training sessions. The conditioning groups were the same D280, D880, and T500 animals described in Experiment 1.

Data Analysis. Percentage CRs and amplitude of CRs and URs were obtained as in Experiment 1 and ana-
analyzed using between groups, repeated measures ANOVA.

Results

Percent CRs. No gender differences were observed, thus gender was excluded as a factor in statistical analyses. The data for percentage of CRs are presented in Figure 3. There was a significant increase across age and session for each group. All three groups reached asymptote by the fourth session of conditioning. Overall, the strongest conditioning was observed in the D280 group whereas conditioning was weaker and similar in the T500 and D880 groups. There was no difference between the groups on postnatal Day 19. Conditioning improved significantly between Days 19 and 23 for the D280 group only, and between Days 23 and 30 for the D880 group only. Trace conditioning, instead, improved gradually so that differences between successive ages were insignificant whereas a significant change occurred between Days 19 and 30. By Day 30, all groups achieved similar asymptotic levels of conditioning at the end of training.

A 3 × 3 × 6 (Group × Age × Session) repeated measures ANOVA revealed significant, \( p < .001 \), main effects of group, \( F(2, 90) = 13.78, \text{age}, F(2, 90) = 19.71 \), and session, \( F(5, 450) = 185.05 \). The interaction of Group × Session was highly significant, \( F(10, 450) = 3.61, p < .001 \), whereas Group × Age and Group × Age × Session interactions approached significance, \( F(4, 90) = 2.28, p = .07 \) and \( F(20, 450) = 1.52, p = .07 \), respectively. Newman-Keuls analyses of the group main effect showed that the percentage of CRs was significantly higher, \( p < .01 \), for the D280 group (71%) than the T500 (52%) and D880 (50%) groups, which were not significantly different from each other. Post hoc Newman-Keuls analyses indicated that the Group × Session interaction reflected slower acquisition of the T500 and D880 groups relative to the D280 group. Sessions 1–5, \( p < .01 \). The T500 and D880 groups, however, were not significantly different from one another on any session. The post hoc Newman-Keuls analyses of the Group × Age interaction showed that significant improvement in conditioning occurred for the D280 group earlier in ontogeny than for either long ISI group. The D280 group improved significantly between postnatal Days 19 and 23, \( p < .01 \) but not between Days 23 and 30, whereas the D880 group did not change between Days 19 and 23 but improved between Days 23 and 30, \( p < .01 \). In contrast, although there did not appear to be significant improvement in the T500 group at either age increment, the change between Days 19 and 30 was significant, \( p < .05 \).

CR Amplitude. A similar picture emerged with CR amplitude (see Figure 4). Although there was no difference in CR amplitude between the groups at postnatal Day 19, the D280 group demonstrated much higher amplitude CRs relative to the T500 and D880 groups at older ages. The long ISI groups, however, did not differ from each other. All groups exhibited an increase in CR amplitudes across age and sessions. There was a larger increase in CR amplitudes across
sessions for the D280 group than the other groups and for all groups on Day 30 as compared to younger ages.

No gender differences in CR amplitudes were observed, therefore gender was excluded as a factor in statistical analyses. A $3 \times 3 \times 6$ (Group $\times$ Age $\times$ Session) repeated measures ANOVA yielded significant main effects, $p < .001$, of group, $F(2, 90) = 24.52$, age, $F(2, 90) = 15.64$, and session, $F(5, 450) = 134.15$, and a significant three-way interaction, $F(20, 450) = 1.84$, $p < .05$. The developmental trend which emerged with age was a significant increase in CR amplitude for the D280 group only. Post hoc Newman-Keuls indicated that there was no difference in CR amplitude between the groups on postnatal Day 19. At Day 23, amplitudes increased for the D280 group and were significantly higher than the other groups, $p < .01$, which did not differ. Amplitudes increased again at Day 30 for the D280 group relative to younger ages, $p < .01$. There were no significant changes from Days 19 to 23 or from Days 23 to 30 in the other two groups. In contrast to CR percentage, there also was no change in CR amplitude for the T500 and D880 groups between Day 19 and Day 30. The gradual change in CR percentage from Days 19 to 30 was not accompanied by changes in CR amplitude for the long ISI groups.

*UR Amplitude.* Differences in CR percent and amplitude were not attributable to differences in performance as determined by UR amplitude. There were no gender differences in UR amplitude. A $3 \times 3$ (Group $\times$ Age) ANOVA on UR amplitudes during Block 1 of conditioning indicated that UR amplitudes did not differ by conditioning paradigm or age. UR amplitudes are presented by age and group in Table 4.

**Discussion**

Learning, as measured by an increase in percentage of CRs across sessions, was observed in all groups at all

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Table 4. Mean (±SE) UR Amplitudes for the First Block of Conditioning Presented by Age and Group for Experiment 2

<table>
<thead>
<tr>
<th>Age</th>
<th>Conditioning Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D280</td>
</tr>
<tr>
<td>Postnatal Day 19</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>Postnatal Day 23</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>Postnatal Day 30</td>
<td>4.4 ± 0.6</td>
</tr>
</tbody>
</table>

*Note.* Amplitude, in arbitrary EMG units, was determined using paired CS–US trials.
ages, but the developmental changes were different for the different training paradigms. Whereas there was no difference between groups at postnatal Day 19, there was improvement in the D280 group at Day 23 that was not matched by the T500 and D880 groups. Consistent with Experiment 1, the T500 and D880 groups were slower to acquire CRs than the D280 group.

Together, these data suggest that there may be a developmental lag in maturation of processes which support conditioning over long ISIs. Consequently, it appears that ISI influences the ontogeny of trace eyeblink conditioning in these studies and may mask the development of short-term memory processes involved in trace conditioning. Because trace conditioning was always worse than standard delay but not different from long-delay, in terms of both CR percent and amplitude, the apparent deficits in trace conditioning were clearly a function of ISI rather than trace interval.

GENERAL DISCUSSION

To summarize the findings, acquisition of the eyeblink CR was studied following six sessions of delay, trace, and long-delay conditioning procedures in 23- and 30-day-old rats in Experiment 1 and in 19-, 23-, and 30-day-old rats in Experiment 2. The D280 group was superior to the T500 and D880 groups in terms of both CR percent and amplitude, and there was no difference between the T500 and D880 groups. Acquisition did not improve between postnatal Days 23 and 30 in Experiment 1, but there was a significant increase in CR acquisition between Days 19 and 30 in Experiment 2. This was primarily due to a distinct and large improvement in acquisition in the D280 group between Days 19 and 23 whereas trace eyeblink conditioning emerged more slowly between Days 19–30. The relative relationship between standard delay (D280) and trace (T500) remained the same across Days 23–30. Comparable development of the T500 and D880 groups indicated that the apparent deficit in trace conditioning was due to the ISI rather than the trace interval (discussed later).

These results only begin to address the question of whether there are ontogenetic differences between standard delay and trace paradigms of eyeblink conditioning. There is a developmental dissociation between standard delay versus trace eyeblink conditioning in that the former increases dramatically between postnatal Days 19 and 23 whereas the latter does not. However, the inherently greater difficulty of trace and long-delay conditioning (discussed later) make it unclear whether trace conditioning develops fully over the postnatal Days 19–30 age range. Previous fear conditioning studies suggest that trace conditioning is a later-developing form of learning (Brasser & Spearr, 1998; Moye & Rudy, 1987). Using a tone CS and footshock US, Moye and Rudy (1987) established that by postnatal Day 15 rat pups exhibited conditioned freezing behavior following conditioning with a 0-s CS–US delay, but not with a 10- or 30-s CS–US trace interval. By Day 21, animals in that study showed conditioning at 10- or 30-s trace intervals that was comparable to that observed with delay fear conditioning. Brasser and Spearr (1998) also demonstrated that Day-17 rat pups could acquire the same tone–footshock association with a 0-s delay but not a 10- or 20-s trace interval unless the training/testing context was enhanced. In an enhanced context, Day-17 pups were able to condition using a 10-s trace interval but not a 20-s trace interval. Fear conditioning has developmental properties that differ from eyeblink conditioning (Carter & Stanton, 1996, 1998; Paczkowski et al., 1999) and neither of the fear conditioning studies mentioned here included a long-delay condition in which the CS and US were contiguous but delayed so that the ISI would match that of the trace groups. All of these factors warrant further study.

Another result of the present studies was a clear difference in acquisition between standard delay and trace and long-delay conditioning at postnatal Days 23 and 30. Consistent with previous studies, the greater difficulty of the trace conditioning paradigm was confirmed by slower acquisition rates and smaller CR amplitudes (e.g., Solomon & Groccia-Ellison, 1996; Solomon et al., 1986; Thompson et al., 1996; Woodruff-Pak et al., 1985). Also consistent with previous studies was the observation that CR performance declined at longer delay intervals (Finkbiner & Woodruff-Pak, 1991; Freeman et al., 1993; Gormezano et al., 1983; Gregg, Kintrell, Donjan, & Ansell, 1978; Smith, Coleman, & Gormezano, 1969). The optimal ISI for developing rat pups at postnatal Day 24 was determined to be 280 ms (Freeman et al., 1993) with performance progressively declining at delays of 560, 1,120, and 1,960 ms. The present results are consistent with these findings as demonstrated by the poorer conditioning of the D880 group relative to the D280 group. The novel result, however, was the similarity between the T500 and D880 groups. We expected the trace conditioning deficit to be greater than the ISI deficit.

As stated in the introduction, the main purpose of the D880 group was to help interpret acquisition dif-

short
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long
ferences between standard delay and trace eyelink conditioning. The results in this article showed a similar impairment in acquisition of trace and long-delay conditioning relative to standard delay eyelink conditioning. This suggests that the difference in acquisition between standard delay and trace was not due to a deficit in short-term memory, but rather in the ability to form associations across long ISIs. This finding was interesting in light of previous studies which have included the long-delay group in comparisons of delay and trace eyelink conditioning. We found three such studies, using rabbits, which compared acquisition on a simple delay paradigm with trace and delay paradigms matched for ISI. Two of these studies found that in young adult rabbits, at 4 or 6 months of age, the long-delay condition was more similar to standard delay than to trace in terms of trials-to-criterion (Thompson et al., 1996) and total number of CRs (Solomon & Groccia-Ellison, 1996). This was contrary to our findings with %CRs across training sessions even though the previous studies used similar ISIs: 600 and 900 ms (Thompson et al. and Solomon & Groccia-Ellison, respectively). The third pair of studies (Sasse, Coffin, & Woodruff-Pak, 1991; Sasse & Woodruff-Pak, 1990) yielded results more similar to ours by demonstrating that 4-month-old rabbits were impaired on long-delay conditioning with a 750-ms delay compared to a shorter 400-ms delay, and that performance did not differ between animals trained with the 750-ms long-delay and a 750-ms trace paradigm. In addition, none of the hippocampal lesion studies of trace conditioning have included long-delay control groups to determine what the respective roles of cerebellum and/or hippocampus might be during eyelink conditioning over long ISIs. Both the cerebellar cortex and the hippocampus have been implicated in CR timing (Akase, Alkon, & Disterhoft, 1989; Perrett, Ruiz, & Maas, 1993). Our data suggest that the question of whether there are differences between delay and trace eyelink conditioning in developing rats appears to be confounded with ISI. If cerebellar cortex is important for long-delay eyelink conditioning, it is possible that the protracted development of the cerebellar cortex results in ISI functions in conditioning that “mask” the ontogenetic course of trace conditioning.

The present studies begin to describe the developmental behavioral profiles of delay and trace conditioning, but also create new questions about the maturation of the hippocampus and the cerebellum and their relative roles in the tasks being studied. This is especially true because of the close similarity in conditioning performance between the T500 and D880 groups. Hippocampal lesion studies suggest that if hippocampal development were to lag behind that of the cerebellum, we would observe a developmental lag in trace eyelink conditioning. The hippocampus does appear to be involved in trace eyelink conditioning in adult rats (Weiss et al., 1999) and rabbits (Moyer et al., 1990; Port et al., 1986; Solomon et al., 1986). Studies are under way to examine the effects of early hippocampal aspirations on the subsequent development of delay and trace conditioning. Further developmental studies of delay and trace conditioning are needed to help characterize the ontogeny of cerebellar–hippocampal functional interactions during eyelink conditioning.

NOTES

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