CS–US Preexposure Effects on Trace Eyeblink Conditioning in Young Rats: Potential Implications for Functional Brain Development

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Recent studies of delay eyelblink conditioning (EBC) in young rats have demonstrated different effects of various conditioned and unconditioned stimulus (CS–US) preexposure conditions on learning at different ages. The present study extends this research to trace EBC. Subjects experienced 1 of 3 preexposure conditions (paired CS–US, unpaired CS–US, or no stimuli) at either 20 or 24 days of age. Four days later, they were conditioned using either trace (Experiment 1) or delay (Experiment 2) EBC parameters. Results were similar at both ages tested. Paired preexposure facilitated acquisition of delay but not trace relative to context preexposure. Unpaired preexposure impaired acquisition of both delay and trace. These behavioral findings provide a foundation for hypotheses about the functional maturation of cerebellar, hippocampal, and entorhinal learning circuits.

Keywords: eyelblink conditioning, trace conditioning, preexposure, learned irrelevance, development

Developmental studies using classical conditioning are an important means of studying the maturation of behavioral processes and the neural substrates underlying associative learning (e.g., Hall, 1987; Ivkovich & Stanton, 2001; Nicolle, Barry, Veronesi, & Stanton, 1989; Rudy, 1992; Sananes, Gaddy, & Campbell, 1988; Stanton & Freeman, 2000; Weber & Richardson, 2004). Different forms of associative learning emerge at different times during development. For example, simple conditioning, or learning to associate two stimuli with each other, appears earlier in development than do higher order conditioning processes (e.g., trace conditioning, discrimination). Such higher order stimulus–stimulus associations are influenced by attention, memory demands, retention intervals, and temporal and configural processes (Green & Stanton, 1989; Hunt, Richardson, & Campbell, 1994; Rudy, 1992). The different developmental profiles for these various types of associative learning correspond to functional maturation of brain mechanisms necessary for the association, retention, and response systems that produce the conditioned response (CR; Freeman, Barone, & Stanton, 1995; Ivkovich & Stanton, 2001; Rudy, 1992; Weber & Richardson, 2004).

Our understanding of the development of various learning processes and their underlying neural mechanisms has been greatly aided by comparison of two forms of classical eyelblink conditioning (EBC): delay and trace. The standard procedure for EBC with developing rat pups (Stanton, Freeman, & Skelton, 1992) involves an audible tone conditioned stimulus (CS) and a shorter mild periorbital shock unconditioned stimulus (US). The US elicits a reflexive eyelinerk as the unconditioned response (UR). For delay conditioning, the CS is presented first and followed by the US such that the stimuli overlap and coterminate. For trace conditioning, the same CS and US are presented except that the stimuli are separated by a stimulus–free trace interval (see Figure 1). Repeated pairings of the CS and US result in an eyelinerk that is a CR. The CR is elicited by the tone CS and precedes or anticipates the shock US.

Delay EBC emerges between Postnatal Day (PND) 17 and 24 in the rat. Because the nonassociative, reflexive eyelinerk is present at a very young age, this late development of learning is not due to underdeveloped sensory or motor function (Andrews, Freeman, Carter, & Stanton, 1995; Stanton et al., 1992). Rather, it has been demonstrated that the emergence of delay EBC relies on maturation of cerebellar–brainstem learning circuits (Freeman, Barone, et al., 1995; Freeman, Carter, & Stanton, 1995; Freeman & Nicholson, 2004; Freeman, Rabinak, & Campolattaro, 2005; Stanton & Freeman, 2000).

Trace EBC, using the same CS and US as in delay EBC but separated by a 500-ms trace interval, is evident at PND 20 but does not become robust until about 30 days of age (Ivkovich et al., 2000). Disrupting hippocampal development around PND 10 impairs subsequent trace EBC, indicating a critical role for this brain region (Ivkovich & Stanton, 2001). It has been suggested that introducing a trace interval between the CS and US adds a short-term memory component to the associative task. This and other higher order learning processes appear to mature later in development and engage the hippocampus and potentially other forebrain regions (Ivkovich et al., 2000; Ivkovich & Stanton, 2001; Nicolle et al., 1989; Rush, Robinette, & Stanton, 2001).

When a learning process first becomes evident in development may depend on the maturation of underlying acquisition processes...
or, alternatively, on an ability specific to the expression of the learned response. Recent studies using a preexposure paradigm have shed light on the relative development of acquisition versus expression for delay EBC. Stanton, Fox, and Carter (1998) demonstrated that rat pups can learn a CS–US association using delay procedures prior to PND 20, even though they may be unable to express that learning. In that study, young rats were given delay EBC (paired CS–US exposure) on PND 17, an age when they were unable to demonstrate learning. However, when conditioned again on PND 20 these rats learned faster than training naive, chamber preexposed controls (context preexposure). The facilitation of learning following paired CS–US preexposure is referred to as savings. This finding suggests that the rats acquired the CS–US relationship on PND 17 and that the brain systems necessary for forming and remembering the association were functional. However, because they were not able to produce CRs on PND 17, the output mechanisms responsible for expressing the learning were not functionally mature. Further, Rush et al. (2001) showed that older rats given delay EBC on PND 27 produced robust CRs (about 80%) and demonstrated near-perfect retention 3 days later (on PND 30). They produced about 85% CRs within the first 10 trials of testing. This suggests that once acquisition mechanisms are mature, the learning is stored in long-term memory for later access. To our knowledge, similar developmental studies using preexposure with trace EBC procedures have not been conducted until now. Studies of savings in trace EBC are important for better understanding neural differences between learning and memory mechanisms in delay and trace EBC.

Another feature of the preexposure paradigm is the ability to examine a second higher order acquisition process known as learned irrelevance (LIR). LIR is the slower acquisition of a CS–US relationship that is the result of preexposure to the CS and US in a random unpaired fashion. It is suggested that, during unpaired CS–US preexposure, subjects learn that there is no relationship between the stimuli (learned irrelevance) and are slower to realize that the stimulus contingency is relevant in later paired training. For example, Rush et al. (2001) demonstrated in the rat that unpaired preexposure on PND 27 impaired subsequent delay EBC on PND 30 relative to naive rats that had no stimulus preexposure and were only preexposed to the context of the testing chamber.

It is interesting that Stanton et al. (1998) found that very young rats, preexposed on PND 17 and tested on PND 20, do not yet show LIR. Instead, unpaired CS–US preexposure on PND 17 slightly facilitated acquisition of delay EBC. However, this facilitation was significantly less than that observed following paired CS–US preexposure. This finding suggests that LIR emerges sometime between PND 17 and 27. Research that has examined the neural mechanisms involved in LIR in the rat is lacking, but recent data from adult rabbits indicate that lesions of the entorhinal cortex impair LIR (Allen, Chelius, & Gluck, 2002). Therefore, it is possible that functional maturation of the entorhinal cortex is correlated with the postnatal onset of LIR. The current experiments examined LIR at points between PND 17 and 27 to identify when this phenomenon emerges and how the expression of LIR is affected by testing with the trace versus delay paradigms.

In sum, the present study compares the effects of preexposure on trace (Experiment 1) and delay (Experiment 2) EBC. Subjects were assigned to one of three preexposure treatments: paired, unpaired, or context (chamber exposure). Four days later, all rats received paired EBC using either trace or delay procedures. The study was designed to further our understanding of the relative ontogeny of acquisition, expression, and retention of associative learning using trace versus delay procedures, as well as potential differences in LIR with each of these conditioning paradigms. Further, the findings from this study were intended to shed additional light on the functional development of putative underlying cerebellar–brainstem (delay EBC), hippocampal (trace EBC), and entorhinal (LIR) circuits.

**Experiment 1**

Previous findings indicate that trace EBC becomes more robust as rats mature from PND 19 to 30, and the ages for preexposure in the current study were chosen accordingly (Ivkovich et al., 2000). To examine the effects of preexposure at an age when little or no acquisition of trace EBC would be observed, we chose PND 20. PND 24 was chosen as an older age for preexposure when the acquisition of trace EBC would be more consistent and was expected to reach a higher asymptote. The main question of interest was whether paired CS–US preexposure would be beneficial to young rats, thus leading to a savings effect when rats were conditioned 4 days later. When compared with a training naive control group that received no stimulus preexposure but spent time in the testing context, the paired preexposure group would be expected to exhibit superior conditioning in the later testing phase if the preexposure experience is retained. An unpaired preexposure group controlled for nonassociative effects of repeated stimulus presentations during the preexposure phase.

**Method**

**Subjects**

In the following experiment, 53 Long–Evans male and female rat pups were sampled from 19 litters born of time-bred pregnant female dams obtained from Charles Rivers Laboratory (Raleigh, NC). On PND 4, litters were culled to 10 pups (5 male and 5 female, whenever possible). Pups were housed with their dam until weaning on PND 19. About half of the rats (n = 24) underwent surgery on PND 19 to implant electrodes for stimulus delivery and behavioral recording, whereas the others (n = 29) were housed with same sex littermates until surgery on PND 23. Following surgery, all rats were housed individually for the duration of testing. Ad lib access to food and water was available except during training sessions. Rats were kept on a 12-hr light–dark cycle with lights on at 0700. Rats were housed in a colony room accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and procedures were
approved by the Laboratory Animal Care and Use Committee of Wright State University.

Surgery

One day prior to the beginning of behavioral procedures, on PND 19 or PND 23, pups were anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg), and two electrodes were implanted according to procedures described previously (Stanton et al., 1992). A bipolar stimulating electrode (Plastics One, Roanoke, VA) was implanted subdermally caudal to the left eye for delivery of the shock US, and a differential electromyograph (EMG) electrode was implanted in the upper eyelid muscle to record eyelid activity. The electrodes were secured to the skull with dental acrylic. Following surgery, rats were allowed to recover from anesthesia and were returned to individual home cages.

Apparatus

All behavioral procedures took place in the same environment: a stainless-steel and Plexiglas cage, measuring 28 cm × 24 cm × 30 cm, contained within a sound-attenuated chamber (Med Associates, St. Albans, VT). The chamber was equipped with a 15-W light, a fan, and speakers (2–12 kHz range) to deliver the tone CS. The US was a 2.8-kHz, 90-dB tone. The US was a 100-ms, 1.5-mA constant current electrical shock produced by a 60-Hz wave square stimulator (World Precision Instruments, Sarasota, FL). Rats were allowed to freely move around this space, and electrodes were connected to a commutator suspended above the chamber. A custom-built EBC system (JSA Designs, Raleigh, NC) controlled stimulus presentations and recorded EMG activity from the eyelid.

Design and Procedure

The procedure consisted of two phases: the preexposure phase and the training phase. Whereas rats experienced different preexposure conditions, subsequent training was the same for all.

Preexposure phase. On PND 20 or PND 24, rats within each age group were randomly assigned to one of three preexposure conditions: paired (paired CS and US), unpaired (explicitly unpaired CS and US), or context (no CS or US but comparable exposure to the testing chamber). All rats received three sessions of preexposure in 1 day (three, 1-hr sessions at 4.5-hr intervals). No more than 1 male and 1 female from the same litter were assigned to the same experimental group. Data are presented below for 24 younger rats (paired, n = 7 [5 female, 2 male]; unpaired, n = 8 [3 female, 5 male]; context, n = 9 [5 female, 4 male]) and 29 older rats (paired, n = 10 [6 female, 4 male]; unpaired, n = 10 [5 female, 5 male]; context, n = 9 [6 female, 3 male]).

Paired CS–US trials in this experiment were presented using standard trace conditioning parameters. Trials consisted of a 380-ms tone CS followed by a 500-ms silent trace period and then a 100-ms periorcular shock US (see Figure 1). Each session consisted of 100 trials (90 paired CS–US trials and 10 CS-alone probe trials). The average intertrial interval was 30 s. For unpaired sessions, the CS and US were explicitly unpaired and neither stimulus was presented more than three times consecutively. The number of CS and US presentations matched that of paired sessions, and the session duration was made comparable by setting the average intertrial interval to 15 s. For context sessions, rats were placed in the testing chamber and connected to recording equipment in the same way as all the other rats in this experiment. No CS or US was presented, but EMG was sampled and recorded during 100 blank trial epochs that matched data collection during paired EBC.

Training phase. Four days after preexposure on PND 24 or 28, all rats received paired training using standard trace EBC procedures as described above. For rats in the paired preexposure group, this was a continuation of the same training procedures following a 3-day break. During the training phase, rats received six sessions of conditioning over 2 days (three sessions/day at 4.5-hr intervals).

EMG Analysis

The raw EMG signal was amplified (5 K), rectified (500 Hz to 5 kHz bandwidth with a 12-dB/octave rolloff), and integrated (20-ms time constant) for quantitative analysis. The threshold for registering an EMG response was set 0.4 arbitrary units above the average baseline amplitude during the pre-CS period (200 ms prior to CS onset).

EMG signals were sampled in 400, 3.5-ms bins within each 1400-ms trial epoch. Each trial was divided into three time periods to categorize response types: (a) startle response (SR), EMG activity within the first 80 ms after tone onset; (b) adaptive CR, a response occurring in the 200 ms just prior to US onset; and (c) UR, activity in the 140 ms following the US (see Ivkovich et al., 2000, for further detail). In the case of unpaired or context preexposure, in which conditioning does not occur, CR measures reflect the spontaneous responses or pseudo-CRs that occur during the same trial period as the adaptive CRs of paired training.

Data Analysis

Measures of learning included the percentage of CRs and the amplitude of CRs over the course of preexposure and training. These measures were analyzed using between-groups repeated measures analyses of variance (ANOVAs) separately for preexposure and training phases, Age (2) × Sex (2) × Preexposure Condition (3) × Sessions (3 preexposure vs. 6 training sessions). The analyses reported below focus on paired CS–US trials when appropriate. During preexposure, the percentage and amplitude of CRs reflect genuine CRs as well as spontaneous or pseudo-CRs that occurred in the absence of conditioning or stimuli. In other words, during preexposure, CR measures for the unpaired group were based on matching tone trials, and analyses for the context group were based on matching blank trials. Analyses of CS-alone probe trials were used to evaluate potential group differences in the latencies of CRs. These analyses did not yield additional information and are not reported below.

Control measures included the percentage of SRs to the tone and the amplitude of URs to the shock. These measures establish that the stimuli have similar effects across groups and training, looking for stimulus effects that might account for the performance differences among groups in learning measures (e.g., CR percentage and amplitude). During the preexposure phase, the context group did not experience any tones or shocks so this group was excluded from analysis of SRs and URs during preexposure. Measures from all three groups were included, however, in analysis of the training phase. SR percentage was analyzed separately for preexposure and training sessions with repeated measures for session. The average UR amplitude was analyzed only for the first session of preexposure and the first session of training, using a one-way, between-groups ANOVA. UR amplitudes sometimes habituate with training, so Session 1 data establish the similar effectiveness of the US across all groups at the onset of conditioning (see also Claffin, Garrett, & Buffington, 2005; Ivkovich & Stanton, 2001).

When data violated assumptions of sphericity, the Huynh–Feldt corrected F values were used. Interactions were analyzed using Bonferroni’s adjustment for analyses of simple main effects. Post hoc t tests were performed, as needed, on main effects using Tukey’s method of adjustment. Results meet the .05 significance level unless otherwise noted.

Results

ANOVA indicated that age was not a significant factor in any of the measures reported below. There were no statistically significant differences between rat pups preexposed on PND 20 and trained on PND 24 versus preexposure and training on PND 24 and
28. Moreover, there were no apparent developmental trends in the data when examined separately for each age group (see Table 1 for CR percentage values). Data in figures represent the combined age groups.

**CR Percentage: Trace Conditioning**

As expected, only the paired group demonstrated some increase in conditioned responding during the preexposure phase. However, this experience did not benefit them during the training phase when compared with training naive, context controls. After a 4-day break, rats in the paired group were no better at trace conditioning than rats in the context preexposure group. Unpaired preexposure did, however, impair subsequent learning; this group lagged behind the other two until Session 4 of training with trace EBC (see Figure 2).

### Table 1

**Conditioned Response Percentage (M ± SE) Separated by Age for Examination of Developmental Trends in Experiment 1 (Top) and Experiment 2 (Bottom)**

<table>
<thead>
<tr>
<th>Day and session</th>
<th>Paired</th>
<th>Unpaired</th>
<th>Context</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 20–24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>15.4 ± 4.5</td>
<td>13.7 ± 4.7</td>
<td>7.9 ± 3.1</td>
</tr>
<tr>
<td>P2</td>
<td>32.6 ± 7.7</td>
<td>14.4 ± 4.3</td>
<td>7.4 ± 1.6</td>
</tr>
<tr>
<td>P3</td>
<td>44.4 ± 10.5</td>
<td>16.8 ± 4.6</td>
<td>7.9 ± 2.2</td>
</tr>
<tr>
<td>T1</td>
<td>58.6 ± 7.8</td>
<td>19.2 ± 6.7</td>
<td>69.2 ± 7.1</td>
</tr>
<tr>
<td>T2</td>
<td>68.1 ± 7.0</td>
<td>29.2 ± 10.2</td>
<td>73.8 ± 8.5</td>
</tr>
<tr>
<td>T3</td>
<td>71.5 ± 6.0</td>
<td>45.8 ± 9.0</td>
<td>70.0 ± 8.1</td>
</tr>
<tr>
<td>T4</td>
<td>75.5 ± 6.0</td>
<td>69.9 ± 11.6</td>
<td>73.2 ± 10.3</td>
</tr>
<tr>
<td>T5</td>
<td>83.0 ± 6.8</td>
<td>70.6 ± 8.8</td>
<td>78.0 ± 7.6</td>
</tr>
<tr>
<td>T6</td>
<td>67.0 ± 9.5</td>
<td>66.0 ± 9.2</td>
<td>74.8 ± 9.0</td>
</tr>
<tr>
<td>PND 24–28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>21.1 ± 6.9</td>
<td>9.8 ± 2.5</td>
<td>8.6 ± 2.0</td>
</tr>
<tr>
<td>P2</td>
<td>40.4 ± 9.1</td>
<td>14.5 ± 3.2</td>
<td>5.5 ± 1.2</td>
</tr>
<tr>
<td>P3</td>
<td>47.0 ± 10.1</td>
<td>14.8 ± 3.3</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td>T1</td>
<td>66.9 ± 9.3</td>
<td>31.2 ± 4.4</td>
<td>65.6 ± 8.3</td>
</tr>
<tr>
<td>T2</td>
<td>73.9 ± 9.1</td>
<td>31.5 ± 6.9</td>
<td>65.5 ± 8.3</td>
</tr>
<tr>
<td>T3</td>
<td>78.5 ± 8.8</td>
<td>44.9 ± 8.9</td>
<td>67.8 ± 9.3</td>
</tr>
<tr>
<td>T4</td>
<td>76.8 ± 8.8</td>
<td>69.0 ± 6.6</td>
<td>73.2 ± 8.7</td>
</tr>
<tr>
<td>T5</td>
<td>76.3 ± 8.5</td>
<td>75.1 ± 7.3</td>
<td>70.3 ± 12.4</td>
</tr>
<tr>
<td>T6</td>
<td>67.1 ± 9.8</td>
<td>72.3 ± 6.6</td>
<td>67.9 ± 13.0</td>
</tr>
</tbody>
</table>

| **Experiment 2** |
|-----------------|--------|----------|---------|
| PND 20–24       |        |          |         |
| P1              | 54.6 ± 8.5 | 29.1 ± 9.0 | 5.5 ± 1.2 |
| P2              | 72.0 ± 4.5 | 27.2 ± 9.8 | 9.1 ± 3.2 |
| P3              | 68.4 ± 3.5 | 27.2 ± 11.1 | 5.4 ± 1.0 |
| T1              | 82.9 ± 6.7 | 49.2 ± 10.7 | 69.0 ± 6.3 |
| T2              | 88.4 ± 5.6 | 56.9 ± 9.1 | 86.0 ± 6.2 |
| T3              | 93.8 ± 1.6 | 73.3 ± 8.4 | 91.6 ± 2.3 |
| PND 24–28       |        |          |         |
| P1              | 33.4 ± 6.1 | 30.0 ± 8.1 | 12.1 ± 1.8 |
| P2              | 53.0 ± 10.3 | 33.3 ± 8.5 | 8.7 ± 1.5 |
| P3              | 65.2 ± 10.4 | 32.6 ± 7.6 | 8.5 ± 2.1 |
| T1              | 92.9 ± 2.1 | 70.2 ± 9.9 | 64.7 ± 9.0 |
| T2              | 91.6 ± 3.2 | 84.6 ± 5.6 | 89.2 ± 2.6 |
| T3              | 92.2 ± 2.3 | 80.3 ± 9.8 | 93.3 ± 1.3 |

**Note.** PND = postnatal day; P1–P3 = preexposure session; T1–T6 = training session.
**CR Amplitude: Trace Conditioning**

Consistent with the percentage of CRs, the paired group showed larger amplitude responses compared with the other groups during preexposure, and males in that group showed a greater change than females. During training, there appeared to be a delay in the increase of CR amplitudes for the unpaired group. This trend, however, was not statistically significant. By the end of training all rats demonstrated increases in CR amplitude consistent with acquisition of the CS–US association.

**Preexposure phase.** During preexposure, there was a significant increase in CR amplitude from Session 1 to Session 2 only in males receiving paired preexposure, Sex × Condition × Session, $F(3.6, 73.6) = 5.9, p = .001$. Females in the paired group gave low amplitude responses throughout preexposure sessions, along with males and females in the unpaired and context preexposure groups. It is interesting that, for CR percentage, the sex effect was an overall effect not specific to the paired group. As with CR percentage, this sex difference did not persist into the training phase.

**Training phase.** Although there was an apparent impairment in the growth of CR amplitude for the unpaired group during training, there were no significant group differences. There was a marginal main effect for condition, $F(2, 41) = 2.84, p = .07$. Examination of Figure 4 indicates that the unpaired group gave consistently lower amplitude CRs during acquisition of trace EBC than did the paired or context groups. All rats demonstrated a significant increase in CR amplitude—session, $F(3.4, 139.0) = 14.51, p = .000$—consistent with the increase in CR percentage observed for all groups. Post hoc analysis indicated that the session effect was due to a significant increase in CR amplitude between the first three and the last three sessions of training that also occurred on separate days (see Figure 4). It is interesting that this also corresponds to a shift in the unpaired group CR amplitudes. Therefore, the lack of a Session × Condition interaction that was seen with CR percentage might be explained by a greater influence of condition, which came close to being significant.

![Figure 4. Mean (± SE) amplitude of conditioned responses (CRs) across three sessions of preexposure (p1–p3) and six sessions of training (t1–t6) in Experiment 1 using trace eyelink conditioning. Separate lines represent preexposure experience: paired (circle), unpaired (square), context (triangle). EMG = electromyograph.](image)

**Control Measures: Trace Conditioning**

**SR percentage.** During both the preexposure and training phases of the study there was significant variation across sessions in the percentage of SRs: preexposure, $F(2, 41) = 14.51, p = .000$; training, $F(3.4, 139.0) = 14.51, p = .000$. During preexposure there was a small but significant increase in SR percentage for the paired and unpaired groups (context group was excluded because no CS was presented) from 60.0% to 70.4% from Session 1 to Session 3. During training, SR percentage held steady around 84.0% for Sessions 1–5 and then dropped significantly to 75.7% in Session 6. These changes in SR percentage were not specific to preexposure experience and cannot account for acquisition differences reported above.

**UR amplitude.** Separate univariate ANOVAs were conducted on UR amplitude during the first session of preexposure for the paired and unpaired groups only (again, no US was presented in context group) and during the first session of training for all three groups. There was no difference in responsiveness to the US between the paired and unpaired groups during Session 1 of preexposure. However, during Session 1 of the training phase, when all three preexposure groups were compared, there was a significant main effect of age, $F(1, 41) = 4.5, p = .041$. Because of the consistency of UR amplitudes, a slightly larger UR in younger rats ($M ± SE = 971 ± 8$ EMG units) than older rats ($M ± SE = 965 ± 12$ EMG units) was significant. However, this pattern was not related to the differences in acquisition reported above that were specific to preexposure experience.

**Discussion**

The findings in Experiment 1 demonstrate that paired CS–US preexposure at 20 or 24 days of age does not yield savings of trace conditioning relative to a training naive context group when rats are tested 4 days later. Although there was a moderate amount of
acquisition during paired preexposure (50% CRs), there was no evidence that learning was being retained at the subsequent testing time. It may be that acquisition mechanisms needed to demonstrate CRs during training may be functional before the memory storage mechanisms for retaining that information over days have developed. That is, rats show evidence of acquiring trace CRs at a young age, but show no more benefit 4 days later than controls that are simply exposed to the training context. There also was evidence of LIR as early as PND 20. So, although the rats did not appear to retain the paired CS–US relationship over a 4-day interval, they did seem to retain something about the irrelevant CS–US relationship that influenced later learning.

Experiment 2

Experiment 2 was identical to Experiment 1, except that paired training consisted of delay EBC. The data reported below enabled us to compare and contrast the effects of various preexposures on trace and delay EBC at the same ages. In addition, the ages studied in this experiment extend previous data on preexposure effects in delay conditioning. Previous studies of delay EBC in developing rats have shown facilitation of acquisition following paired preexposure on PND 17, though no LIR was detected at that age (Stanton et al., 1998). Preexposure on PND 27 resulted in enhanced retention 3 days later and the emergence of LIR (Rush et al., 2001). The present study examined these effects at intervening ages.

Method

Subjects

Subjects were 57 Long–Evans male and female rat pups treated identically to those in Experiment 1. Surgery to implant electrodes for EBC took place on PND 19 for 28 rats and on PND 23 for 29 rats.

Design and Procedure

As in Experiment 1, rats were randomly assigned to the three preexposure groups: paired, unpaired, and context (testing chamber only). In this experiment, paired trials consisted of the same CS and US presented in Experiment 1, but the US now overlapped with the last 100 ms of the tone CS and the two stimuli coterminated (see Figure 1, Delay). The protocol was similar, three sessions of preexposure followed 4 days later by three sessions of delay conditioning (three sessions/day at 4.5-hr intervals). Delay conditioning typically yields faster acquisition than trace conditioning, so only three training sessions were administered. As in Experiment 1, each delay conditioning session consisted of 100 trials, 10 blocks of 9 paired CS–US trials and 1 CS-alone probe trial with an average intertrial interval of 30 s. Data are presented below for 28 younger rats (paired, \( n = 10 \) [5 female, 5 male]; unpaired, \( n = 9 \) [4 female, 5 male]; context, \( n = 9 \) [4 female, 5 male]) and 29 older rats (paired, \( n = 12 \) [6 female, 6 male]; unpaired, \( n = 8 \) [3 female, 5 male]; context, \( n = 9 \) [5 female, 4 male]). No more than 1 male and 1 female from a litter were assigned to the same experimental group.

EMG Analysis

EMG data were recorded and analyzed as in Experiment 1. For delay conditioning, the overlap of the two stimuli yielded a shorter trial epoch, a trial length of 800 ms with 400, 2-ms bins of data collection.

Data Analysis

As in Experiment 1, measures of learning were CR percentage and amplitude. Measures of stimulus effectiveness and performance were SR percentage and UR amplitude. These data are presented in the same way as for Experiment 1 and were analyzed in the same manner.

Results

As in Experiment 1, there was no effect of age on any of the recorded measures, so both age groups are considered together (see Table 1 for values separated by age). Also, there were no significant effects of sex, so this factor is not discussed.

CR Percentage: Delay Conditioning

During preexposure, just as in Experiment 1, the delay paired group showed an increase in CRs across sessions, whereas the context and unpaired groups remained at baseline levels of responding (see Figure 5). During training, however, the paired group did show benefits of preexposure by producing a high percentage of CRs in the very first training session relative to the other groups. The context group reached the same high percentage of CRs as the paired group by Session 2, but the unpaired group continued to produce significantly fewer CRs throughout the three training sessions (see Figure 5).

Preexposure phase. A repeated-measures ANOVA of percentage of CRs during three sessions of preexposure, Age (2) × Sex (2) × Preexposure Condition (3) × Session (3), revealed a significant Session × Condition interaction, \( F(4, 90) = 6.14, p = .000 \). During Session 1, the paired and unpaired groups produced significantly more CRs than the context group, which did not receive any stimulus exposure. During Sessions 2 and 3, all three groups significantly differed from each other as the paired group demonstrated acquisition and produced the most CRs, the nonstimulated context group produced the fewest CRs, and the unpaired group
was in-between with a low level of nonassociative, spontaneous CRs to the tone CS (see Figure 5).

Training phase. The pattern of results during the training phase was different for each of the preexposure groups as supported by a significant Session × Condition interaction, $F(4, 90) = 4.82, p = .001$. Post hoc analyses revealed that during Session 1 of training, the paired group produced significantly more CRs than the other two groups, indicating clear retention of preexposure learning. By Session 2 of training, the context control group reached the level of performance of the paired group. The unpaired group, however, remained impaired relative to the other groups through Sessions 2 and 3 of training (see Figure 5). A more detailed analysis of Session 1 only, Preexposure Condition (3) × Trial Block (10), revealed a significant Condition × Trial Block interaction, $F(13.7, 368.8) = 3.4, p = .000$. Further examination of the interaction confirms that the paired group produced a greater number of CRs than the other groups from the onset of the training phase, but that by Block 5 the context group began to catch up with the paired group and that the unpaired group remained significantly different than the paired group until Block 9 (see Figure 6).

CR Amplitude: Delay Conditioning

During preexposure, the paired group demonstrated acquisition of delay EBC because CR amplitudes increased. During the training phase, the paired group continued to show greater CR amplitudes than the other two groups but only during the first training session. The unpaired and context preexposed groups acquired the same amplitude of CR responses in the later part of training.

Preexposure phase. Consistent with percentage CRs, there was a significant Session × Condition interaction for CR amplitude during preexposure in Experiment 2, $F(3.3, 72.5) = 8.7, p = .000$. Further analysis indicated that CR amplitude increased only in the paired group across the three preexposure sessions. CR amplitude in the paired group exceeded that of the context control group in all three sessions and exceeded the amplitude produced by spontaneous CRs in the unpaired group in Sessions 2 and 3.

Control Measures: Delay Conditioning

SR percentage. Once again, the context group was excluded from the preexposure analyses because no CS was presented. There was a significant Age × Condition interaction for SR percentage during preexposure, $F(1, 31) = 9.3, p = .005$. This was due to a lower percentage of SRs for the older paired group only. The younger rats given paired preexposure (PND 20) produced significantly more SRs (78.3%) than the older paired rats (47.1%). There was no significant age difference for the unpaired groups. A similar significant interaction of Age × Condition was observed in the training phase of delay EBC, $F(2, 45) = 3.7, p = .031$. However, post hoc analysis of the interaction did not reveal any significant comparisons. Neither preexposure nor training phase differences in SR percentage matched observed differences in CR measures. Therefore, SR amplitude cannot account for the differences in acquisition of delay EBC reported above.

UR amplitude. There were no significant effects of age, sex, or preexposure condition on UR amplitude in either the preexposure or training phases of this experiment. Therefore, sensitivity to the US could not account for acquisition differences in learning rates described above.
Discussion

This experiment demonstrated that clear retention of delay EBC is possible from 20 to 24 and 24 to 28 days of age when rats were given paired CS–US preexposure. Consistent with previous studies showing robust conditioning by PND 24 (Stanton et al., 1992), rats in this experiment learned well during the preexposure phase, achieving about 67% CRs in Session 3. In addition, they demonstrated excellent retention and continued improvement of conditioning 4 days later, with 88% CRs in the first session of training relative to training naive context controls and unpaired rats. Although the context group reached similar conditioning levels as the paired group by Session 2, the unpaired group was still lagging in Session 3 of training, despite some improvement in the percentage and amplitude of CRs.

General Discussion

In summary, preexposure had different effects on acquisition of trace versus delay EBC. Rats given paired trials during preexposure in Experiment 1 were able to show some learning of trace EBC but not facilitation of learning 4 days later when compared with context controls. In contrast, robust acquisition and later facilitation of delay EBC were observed for rats at the same ages. This suggests a difference in the maturation of underlying neural mechanisms for initial acquisition of trace and delay EBC, but also potential differences in memory storage over the 4-day retention interval. Further, unpaired CS–US preexposure at 20 or 24 days of age impaired later acquisition of CRs, compared with context controls, using both trace and delay procedures. In view of previous studies (Rush et al., 2001; Stanton et al., 1998), this suggests that LIR emerges sometime between 17 and 20 days of age for delay EBC. That the context group performed so well in acquiring trace conditioning suggests that there may be additional influences at least on trace EBC that need to be examined and considered when defining retention and LIR.

The main goal of this study was to determine whether early exposure to paired CS–US stimuli would produce similar savings and retention of trace EBC as has been previously demonstrated for delay EBC. For savings to take place, we would expect to see clear facilitation of conditioning relative to context controls during the training phase, even though there was little or no conditioning during the preexposure phase. Despite a moderate amount of learning for trace conditioning during the preexposure phase in this study, there was no evidence of facilitation during the training phase. There was no difference between pretrained rats (paired group) and training naive rats (context group). Even close examination of the first training session indicated identical learning curves for these two groups. This was in stark contrast to delay EBC at these same ages (Experiment 2) or at younger ages (Stanton et al., 1998). For delay EBC, even unexpressed learning between 17 and 20 days of age facilitates acquisition at the later age. Paired preexposure at 20, 24, or 27 days of age yields moderate to robust delay conditioning that is preserved and even strengthened when training resumes several days later. Such continued improvement after a break in training was not observed for trace conditioning in the present study.

Previous studies comparing the development of delay versus trace have focused only on acquisition and it has been difficult to pinpoint a specific age when trace EBC first emerges. Low levels of trace conditioning can be observed from the time that delay conditioning begins to emerge, but trace EBC reaches asymptotic levels much later (PND 30) than delay EBC (PND 24; Claflin et al., 2005; Ivkovich et al., 2000; Stanton et al., 1992). The present data from paired preexposure are consistent with the previously reported differences in the amount of acquisition for trace and delay at PND 20 and 24. As has been reported previously (Ivkovich & Stanton, 2001), an intact hippocampus is necessary for trace EBC in young rats, and it is likely that functional maturation of the hippocampus, or some of its connections, plays a role in the slower development of trace EBC. In the present study, moderate levels of acquisition during preexposure and robust acquisition during training with trace procedures suggest that the hippocampus was sufficiently mature to support expected levels of acquisition.

A novel finding in our study, revealed by using the preexposure paradigm, was that there may be differences in retention in memory storage processes between trace and delay. Perhaps, although the hippocampus is sufficiently mature to support acquisition of trace conditioning with regular training, memory consolidation mechanisms that enable rats to retain the learning and improve between preexposure and training are not yet mature. One possibility is that different areas of the hippocampus are involved in the different stages of processing and are reaching functional maturity at different rates. Evidence from human functional magnetic resonance imaging during hippocampal-mediated episodic learning and memory indicate that different regions of the hippocampus itself are involved in the encoding versus retrieval processes (El-Edrige, Engel, Zeineh, Bookheimer, & Knowlton, 2005). Another possibility is that, during consolidation, the memory is being transferred from the hippocampus to another brain region for later retrieval. Some evidence for such transfer has been found for trace EBC in mice. In fact, several studies have demonstrated a time-limited role in memory storage for the hippocampus (e.g., Kim, Clark, & Thompson, 1995; Takehara, Kawahara, Takekatsu, & Kirino, 2002; Zola-Morgan & Squire, 1990). For mice acquiring trace EBC, as time elapses, the circuitry is reorganized to shift reliance from the hippocampus to the medial prefrontal cortex (mPFC; Takehara, Kawahara, & Kirino, 2003; Takehara et al., 2002). It is possible, then, that delayed maturation of the mPFC or connections to that area from the hippocampus prevent consolidation of trace EBC early in development. Alternatively, it may be that biochemical mechanisms responsible for memory consolidation, in conjunction with late-phase, long-term potentiation in the hippocampus, are not being recruited effectively. A critical role for protein synthesis has been demonstrated for acquisition, consolidation, and extinction of trace conditioned eyelblink responses in mice (Inda, Delgado-Garcia, & Carrion, 2005). The precise mechanisms involved in retention of trace versus delay EBC remain to be examined.

Further evidence for time-limited memory storage in the hippocampus, or connected structures, comes from our results of unpaired preexposure. The studies of Stanton et al. (1998) indicated that LIR in delay EBC did not occur with unpaired CS–US preexposure on PND 17 and training on PND 20. Preexposure at PND 27 and training on PND 30 did yield an LIR effect (Rush et al., 2001). Further, Rush et al. (2001) did not observe LIR in delay conditioning when both preexposure and training took place on PND 20. The present study, then, enabled us to refine the estimate
of when LIR emerges in the developing rat and observe the effect of preexposure on PND 20 and 24 on acquisition several days later. The present study demonstrated that unpaired preexposure on PND 20 and 24 can impair later acquisition of both delay and trace EBC, compared with context controls, if there are several days between preexposure and training. Again, perhaps time is needed for consolidation of the preexposure experience and the transfer of learning from one brain region to another. Such a time-limited role for memory transfer has been demonstrated for other forms of hippocampal-dependent learning. For example, recently acquired memories for trace EBC and object discrimination can be impaired by lesions of the hippocampus, thereby preventing the memory storage process from taking place (Kim, Clark, & Thompson, 1995; Zola-Morgan & Squire, 1990). The more time between learning and lesion, the better the retention. It is unclear whether a similar time-limited memory storage mechanism applies to LIR and its putative entorhinal cortex substrates (Allen et al., 2002) or whether the hippocampus also plays a role in LIR through its entorhinal connections. There is only one published study examining the neural substrates of LIR in EBC (Allen et al., 2002).

The present study used a context control group that is typical of studies examining preexposure effects. The level of performance of the context group, particularly in trace EBC, suggests that preexposure to context alone may have had a facilitative effect. Indeed, one earlier study found that context preexposure enhanced later acquisition, though only under certain conditions. Katz, Rogers, and Steinmetz (2002) found that four sessions (over 4 days) of exposure to the conditioning chamber facilitated acquisition of delay EBC in adult rabbits. Groups exposed for 0 or 8 days did not show such facilitation. In the present study, comparisons of the paired group’s initial acquisition during preexposure and the context group’s initial acquisition during training suggests possible context facilitation as well (paired < context): trace, $F(1, 33) = 21.3, p < .001$; delay, $F(1, 38) = 16.7, p < .001$. However, it is not clear whether such a comparison is confounded by the increase in age between preexposure and training, despite the lack of significant age differences in the study. If context preexposure did in fact facilitate acquisition of both trace and delay EBC, the lack of further enhancement of performance in the trace paired group suggests that this group may be demonstrating some contextual effects but no retention of CS–US pairings. On the other hand, the clear difference between the delay paired and context groups indicates additional facilitation from CS–US pretraining. Nonetheless, further resolution of this issue will require more research as the role of context in both trace and delay EBC is poorly understood.

Our operational definition of LIR as representing learning that stimuli are irrelevant during preexposure may be also affected by context facilitation in the context control group. Using an alternative paradigm for retarding acquisition, Katz et al. (2002) suggest that latent inhibition of delay conditioning, resulting from CS-alone preexposure, is not about learning to ignore a stimulus but rather is the result of the stimulus experience blocking normal context facilitation. Once again, this context-based interpretation might be applied to our studies. What appears as learning that stimuli are irrelevant may be a lack of context facilitation. For example, a statistical comparison of acquisition between our unpaired groups during the first three sessions of training phase and the paired groups during preexposure indicated similar rates of acquisition: trace, $F(1, 33) = 0.0, p = .99$; delay, $F(1, 37) = 2.1, p = .15$. This suggests that once given paired trials, the unpaired groups acquired at normal, not retarded rates, regardless of conditioning paradigm. Thus, although unpaired preexposure clearly impairs learning relative to the other preexposure conditions, the reason for this impairment will require further experimentation.

Despite the need for further experiments focusing on context, the behavioral data in this paper can be used to develop working hypotheses about the relative maturation of presumed neural substrates for the different types of learning studied. Through the use of conventional interpretations of the data on the basis of comparison with context controls in the present study, retention of information about CS–US pairings, especially trace EBC, was poorer than retention of unpaired CS–US experience, as in LIR. Therefore, it is likely that memory storage for trace EBC and LIR is mediated by different brain regions. This is consistent with other research indicating that lesions of the entorhinal cortex impair LIR in adult rabbits but selective lesions of the hippocampus do not (Allen et al., 2002), whereas hippocampal lesions do clearly impair trace EBC in several species (Ivkovich & Stanton, 2001; Moyer, Deyo, & Disterhoft, 1990; Weiss, Bouwmeester, Power, & Disterhoft, 1999). Therefore, viewing our data in light of other developmental studies, it is probable that cerebellar–brainstem circuits responsible for acquisition and retention of delay EBC mature first, around PND 17–20. Entorhinal circuits supporting LIR follow, around PND 20. Acquisition of trace EBC which relies on the basic cerebellar–brainstem circuit, as well as the hippocampus, emerges with the acquisition of delay EBC, but retention mechanisms for trace EBC, whether in different areas of the hippocampus or in the mPFC or based on later-developing protein synthesis mechanisms, mature after PND 24. Future preexposure studies will enable us to examine separately the acquisition and retention mechanisms of different EBC paradigms and various stimulus timing parameters, as well the effects of context. It will be important to distinguish whether it is the different EBC paradigms (e.g., delay vs. trace) that rely on different neural mechanisms or whether other factors, such as context or timing, engage different higher order brain systems. Such parametric studies comparing delay and trace EBC during development will enable us to continue studying the building blocks of higher order associative learning.

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