Effects of Early Hippocampal Lesions on Trace, Delay, and Long-Delay Eyeblink Conditioning in Developing Rats

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The effects of bilateral hippocampal aspiration lesions on later acquisition of eyeblink conditioning were examined in developing Long-Evans rat pups. Lesions on postnatal day (PND) 10 were followed by evaluation of trace eyeblink conditioning (Experiment 1) and delay eyeblink conditioning (Experiment 2) on PND 25. Pairings of a tone conditioned stimulus (CS) and periocular shock unconditioned stimulus (US, 100 ms) were presented in one of three conditioning paradigms: trace (380 ms CS, 500 ms trace interval, 880 ms interstimulus interval [ISI]), standard delay (380 ms CS, 280 ms ISI), or long delay (980 ms CS, 880 ms ISI). The results of two experiments indicated that hippocampal lesions impaired trace eyeblink conditioning more than either type of delay conditioning. In light of our previous work on the ontogeny of trace, delay, and long-delay eyeblink conditioning (Ivkovich, Paczkowski, & Stanton, 2000) showing that trace and long-delay eyeblink conditioning had similar ontogenetic profiles, the current data suggest that during ontogeny hippocampal maturation may be more important for the short-term memory component than for the long-ISI component of trace eyeblink conditioning. The late development of conditioning over long ISIs may depend on a separate process such as protracted development of cerebellar cortex.

Key Words: learning; memory; ontogeny; development; hippocampus; classical conditioning.

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INTRODUCTION

The development of associative learning processes has been studied for some time using rodent models and behavioral procedures that take advantage of pairing simple sensory stimuli with the young organism’s simple reflexive responses such as mouthing, freezing, and exploration (Rudy, 1992). In these studies, the ontogeny of associative learning is sometimes constrained by sensory or motor system development as well as by temporal properties of the paradigm (Rudy, 1992). A better understanding of these constraints may lie in a better understanding of the underlying neurobiological mechanisms supporting these processes. With this in mind, Stanton and colleagues (Freeman, Barone, & Stanton, 1995; Freeman, Carter, & Stanton, 1995; Ivkovich, Paczkowski, & Stanton, 2000; Stanton, Freeman, & Skelton, 1992) have begun to study the developmental psychobiology of a well-studied behavioral learning paradigm that also has the advantage of being understood in terms of a well-defined neural circuit—classical conditioning of the eyeblink response (Stanton, 2000; Stanton & Freeman, 2000).

Classical eyeblink conditioning has advantages for application to developmental studies at both the behavioral and neural levels (Ivkovich, Eckerman, Krasnegor, & Stanton, 2000; Stanton, 2000; Stanton & Freeman, 1994, 2000). A long tradition of behavioral studies in adult rabbits and humans gives us a variety of well-described paradigms that can be used to evaluate a range of associative and cognitive processes (Gormezano, Kehoe, & Marshall, 1983; Woodruff-Pak & Steinmetz, 2000a,b). Examples of these paradigms include delay conditioning, trace conditioning, discriminative conditioning and reversal, and latent inhibition. Developmental applications of these paradigms provide a set of “building blocks” for understanding cognition and cognitive development in relation to simpler sensory, motor, and associative processes (Stanton, 2000).

At the neural level, we have come to understand the essential brain circuit underlying classical eyeblink conditioning in adult animals (e.g., Lavond, Kim, & Thompson, 1993; Thompson, 1986). This circuit critically involves the brain stem and cerebellum and supports simple associative learning of the relationship between a sensory conditioned stimulus (CS, typically a tone or light) and a somatosensory unconditioned stimulus that elicits an eyeblink (US, typically an air puff or a periocular shock). Researchers have also examined the roles of limbic and forebrain areas in eyeblink conditioning phenomena. Whereas the basic brain stem–cerebellar circuit is sufficient to support eyeblink conditioning when stimuli are presented in the delay paradigm (overlapping and coterminating), research suggests that other brain areas, especially the hippocampus and prefrontal cortex, may play a role in higher order conditioning phenomena such as trace conditioning (Beylin et al., 1999; McGlinchey-Berroth, Carrillo, Gabrieli, & Disterhoft, 1997; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986), latent inhibition (Solomon & Moore, 1975), conditional discrimination (Daum, Channon, & Gray, 1992), and discrimination reversal (Berger & Orr, 1983), to name a few examples (for reviews, see Green & Woodruff-Pak, 2000; Schmajuk & Di Carlo, 1991, 1992).

Trace eyeblink conditioning is the simplest of the higher order phenomena that is believed to engage the hippocampus. During trace conditioning, the CS and US are separated by a stimulus-free period, and the individual must bridge the interval by maintaining a memory “trace” of the CS long enough to form an association with the subsequent US (see T500 panel in Fig. 1). Other studies (also reviewed in Ivkovich, Paczkowski, &
FIG. 1. Conditioning paradigms. For the standard-delay conditioning group (D280), a 380-ms CS overlapped and coterminated with a 100-ms US, producing a 280-ms interstimulus interval (ISI). For the trace group (T500), the same CS and US were separated by a 500-ms trace interval, producing an 880-ms ISI. For the long-delay group (D880), a 980-ms CS overlapped with a 100-ms US to match the T500 group for ISI. (Taken from Ivkovich, Paczkowski, & Stanton, 2000.)

Stanton, 2000) have demonstrated that lesions of the hippocampus impair the acquisition of trace eyeblink conditioning at trace intervals of 250 ms in rats (Weiss, Bouwmeester, Power, & Disterhoft, 1999) and 500 ms in rabbits (Moyer et al., 1990). Similar lesions made post-acquisition also impair the retention of recently, but not remotely, acquired trace conditioned responses (CRs) (Kim, Clark, & Thompson, 1995). We have begun to examine the ontogeny of trace conditioning in developing rats (Ivkovich, Paczkowski, & Stanton, 2000). Because the cerebellum and hippocampus are structures that continue to develop postnatally in both rats and humans (Altman, 1982; Altman & Bayer, 1975; Kretschmann, Kammradt, Krauthausen, Sauer, & Wingert, 1986), trace eyeblink conditioning has the potential to reveal interesting ontogenetic information that may contribute to our understanding of hippocampal–cerebellar interactions during the ontogeny of eyeblink conditioning (Stanton, 2000).

Previously, we have shown that the ontogeny of trace eyeblink conditioning, with a 500-ms trace interval and an 880-ms interstimulus interval (ISI) (see Fig. 1), shows slower ontogenetic development than does standard-delay eyeblink conditioning (Ivkovich, Paczkowski, & Stanton, 2000). Delay conditioning became robust by postnatal day (PND) 23, whereas both trace and long-delay eyeblink conditioning (a delay paradigm that uses
a longer CS and matches the ISI used in trace conditioning (Fig. 1) had similar slower developmental profiles and became robust by around PND 30. The similarity between the long-delay and trace paradigms raised the question of the role of the hippocampus in the ontogeny of eyeblink conditioning paradigms with long ISIs.

The importance of hippocampal maturation in the ontogeny of spatial learning paradigms has been demonstrated for discrete trials delayed alternation (Freeman & Stanton, 1991; Green & Stanton, 1989; Stanton, Jensen, & Pickens, 1991). Fimbria–fornix transections that disrupt the development of the septohippocampal system on PND 10 produce a developmental delay in the emergence of delayed alternation but not simple position habit learning (Freeman & Stanton, 1991). Consequently, performance on delayed alternation provides a means for evaluating the effectiveness of the early hippocampal lesions independent of potential effects on trace eyeblink conditioning.

Here, in two experiments, we examined the effects of hippocampal lesions produced early in development (PND 10) on subsequent eyeblink conditioning on PND 25 using the same procedures introduced by Ivkovich, Paczkowski, and Stanton (2000). The stimuli were a tone CS and a periocular shock US presented in one of three conditioning paradigms: trace, standard delay, and long delay (Fig. 1). Experiment 1 focused on the trace conditioning paradigm. Animals received bilateral aspiration lesions of either hippocampus and overlying cortex or overlying cortex only (an unoperated control group was also included). Following eyeblink conditioning, animals were trained on delayed alternation in a T-maze apparatus. This task provided independent confirmation that the hippocampal lesions in this experiment were sufficient to produce the profound deficits that would be expected in spatial learning paradigms. If normal development of the hippocampus is critical to the ontogeny of trace eyeblink conditioning, then we would expect hippocampal-lesioned animals to show deficits in conditioned responding relative to cortical and unoperated controls. In Experiment 2, animals with bilateral hippocampal lesions were compared to unoperated controls on standard-delay and long-delay eyeblink conditioning. If the hippocampus is critical for conditioning over long ISIs, then we would expect to see impairments of the long-delay group and not of the standard-delay group. Together, the two studies compare the two factors that differentiate trace conditioning from standard delay conditioning, the trace interval and the ISI, and the contribution of the hippocampus to each at the point in development when trace conditioning first emerges.

**EXPERIMENT 1**

The purpose of this experiment was to determine whether early damage to the hippocampus would interfere with the ontogeny of trace eyeblink conditioning. Animals with cortical lesions served as controls for the effects of nonspecific brain damage, anesthesia, and surgery at a young age. Our previous work showed that trace conditioning develops in rat pups gradually between 19 and 30 days of age (Ivkovich, Paczkowski, & Stanton, 2000). The current study evaluates trace conditioning starting on PND 25. Conditioning was evaluated using criteria for adaptive CRs; those responses that occurred within the 200-ms period immediately preceding the US were counted (Ivkovich, Paczkowski, & Stanton, 2000). Adaptive, long-latency CRs have been shown in adults to be more affected by hippocampal lesions than have short-latency CRs (Moyer et al., 1990; Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; Solomon et al., 1986).
Method

Subjects

Subjects were 45 Long–Evans rat pups (22 males and 23 females from eight litters) balanced for sex across groups. No more than 1 same-sex littermate was assigned to any one condition. Animals were maintained on a 12:12-h light–dark cycle with lights on at 07:00 h. Animals were shipped as litters, culled to 4 male and 4 female pups, on PND 5 from Charles River Laboratories (Raleigh, NC). Pups remained with their dams until weaning on PND 21. During this time, they were housed in an animal colony accredited by the American Association for the Accreditation of Laboratory Animal Care and the animal care committee of the National Health and Environmental Effects Laboratory at the U.S. Environmental Protection Agency (Research Triangle Park, NC). At weaning, pups were separated into groups of 4 same-sex littermates. During the experiment, animals were housed individually. Ad libitum access to food and water was provided except during training sessions.

Surgery

On PND 10, animals underwent surgery to aspirate either hippocampus or overlying cortex bilaterally. Some littermates did not undergo surgery and served as normal control animals. All pups were returned to their dam until weaning and underwent a second surgery on PND 24 to implant electrodes for eyeblink conditioning.

Lesion surgery. For hippocampal or cortical surgeries, all pups were taken from the dam and placed in individual compartments of a clear Plexiglas enclosure placed over two store-bought heating pads. The lower pad was set on low, whereas the upper pad (adjacent to the Plexiglas) was turned off. Pups that were to undergo surgery were anesthetized by administration of a ketamine/xylazine cocktail (0.075 ml/g dose of 10 mg/ml ketamine and 2 mg/ml xylazine), which took effect in about 5 min. Each pup was then placed under a dissecting microscope, and a midline incision on the head exposed the soft skull just anterior to lambda. Two narrow rectangular openings (approximately 3 × 8 mm, medial to lateral) were cut in front and along the medial–lateral extent of lambda using micro-scissors to expose the surface of the brain. The cortex in this area was then aspirated using a fire-polished curved 1-mm glass pipette under vacuum suction to expose the dorsal hippocampus. At this point, cortical surgery was complete. Hippocampal surgeries proceeded to include aspiration of the structure following it dorsally and ventrally as completely as possible. Excessive bleeding was controlled with cold saline and suction. The cavity was packed with Gelfoam (Upjohn, Kalamazoo, MI), the incision was sutured closed, and neosporin disinfectant was applied to the wound. Pups were returned to the incubator, monitored during recovery for about 4 h, and then returned to their dam when they were alert or responsive to tactile stimulation. All littermates were returned to the dam at the same time. Of 18 pups that received hippocampal lesions, 2 did not survive to the behavioral training phase of this study. Of 13 pups that received cortical lesions, 1 did not survive. The overall survival rate was 93%.

Eyeblink preparation surgery. The second surgery took place on PND 24, the day before conditioning procedures began. Pups were separated from their littermates into
individual cages with ad libitum access to food and water, where they remained for the duration of the study. Each animal was anesthetized with Metofane (Pitman–Moore, Mundelein, IL) and implanted with a head stage 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 at the back of the neck. A bipolar stimulating electrode, for delivery of the US, was placed subcutaneously 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**

_Eyeblink conditioning_. 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), dim light (15 W), and two speakers (2- to 12-kHz range), one of which was used 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’ head stages were connected to wire leads that 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 (Health Effects Research Laboratory) controlled stimulus presentations and recorded EMG activity (rectified and integrated) from the eyelid (for details, see Stanton & Freeman, 1994).

_Delayed alternation_. This apparatus, described in detail by Freeman and Stanton (1991), consisted of two Plexiglas T-mazes with a start box and two goal arms, all of equal dimensions (22.0 × 9.0 × 10.5 cm). The area connecting the three arms of a maze, the choice point, was isolated by computer-controlled doors. All walls and doors were opaque, and reduced illumination was provided by a 45-W amber light bulb positioned 30 cm above the choice point. A small metal cup was attached to the end of each goal arm for delivery of a light cream reward. A photoelectric beam was directed across each arm about 2 cm in front of the reward cup, and the breaking of this beam on rewarded trials resulted in an infusion of 0.07 ml of cream into the cup via computer-controlled syringe pumps. An experimenter manually introduced animals into the maze at the start of each trial and moved them from the goal arm to the start box between the forced and choice run phases of the trial (see Design and Procedure below). The opening of maze doors, administration of reward, and data collection all were automated. Between trials, animals were placed nearby in individual “intertrial interval (ITI) compartments” made of clear Plexiglas.

**Design and Procedure**

Animals were randomly assigned to one of three lesion groups: hippocampal (n = 16), cortical control (n = 12), or normal control (n = 14). No more than 1 same-sex littermate was assigned to each of these groups.
Eyeblink conditioning. Starting on PND 25, all animals received six trace conditioning sessions (two sessions/day at 5-h intervals over 3 days) according to general procedures described by Ivkovich, Paczkowski, and Stanton (2000). Conditioning trials consisted of paired presentations of a 380-ms, 2.8-kHz, 90-dB tone CS and a 100-ms, 2-mA periocular shock US separated by a 500-ms stimulus-free trace interval (see T500 panel in Fig. 1). The CS–US interval was 880 ms. Each session consisted of 100 trials: 10 blocks of 9 paired trials and 1 CS-alone trial. The ITI averaged 30 s.

Delayed alternation. The delayed alternation protocol began 1 day after eyeblink conditioning was completed. The procedures were the same as in Freeman and Stanton (1991) except where noted. Deprivation of food and water occurred on PND 29 or 30 at 1600–1700 h (about 48 or 72 h after the last conditioning session). Starting at 0800 h the next day, pups were acclimated to the maze during two pretraining “goal box sessions” and a session of 12 forced runs. The start of each of these three sessions began at 4-h intervals. During goal box sessions, animals were placed in one of the goal arms for 3 min or until they consumed the 0.07 ml cream reward provided. They experienced six such exposures to one goal arm in the first session and six exposures to the other goal arm in the second session. The forced run session consisted of 12 trials in which each animal was placed in the start box and the door to one of the two goal arms was open; which arm was open was controlled by computer and determined pseudo-randomly with six runs occurring to each side (left and right). The animal was required to travel from the start box to the goal arm and to break the photoelectric beam to receive a reward (0.07 ml cream).

The following day, two acquisition sessions of delayed alternation were administered at 5-h intervals, with the first consisting of two 12-trial blocks and the second consisting of three 12-trial blocks. Each trial consisted of a pair of runs: a forced run followed by a choice run. During the forced run, each animal was placed in the start box with one goal arm door open. Once it traveled down the arm and retrieved its reward, the animal was placed back in the start box for the choice run. This time, both goal arm doors were open and the animal had to choose the opposite arm to the one traveled during the forced run in order to receive a reward. Animals were run in squads of 4 and received their trials in rotation. In this way, the ITI for a given animal was determined by the period of time it took the other 3 animals to complete a trial (approximately 3 min). At the end of each training session, supplementary feeding of light cream was provided to equate individual pups for total diet consumed (according to reward earned during the session) and to maintain body weight at 85% of pre-deprivation levels. At the end of training, pups were immediately provided with ad libitum food and water.

Data Analysis

Eyeblink conditioning. Electromyographic signals were sampled in 3.5-ms bins during the 1400-ms trial epoch in the trace conditioning paradigm. 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, the first 80 ms after tone onset (on a small percentage of trials, a nonassociative short-latency startle reaction [SR, alpha response] occurred to the CS); (c) CS period, the 200 ms of tone presentation that immediately preceded onset of the US
(EMG activity during this period constituted a CR; and (d) US period, the time from onset of the US to the end of the trial (240 ms) (EMG activity during 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 described previously (e.g., Skelton, 1988; Stanton et al., 1992).

Percentage of CRs and amplitude of CRs and URs were analyzed using a between-groups, repeated-measures analysis of variance (ANOVA) and a 3 (Lesion) × 6 (Session) design. A CR was defined as any response crossing threshold during the CS period. The average CR amplitude in this experiment was a magnitude measure that included non-CR trials (amplitude = 0) in the average. Post hoc Newman–Keuls analyses were performed as needed. Results were reported using a .05 significance level unless otherwise stated.

Delayed alternation. The percentage of correct responses was analyzed for the three lesion groups over five 12-trial blocks of training, also using a repeated-measures ANOVA. Post hoc Newman–Keuls analyses were performed as needed. Results were reported using a .05 significance level unless otherwise stated.

Histology

At the end of all behavioral testing, animals were overdosed with 3 to 4 cc of sodium pentobarbital, injected subcutaneously, and perfused intracardially with physiological saline followed by 10% formalin. The brains were removed and stored in 30% sucrose formalin for at least 1 week. The brains were then embedded in a 10% gelatin mold and stored in sucrose formalin an additional week before being sectioned on a freezing microtome. Every other 80-μm coronal section through the extent of hippocampal regions was collected and stained with cresyl violet for later lesion reconstruction.

Results

Of the 42 animals, 2 hippocampal and 2 cortical animals were excluded from analyses because histological results showed their lesions to be too small or too large, respectively, to be included with others in their groups. Effective lesions were those that damaged more than 50% of the dorso-ventral extent of hippocampus bilaterally but did not encroach on the thalamus medially. The overall development of hippocampal animals appeared delayed relative to the other groups in terms of body weight measures. Body weights at the beginning of eyeblink conditioning were analyzed using a three-groups ANOVA for the 7 hippocampal, 7 cortical, and 11 control animals whose eyeblink data are presented below. Body weights were 71.5 g (± 4.6 SEM), 65.4 g (± 4.3 SEM), and 56.1 g (± 3.1 SEM) for the normal, cortical, and hippocampal groups, respectively. The main effect of lesion was significant, F(2, 22) = 4.09, p < .05. Post hoc Newman–Keuls analysis revealed that although the cortical group did not differ significantly from either the hippocampal or normal group, pups in the hippocampal group had significantly lower body weights (p < .05) relative to normal controls.
Eyeblink Conditioning

Both normal controls and animals with cortical lesions demonstrated robust acquisition of the trace-conditioned eyeblink response. In contrast, animals with hippocampal lesions were markedly impaired on all measures of conditioning.

CR percentage: The final groups on which this and all other eyeblink analyses were based consisted of 7 animals with hippocampal lesions (3 males and 4 females), 7 with cortical lesions (4 males and 3 females), and 11 unoperated controls (6 males and 5 females). A total of 10 animals (5 hippocampal, 3 cortical, and 2 normal) were excluded from eyeblink analyses because of excessive noise in their EMG records during two or more conditioning sessions. A stepwise outlier analysis was performed on the overall percentage of CRs to exclude animals whose scores deviated from the rest of the group by more than 2 standard deviations. A total of 3 animals were excluded on this basis (1 normal [2.4 SD] and 2 hippocampal [2.9 and 4.9 SD]). 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 in Fig. 2A for all groups. There was a significant increase in CR percentage across sessions for the normal and cortical control groups, but the hippocampal lesion group was significantly impaired on trace eyeblink conditioning.

A 3 (Lesion) × 6 (Session) repeated-measures ANOVA confirmed that there was a significant interaction of Lesion × Session, $F(10, 110) = 4.23$, $p < .001$. There were also significant main effects of lesion, $F(2, 22) = 9.29$, $p = .001$, and session, $F(5, 110) = 74.85$, $p < .001$. Post hoc Newman–Keuls analysis of the lesion effect revealed that the hippocampal group was significantly impaired ($p < .01$) relative to both the cortical and normal control groups, which did not differ from each other. Further Newman–Keuls analyses indicated that although there were no differences between the groups on Sessions 1 and 2 of trace conditioning, the normal and cortical lesion groups produced significantly ($p < .01$) more CRs than did the hippocampal lesion group during the remaining four sessions. Cortical and normal groups failed to differ across sessions except for a transient decrement in the cortical group on Sessions 3 and 4. Although hippocampal animals

![FIG. 2](image-url) Mean (± SE) percentage CRs (A) and CR amplitude (B) during trace eyeblink conditioning as a function of lesion group and training session (Sessions 1–6). HIPP, bilateral hippocampal lesion; CTX, bilateral cortical lesion; NORM, unoperated control. Conditioned response amplitude was measured in arbitrary EMG units.
were significantly impaired on trace eyeblink conditioning relative to the control groups, hippocampal animals did demonstrate some conditioning, as indicated by a significant increase in the percentage of CRs from Session 1 to Session 6 (Newman–Keuls, \( p < .01 \)) for that group.

**CR amplitude.** Because there were no gender differences in CR amplitude, further analyses excluded gender as a factor. Mean CR amplitudes are presented by group in Fig. 2B and were similar to trends in CR percentage. Conditioned response amplitudes increased across sessions for the normal and cortical control groups, whereas there was significant impairment in amplitude across sessions for the hippocampal group.

A 3 (Lesion) \( \times \) 6 (Session) repeated-measures ANOVA indicated that although there was no main effect of lesion, \( F(2, 22) = 2.45, p = .11 \), there was a significant main effect of session, \( F(5, 110) = 21.9, p < .001 \), and a significant Lesion \( \times \) Session interaction, \( F(10, 110) = 2.69, p < .01 \). Post hoc Newman–Keuls analysis of the Lesion \( \times \) Session interaction indicated that on Session 1 there was no difference in CR amplitudes across lesion groups but that on Session 6 CR amplitudes for the hippocampal group were significantly lower than those for both the cortical (\( p < .01 \)) and normal groups (\( p < .01 \)). These differences emerged on Session 2 (hippocampal vs cortical: \( p < .01 \); hippocampal vs normal: \( p < .05 \)).

**Performance measures: UR and SR amplitude.** To verify that the CR effects observed above were due to conditioning rather than to CS or US efficacy or motor ability, we analyzed UR and SR amplitudes. Unconditioned response amplitudes were compared for all animals on Session 1 of conditioning. There were no main or interaction effects involving gender in the UR analysis. There were also no group differences in amplitude that could account for the reported differences between groups on CR measures. Amplitudes were 3.72 ± 0.76, 4.41 ± 0.89, and 3.24 ± 0.59 arbitrary EMG units for the hippocampal, cortical, and normal groups, respectively. A one-way between-groups ANOVA indicated that there was no significant effect of lesion (\( F < 1 \)) on UR amplitude.

The amplitude of short-latency responses to the tone was analyzed using a 3 (Lesion) \( \times \) 6 (Session) repeated-measures ANOVA. There was no main effect of lesion, but the main effects of session and Lesion \( \times \) Session interaction were significant, \( F(5, 110) = 7.60, p < .001 \), and \( F(10, 110) = 2.15, p < .05 \), respectively. Post hoc Newman–Keuls indicated that there was no difference in the amplitude of SRs on Session 1 (range = 17.1–23.8 arbitrary EMG units). By Session 6, there was significant growth in SR amplitudes for the control groups (reaching 98.0 and 105.2 arbitrary EMG units, respectively) relative to the hippocampal group, which did not change significantly (Session 6 amplitude = 30.1 arbitrary EMG units).

**Delayed Alternation**

**Percentage correct.** The percentage of correct choices over five blocks of delayed alternation training in the T-maze are presented by group in Fig. 3. There were 16 animals with hippocampal lesions, 8 with lesions of the overlying cortex, and 14 unoperated controls. (This analysis includes all animals in the data set regardless of their inclusion or exclusion in eyeblink analyses.) There were no main effects or interaction effects involving gender, so this factor was excluded from further analyses. Whereas animals in
the normal and cortical control groups learned the delayed alternation task and increased their percentage of correct responses over the course of training, animals with hippocampal lesions remained at chance levels throughout.

A 3 (Lesion) × 5 (Block) repeated-measures ANOVA revealed a significant Lesion × Session interaction, $F(8, 140) = 2.97, p < .01$, as well as significant main effects of lesion and session, $F(2, 35) = 82.73, p < .001$, and $F(4, 140) = 16.30, p < .001$, respectively. Post hoc Newman–Keuls analysis of the main effect of lesion yielded a significant impairment ($p < .01$) of the hippocampal group relative to the cortical and normal groups, which did not differ from one another. Block-by-block analyses show that although the three groups differed from one another on Block 1 (normal, cortical, and then hippocampal in descending order of percentage correct responses), the difference between the normal and cortical groups disappeared in Block 2 and throughout the rest of training. The hippocampal group was significantly impaired relative to the other two groups throughout delayed alternation training.

**Histology**

Cresyl violet stained coronal sections were used to reconstruct the hippocampal and cortical lesions for each animal. The typical hippocampal and cortical lesion are presented in Fig. 4. Hippocampal lesions typically included all of the dorsal hippocampus and extended to the ventral cortex, sparing only the most ventral aspects of the hippocampus. The corpus callosum and parietal cortex were also significantly damaged. Cortical lesions included the parietal areas affected in the hippocampal lesion and some damage to the corpus callosum.

**Discussion**

The findings of this experiment show that early hippocampal lesions do impair trace eyeblink conditioning on PND 25 relative to animals with lesions of the overlying cortex.
and unoperated controls. Normal and cortical animals demonstrated conditioning levels consistent with those observed previously in rat pups between 23 and 30 days of age (Ivkovich, Paczkowski, & Stanton, 2000). Unconditioned responses did not differ between lesion groups at the start of conditioning and cannot account for the differences in learning behavior. Interestingly, SR amplitudes, which are typically used to evaluate CS salience, did increase with conditioning. Because this outcome is typically not observed in delay conditioning, this increase in startle response may reflect a greater amount of orienting to the tone either as a result of trace conditioning or as a precursor for trace acquisition.

As was expected from animals with disruptions in septohippocampal development (Freeman & Stanton, 1991), performance on delayed alternation was dramatically impaired in the hippocampal group relative to cortical and unoperated controls. This finding demonstrated that the hippocampal lesions in this experiment profoundly impaired performance on this spatial learning task. That the hippocampal deficits in eyeblink conditioning were less dramatic suggests that this task is less sensitive to potentially incomplete disruptions in hippocampal function.

**EXPERIMENT 2**

There are two features of trace eyeblink conditioning that distinguish it from standard-delay eyeblink conditioning: the addition of a trace interval and the extension of the ISI (Fig. 1). To examine whether the conditioning deficits observed in Experiment 1 were unique to trace conditioning, Experiment 2 was designed to evaluate the effects of similar hippocampal lesions on delay eyeblink conditioning. In particular, this experiment compared the effects of hippocampal lesions on delay conditioning at two ISIs: the standard 280 ms and a long delay of 880 ms, comparable to the CS–US interval in trace conditioning in Experiment 1. A deficit in long-delay conditioning but not in standard-delay conditioning would suggest that the hippocampus plays a role in associations that take place over long CS–US intervals. The data from this experiment also enabled us to make comparisons between the long-delay group in this experiment and the trace conditioning group in Experiment 1. The hippocampal deficits in trace conditioning that we observed in Experiment 1 may be related either to the trace interval or to making associations over long ISIs in general. If this experiment yields a lesser effect of hippocampal lesions on long-delay conditioning, then it would suggest that the hippocampal deficits in Experiment 1 were unique to trace eyeblink conditioning and must be related to the presence of a trace interval in the paradigm.
Methods

Subjects. Subjects were 39 Long–Evans rat pups (20 males and 19 females from seven litters) balanced for sex across groups. No more than 1 same-sex littermate was assigned to any one condition. Animals were maintained and treated as in Experiment 1.

Surgery. Because cortical and normal controls performed similarly in the previous experiment, only hippocampal lesions were performed for animals in this experiment. Littermates served as normal unoperated controls.

Apparatus. The eyeblink conditioning apparatus in this experiment was the same as in Experiment 1.

Design and procedures. Animals were randomly assigned to one of four groups. These groups resulted from two lesion conditions (hippocampal or normal control) and two delay conditioning paradigms (standard delay [D280] or long delay [D880]). No more than 1 same-sex littermate was assigned to each of these groups. The training protocol consisted of six conditioning sessions (two sessions/day at 5-h intervals over 3 days). Paired CS–US acquisition trials were presented using parameters for standard-delay or long-delay conditioning (Fig. 1). For the standard-delay conditioning group, trials consisted of the same stimuli as those used for trace conditioning in Experiment 1: a 380-ms, 2.8-kHz, 90-dB tone CS and a 100-ms, 2-mA periocular shock US (Stanton et al., 1992) that overlapped and coterminated to produce a delay interval of 280 ms between CS and US onset. For the long-delay conditioning group, trials consisted of a longer tone (980 ms) that overlapped and coterminated with the same 100-ms periocular shock to produce an 880-ms delay interval matching the ISI of the trace procedure (Fig. 1).

Data analysis. Electromyographic signals were sampled in 2.5-ms bins during the 1000-ms trial epoch in the D280 group and in 3.5-ms bins during the 1400-ms trial epoch in the D880 group. Each trial epoch was divided into four time periods as described in Experiment 1.

Percentage of CRs and amplitude of CRs and URs were analyzed using a between-groups, repeated-measures ANOVA and a 2 (Lesion) × 2 (Group) × 6 (Session) design. Post hoc Newman–Keuls analyses were performed as needed. Results were reported using a .05 significance level unless otherwise stated.

Histology. Brain tissue was collected, treated, and reconstructed as in Experiment 1.

Results

As in Experiment 1, conditioning and body weight measures in this experiment suggested a developmental delay in the 16 hippocampal animals relative to the 15 unoperated controls. Body weights were 73.0 g (± 3.4 SEM), 72.8 g (± 3.5 SEM), 62.8 g (± 3.0 SEM), and 62.4 g (± 2.9 SEM) for the normal D280, normal D880, hippocampal D280, and hippocampal D880 groups, respectively. Body weights at the beginning of eyeblink conditioning were analyzed using a 2 (Lesion) × 2 (Group) ANOVA. There was a significant main effect of lesion (hippocampal < normal), $F(1, 27) = 10.02, p < .01$, but there was no significant interaction involving the group factor. A total of 4 pups that received hippocampal lesions did not survive to the eyeblink conditioning phase of this study. No pups were excluded on the basis of histology.
Another 2 (Lesion) × 2 (Group) ANOVA was run to compare body weights of the T500 animals from Experiment 1 and the D880 group from this experiment (which are compared behaviorally below). The ANOVA yielded a significant lesion effect (hippocampal < normal), \( F(1, 29) = 12.71, p < .01 \), but there was no difference between the delay and trace tasks, and there was no Lesion × Task interaction. Hence, the task differences observed between the D880 and T500 groups across experiments cannot be attributed to differences in the effect of the lesions on body weight.

As in Experiment 1, a stepwise outlier analysis was performed on the overall percentage of CRs to exclude 4 animals whose scores deviated from the rest of the group by more than 2 standard deviations (2 normal D880 and 2 hippocampal D880). The final groups on which this and all other eyeblink analyses were based consisted of 16 animals with hippocampal lesions (7 D880 and 9 D280) and 15 unoperated controls (8 D880 and 7 D280).

**CR percentage.** Because there were no gender differences in percentage of CRs, further analyses excluded gender as a factor. The percentages of CRs across sessions are presented in Fig. 5A for all groups. All animals demonstrated a significant increase in CR percentage across sessions, but the hippocampal-lesioned groups were significantly impaired on both D880 and D280 eyeblink conditioning relative to the normal control groups. The impairment was similar for both delay conditioning groups, but a comparison of the D880 group with the T500 group from Experiment 1 revealed a task dissociation as demonstrated by a greater impairment of trace conditioning relative to long-delay conditioning with hippocampal lesions (Fig. 5B).

A 2 (Lesion) × 2 (ISI) × 6 (Session) repeated-measures ANOVA on the data in Fig. 5A confirmed that there were significant main effects of lesion (hippocampal < normal), \( F(1, 27) = 9.21, p < .01 \), and ISI (D880 < D280), \( F(1, 27) = 24.73, p < .001 \), but there was no interaction (Lesion × ISI: \( F < 1 \)). The main effect of session was also significant, \( F(5, 135) = 165.74, p < .001 \). There was also a significant Lesion × ISI × Session interaction, \( F(5, 135) = 3.07 \). Post hoc Newman–Keuls of the three-way interaction revealed that the differences between the hippocampal and normal groups for the D280

![FIG. 5. Mean (± SE) percentage CRs as a function of lesion (H = hippocampal lesion, N = unoperated control), training session (Sessions 1-6), and conditioning paradigm. (A) Comparison of standard-delay (D280) and long-delay (D880) conditioning paradigms. (B) Comparison of long-delay (D880) and trace (T500 from Experiment 1) conditioning paradigms.](image-url)
condition were present from Session 1 and disappeared by Session 5. In contrast, a similar comparison between lesion groups for the D880 task showed that the two lesion groups performed similarly on Sessions 1 and 2, followed by a transient difference (hippocampal < normal) on Sessions 3 and 4, with the difference disappearing again on Session 5.

To directly contrast the effects of hippocampal lesions on delay versus trace eyeblink conditioning, a cross-experiment comparison between similar ISI groups was performed (Fig. 5B). The D880 groups in this experiment were compared to comparable T500 groups from Experiment 1. A 2 (Lesion) × 2 (Task) × 6 (Session) repeated-measures ANOVA indicated that the three-way interaction was not significant, \( F(5, 145) = 1.67, p = .14 \), but that there were significant interactions of Lesion × Task, \( F(1, 29) = 7.4, p = .01 \), and Lesion × Session, \( F(5, 145) = 8.41, p < .001 \). Main effects of lesion, task, and session were also significant, \( F(1, 29) = 24.22, p < .001; F(1, 29) = 4.12, p = .05; \) and \( F(5, 145) = 132.72; p < .001 \), respectively. Post hoc Newman–Keuls analysis of the Lesion × Session interaction indicated that it was a result of a difference between lesion groups \( p < .01 \) on all but Session 1. The Lesion × Task interaction occurred because there was no reliable difference between the hippocampal and normal groups during long-delay conditioning, but there was a significant difference between these groups during trace conditioning \( p < .01 \). These data, therefore, indicate that there was much greater impairment of trace conditioning following early hippocampal lesions as compared to long-delay conditioning.

**CR amplitude.** Because there were no gender differences in CR amplitude, further analyses excluded gender as a factor. Mean CR amplitudes are presented in Fig. 6A. CR amplitudes increased across sessions for all groups, although there was a much larger increase for the D280 groups than for the D880 groups. Compared to the D280 hippocampal and control groups, CR amplitudes were lower for the D880 groups regardless of lesion. Hippocampal lesions produced a greater impairment of CR amplitudes over sessions for the D280 group than for the D880 group, which showed little change across sessions.

As with CR percentage, a 2 (Lesion) × 2 (ISI) × 6 (Session) repeated-measures ANOVA on CR amplitude revealed significant main effects of lesion (hippocampal <

![FIG. 6. Mean (± SE) CR amplitude as a function of lesion (H = hippocampal lesion, N = unoperated control), training session (Sessions 1-6), and conditioning paradigm. (A) Comparison of standard-delay (D280) and long-delay (D880) conditioning paradigms. (B) Comparison of long-delay (D880) and trace (T500 from Experiment 1) conditioning paradigms.](image-url)
normal), $F(1, 27) = 4.12, p = .05$, ISI (D880 < D280), $F(1, 27) = 30.08, p < .001$, and session, $F(5, 135) = 49.70, p < .001$, and the Lesion $\times$ ISI interaction was marginally significant, $F(1, 27) = 3.14, p = .09$. The apparent difference in hippocampal effects on CR amplitude for the two different ISI groups was not confirmed by the three-way interaction, $F(5, 135) = 92, p = .46$, but Lesion $\times$ Session and ISI $\times$ Session interactions were again significant, $F(5, 135) = 2.35, p < .05$, and $F(5, 135) = 13.79, p < .001$, respectively. These interactions probably reflect the greater increase in CR amplitude across sessions for unoperated versus hippocampal-lesioned animals and the smaller change in CR amplitude across sessions for D880 versus D280 conditioning paradigms.

The D880 groups in this experiment were compared to the T500 groups in Experiment 1 (Fig. 6B). A 2 (Lesion) $\times$ 2 (Task) $\times$ 6 (Session) repeated-measures ANOVA revealed a significant three-way interaction, $F(5, 145) = 3.13, p < .05$. Main effects of lesion and session, but not of task, were also significant, $F(1, 29) = 7.91, p < .01; F(5, 145) = 51.63, p < .001$; and $F(1, 29) = 1.27, ns$, respectively. Post hoc Newman–Keuls analysis of the three-way interaction indicated that although there was no lesion-related difference in CR amplitude across sessions for the long-delay group, a difference between lesion groups emerged on Session 3 for the trace conditioning group. These data show that the greatest impairment in CR amplitudes was demonstrated by the hippocampal-lesioned animals during trace conditioning. Hippocampal lesions did not significantly impair CR amplitude in the long-delay conditioning group.

**Performance measures: UR and SR Amplitude.** As in Experiment 1, UR and SR amplitudes were used to determine whether the observed effects on learning measures for delay conditioning could be attributed to CS or US efficacy or motor ability. Unconditioned response amplitudes were compared for all animals on Session 1 of conditioning. There were no main or interaction effects involving gender in the UR analysis. There were also no group differences in UR amplitude that could account for the reported differences between groups on CR measures. Amplitudes, in arbitrary EMG units, were $5.81 \pm 0.72$ (hippocampal D880), $4.86 \pm 1.03$ (normal D880), $5.57 \pm 0.59$ (hippocampal D280), and $4.95 \pm 0.75$ (normal D280). A 2 (Lesion) $\times$ 2 (ISI) between-groups ANOVA on Session 1 UR amplitudes indicated that there were no significant main or interaction effects (all $F$s < 1).

Startle response amplitude was analyzed for the two delay groups using a 2 (Lesion) $\times$ 2 (ISI) $\times$ 6 (Session) repeated-measures ANOVA. There were no main effects or interactions involving lesion or ISI (all $F$s < 2.1). The main effect of session was significant, $F(5, 135) = 8.94, p < .001$, indicating that the amplitude of SRs increased across sessions for all groups. Session interactions were not significant (all $F$s < 1.4). Session 1 amplitudes averaged $4.3 \pm 1.0$ and grew to $34.4 \pm 7.6$ arbitrary EMG units by Session 6.

**Histology.** Cresyl-stained coronal sections were used to reconstruct the hippocampal lesions for each animal. Hippocampal lesions typically included all of the dorsal hippocampus and extended to the ventral cortex, sparing only the most ventral aspects of the hippocampus. The corpus callosum and parietal cortex were also significantly damaged. The typical hippocampal lesion presented for Experiment 1 in Fig. 4 is also representative of lesions in this experiment.


Discussion

Hippocampal lesions on PND 10 impaired delay conditioning at both 280-ms and 880-ms ISIs relative to unoperated controls. A comparison of the long-delay group from this experiment and the trace conditioning group from Experiment 1, however, showed that trace conditioning is differentially more affected by the hippocampal lesions. The UR and SR amplitudes did not differ systematically between tasks or between lesions and cannot account for the observed trends in learning measures. Because the trace and long-delay groups were matched for ISI, it suggests that the role of the hippocampus in trace conditioning is related to the trace interval and not the ISI.

GENERAL DISCUSSION

The effects of early bilateral hippocampal lesions on the acquisition of eyeblink conditioning in weanling rats were examined in two experiments. The paradigms used were: trace eyeblink conditioning with a 500-ms trace interval (Experiment 1) and delay eyeblink conditioning with either a 280-ms or an 880-ms ISI (Experiment 2). Together, these two experiments have demonstrated that hippocampal lesions on PND 10 impair trace eyeblink conditioning on PND 25 more than they do standard-delay (280 ms) or long-delay (880 ms) eyeblink conditioning. Impairment of acquisition was observed for hippocampal-lesioned animals on all tasks. However, the impairment in acquisition of trace conditioning was significantly greater than that for either of the delay groups. Although body weights were lower for hippocampal animals compared to unoperated controls, there were no differences in UR performance that could account for the differences in learning-related measures.

This dissociation between the effects of early hippocampal lesions on delay versus trace eyeblink tasks, in weanling rats at a stage of development when these tasks are just beginning to emerge, is generally consistent with the literature on similar lesions in adult animals (e.g., Moyer et al., 1990; Solomon et al., 1986). The lesion effect for developing organisms is less selective than that for adults, however, as some conditioning deficits were observed during delay conditioning in the rat pups that are not seen after similar lesions in adult animals. This outcome may reflect the age at which we evaluate the effects of the early lesion, the age at which the lesion is made, or perhaps a developmental delay associated with the lesion. To address the first possibility, there may be developmental differences between infants and adults such that the hippocampus plays a larger role in simple associative processes early in development but not during adulthood. If so, then testing at later stages of development after the same early lesion might reveal the adult pattern of lesion effects across tasks. Early exposure to drugs has been shown to disrupt simple associative learning early but not later in development, when only “higher order” learning is affected (e.g., Heyser, Chen, Miller, Spear, & Spear, 1990). On the other hand, it is also possible that this lesion produces different effects when it occurs early in development than when it occurs during later stages of development. Perhaps the early lesion of hippocampus produces secondary effects on the development of other brain systems. For example, in this case the lesion could somehow alter the development of the cerebellum. Previously, we have found that lesions of contralateral cerebellar cortex that do not impair delay conditioning when performed during adulthood, or even on PND
20, do impair conditioning when performed on PND 10. This impairment may be a result of secondary effects on the rubral projections that are sensitive to damage at this age (Freeman, Carter, & Stanton, 1995). Perhaps a similar principle applies to the case of early hippocampal damage in this study. If so, then a similar protocol of lesion at around PND 20 and testing at around PND 36 may result in the adult pattern of lesion effects across task. Finally, the lower body weights for hippocampal-lesioned animals suggest that growth and development in general may have been delayed for these animals. This is another factor that would prevent the lesion effect from being completely selective across these tasks. However, the present data still demonstrate a lesion by task dissociation between delay and trace conditioning. In light of our earlier study showing that long-delay and trace eyeblink conditioning have similar ontogenetic profiles, with both emerging later than D280 (Ivkovich, Paczkowski, & Stanton, 2000), the current dissociation between long-delay and trace conditioning indicates that a developmental delay cannot account for all aspects of the current findings. Although the factors responsible for nonspecific lesion effects are potentially interesting and warrant further study, the current findings indicate that trace conditioning is more severely impaired by early hippocampal damage than is either delay conditioning procedure.

Comparison of the trace and long-delay groups showed that trace conditioning was differentially more affected by the hippocampal lesions. Because these two groups are matched for ISI, it suggests that the role of hippocampus in trace conditioning is related to the trace interval and not the ISI. In our previous report on the ontogeny of long-delay and trace conditioning (Ivkovich, Paczkowski, & Stanton, 2000), we suggested that the similarity between the ontogenetic profiles of these two groups may mean that the delayed development of trace eyeblink conditioning reflects the long ISI and not the presence of a stimulus-free trace interval. We also speculated that slower cerebellar development could be “masking” the effects of hippocampal maturation. Those developmental data could not address the contribution of hippocampal development to the emergence of delay versus trace conditioning tasks. The current study extends our previous findings in two ways. First, it suggests that the hippocampus is more important to the development of trace eyeblink conditioning than to that of long-delay eyeblink conditioning and that the hippocampus is functionally mature before cerebellar development is able to support learning over long ISIs (Ohyama & Mauk, 2000; Perrett, Ruiz, & Mauk, 1993). Second, it confirms that the developmental delay in the emergence of long-delay and trace eyeblink conditioning that we observed previously is a feature of developmental changes in ISI functions that are not dependent solely on the hippocampus. Given the delayed alternation data confirming that all hippocampal animals were profoundly impaired on a spatial learning task, we can rule out the possibility of ineffective lesions producing the differences in deficits observed between behavioral conditions matched for ISI as well (greater impairment of T500 than of D880).

The eyeblink conditioning procedure may be valuable for the study of developmental disorders (Goodlett, Stanton, & Steinmetz, 2000; Stanton & Freeman, 1994). Eyeblink conditioning is a procedure with simple sensory and response demands that can be used similarly in humans and laboratory animals and over a wide range of ages and abilities (Ivkovich, Collins, Eckerman, Krasnegor, & Stanton, 1999; Ivkovich, Eckerman, et al., 2000). Eyeblink conditioning also has the special advantage that it can identify links between behavioral processes and underlying neurological systems. Recent research with
adult human clinical populations has demonstrated that eyeblink conditioning procedures can identify people with early-onset Alzheimer’s disease, autism, obsessive–compulsive disorder, and temporal lobe amnesia (for reviews, see Woodruff-Pak & Steinmetz, 2000a). We have recently adapted eyeblink procedures for use in 4- and 5-month-old human infants and are beginning to chart the normative developmental course of various conditioning paradigms in human infants as a complement to ongoing work in developing rat pups (Ivkovich et al., 1999; Ivkovich, Eckerman, et al., 2000). The hope is that, together, the human and rodent models of the early ontogeny of eyeblink conditioning can aid in the early identification of perinatal central nervous system injury. This identification is critical if available intervention strategies that promote recovery are to be effective. Our data indicate that trace eyeblink conditioning can identify cognitive deficits produced by early hippocampal damage at the time when this type of learning is first observed developmentally. This encourages the use of this paradigm in studies of human infants who may be at risk for damage to the hippocampal system.

In summary, early hippocampal lesions produce a deficit in trace eyeblink conditioning that appears at the time that performance on this task also develops. This lesion also produced a transient impairment of delay conditioning at short and long ISIs, indicating that lesion effects were less selective than similar lesions in adult animals. However, lesion effects on trace conditioning were more severe, suggesting that dissociations between behavior and underlying mechanisms can be identified using this rodent model of eyeblink conditioning during development. Trace conditioning may be useful for studying the functional development of the hippocampal system in rodents and humans. It may prove useful for the study of neonatal ischemic insults and for better detection and understanding of neurodevelopmental disorders in general.

REFERENCES


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