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Post-Training Excitotoxic Lesions of the Dorsal Hippocampus Attenuate Forward Trace, Backward Trace, and Delay Fear Conditioning in a Temporally Specific Manner

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ABSTRACT: The present study sought to determine whether post-training excitotoxic lesions of the dorsal hippocampus would disrupt retention of fear conditioned using a trace procedure. Rats were trained using one of six procedures. Forward trace conditioning consisted of 10 trials in which a 16-s tone conditional stimulus (CS) was followed by a 28-s stimulus-free trace interval and then a mild footshock unconditional stimulus (US). We used two forms of delay conditioning where the tone and footshock co-terminated. Short delay used a 16-s tone and long delay used a 46-s tone. Backward trace conditioning was the same as forward trace, except that the order of the CS and US was reversed. CS-only and US-only were similar to forward trace except that the footshock or tone, respectively, was eliminated. One day later, animals received either an N-methyl-D-aspartate (NMDA)-induced lesion of the dorsal hippocampus or sham surgery. One week later, the rats were tested for freezing to the tone in a novel context. The next day, they were tested for freezing to the original training context. Hippocampal lesioned trace conditioned rats showed significantly less freezing during the tone compared with their sham lesioned controls. The lesion did not affect freezing during the tone in delay conditioning, nor in the other training conditions. During the 1-min period after tone offset, there was a trend in all hippocampal lesioned animals toward a deficit in freezing, compared with their corresponding sham lesioned controls, although only short delay, forward and backward trace groups showed a significant deficit. Hippocampal lesions also attenuated contextual conditioning. Thus, the hippocampus is critical for the consolidation and/or expression of a trace fear conditioned stimulus. *Hippocampus 2002;12:495–504.*

KEY WORDS: learning; memory; consolidation; freezing; rats

INTRODUCTION

Pavlovian fear conditioning is a useful model for examining the neural substrates underlying learning and memory. In a typical delay fear conditioning procedure, a series of temporally contiguous tone–footshock pairings are given. The tone conditional stimulus (CS) comes to predict the footshock unconditional stimulus (US) and elicit an array of conditional responses (CRs), such as freezing (Bouton and Bolles, 1980), elevated blood pressure (Iwata and LeDoux, 1988), potentiated startle (Davis, 1986), enhanced eyeblink (Lam et al., 1996), and analgesia (Fanselow and Bolles, 1979). In addition, the contextual cues that are present during conditioning come to elicit these CRs.

It has been shown that the hippocampus is critical for the consolidation of fear to a context, but not to a discrete delay conditioned cue such as a tone (Selden et al., 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992). However, in Pavlovian trace conditioning, a stimulus-free "trace" interval is inserted between the CS and US, forming a temporally non-contiguous relationship between the two stimuli. In contrast to delay conditioning, trace conditioning to a discrete CS depends on an intact hippocampus. Most of the work relating the hippocampus and trace conditioning has used rabbit eyeblink conditioning (e.g., Moyer et al., 1990; Kim et al., 1995). More recently, however, investigators have begun using the fear conditioning paradigm in understanding the role of the hippocampus in trace conditioning (e.g., Crestani et al., 1999; Huerta et al., 2000; McEchron et al., 1998; McEchron et al., 2000). All of the investigations of trace fear conditioning thus far have examined pre-training manipulations of hippocampal function. Such procedures do not allow us to make a distinction between hippocampal involvement during acquisition, consolidation, and/or expression of the CR. Kim et al. (1995) used post-training lesions of the hippocampus after eyeblink conditioning to show that the hippocampus is specifically critical for the consolidation, but not expression, of trace eyeblink conditioning. Such a conclusion could not have been reached based on pre-training manipulations.

In addition, several of the studies looking at the role of the hippocampus in trace fear conditioning have used aspiration lesions (McEchron et al., 1998; Rawlins and

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Tanner, 1998). Hippocampal aspiration lesions often result in damage to adjacent structures, such as the dorsal thalamus, subicular complex, and cingulate gyrus, as well as fibers of passage through the region (Jarrard, 2001; Jarrard and Davidson, 1991). Compared with more selective neurotoxic lesions, aspiration lesions of the hippocampus have been shown to produce greater deficits in hippocampal-dependent tasks, presumably because of additional extrahippocampal damage (e.g., Jarrard and Davidson, 1991). Thus, it is important to consider whether selective damage to hippocampal neurons produced by neurotoxicity has a similar detrimental effect on trace conditioning, compared with deficits reported with more extensive aspiration lesions.

The purpose of the present study was to establish whether post-training neurotoxic lesions of the dorsal hippocampus, which severely attenuate fear conditioning to a context that has been paired with shock, attenuate trace fear conditioning to a tone CS. Such a finding would indicate that the hippocampus is critical for the consolidation and/or expression of fear conditioning to a discrete CS when a temporal gap is imposed between the CS and US.

MATERIALS AND METHODS

Subjects

A total of 112 experimentally naive male Long-Evans rats (approximately 130 days of age; average weight 467 g) born and raised in the Herbert L. Washington Psychology Department vivarium at UCLA were used for this experiment. Animals were housed in large stainless steel cages (8 rats/cage) before the experiment. At the start of the experiment, rats were individually housed in wire mesh cages, had ad libitum access to food and water, and lived on a 14:10-h light/dark cycle. All procedures occurred during the light cycle. Animals were handled for approximately 20 s each for the 5 consecutive days before conditioning and testing.

Surgery

Animals were anesthetized with 55 mg/kg sodium pentobarbital i.p. The animal's head was shaved and mounted into a standard stereotactic instrument. The scalp was incised and the skin retracted. The head was leveled by equating bregma and lambda in the dorsoventral plane. Four small holes were drilled into the skull (two per side), and a stainless steel injection cannula (28-gauge) was lowered according to the following coordinates measured from bregma: rostral sites (AP −2.8 mm; ML ±1.6 mm; DV −4.3mm from skull) and caudal sites (AP −4.2 mm; ML ±2.6 mm; DV −4.0 mm from skull). These coordinates were established according to Paxinos and Watson (1997), with correction for the strain and age of our animals. Sham surgery was similar except that no injection cannula was lowered, and no drug infused. The injection cannula was connected to a 10-μl Hamilton syringe, using clear polyethylene tubing; the syringe was mounted onto an infusion pump (Harvard Apparatus, South Natick, MA). N-methyl-D-aspartate (NMDA) (20 μg/μl; Sigma, St. Louis, MO), dissolved in phosphate-buffered saline (PBS), was infused across 4 min (0.1 μl/min), and the injector remained in place for an additional 5 min after the infusion at each site to allow for diffusion of the drug. After the last infusion, the incision was closed with stainless steel wound clips. The wound was wiped with alcohol and Betadine. Rats were placed on a heating pad until they awoke from anesthesia, at which time they were placed back into their home cages.

Apparatus

Eight similar conditioning chambers (28 × 21 × 21 cm) from Lafayette Instrument Co. (Lafayette, IN) were used for conditioning. The side walls of each chamber were made of aluminum. The front door, back wall, and ceiling were all made of clear Plexiglas. The floor of each chamber consisted of 18 stainless steel rods, separated by 1.5 cm, that were wired to a shock generator and scrambler (Med Associates). A stainless steel pan coated with a 60% citrus-scented air freshener solution (Smart and Final, Los Angeles, CA) was inserted under the grid floor in each box. The entire chamber was cleaned with an odorless 5% sodium hydroxide solution before the placement of each animal into the chamber for both conditioning and context testing sessions. Background noise (65 dB) was provided by a fan located inside the room. The overhead room lights remained on.

Tone testing took place in a completely separate context. Again, eight similar chambers were used (same dimensions as above). These chambers, however, had white Plexiglas triangular inserts (with each side of the triangle placed at a 60-degree angle from the floor). The inserts had holes drilled in them so that the speaker, located above each chamber, could be heard clearly. The floor of each chamber consisted of 17 stainless steel rods staggered, forming two rows 1cm apart vertically. Within each row, rods were located 2.6 cm apart. A stainless steel pan coated with a 1% acetic acid solution was inserted under each grid floor. Also, this solution was used to clean each chamber before placement of the animal into the chamber for the tone testing session. A white noise generator provided background noise (65 dB). The room lighting consisted solely of a 30-W red light located in a corner of the room across from the conditioning chambers.

Procedure

Rats were randomly assigned to one of six training procedures: trace, short delay, long delay, backward trace, US-only, or CS-only (Fig. 1). Trace consisted of a 16-s tone, followed by a 28-s stimulus-free trace interval, and then a footshock. Short delay (SD) used a 16-s tone that co-terminated with a 2-s footshock. Long delay (LD) used a 46-s tone that co-terminated with a footshock. The two different delay procedures were used to control for CS duration and inter-stimulus interval (ISI; CS-onset to US-onset). That is, in the short delay procedure the duration of the CS was equivalent to that used in trace conditioning. In the long delay procedure, the time between CS-onset and US presentation was equal to that used in the trace procedure. Backward trace (Bkwd) was the same as trace, except that the order of the CS and US was reversed. US-only and CS-only were similar to trace, except that the tone

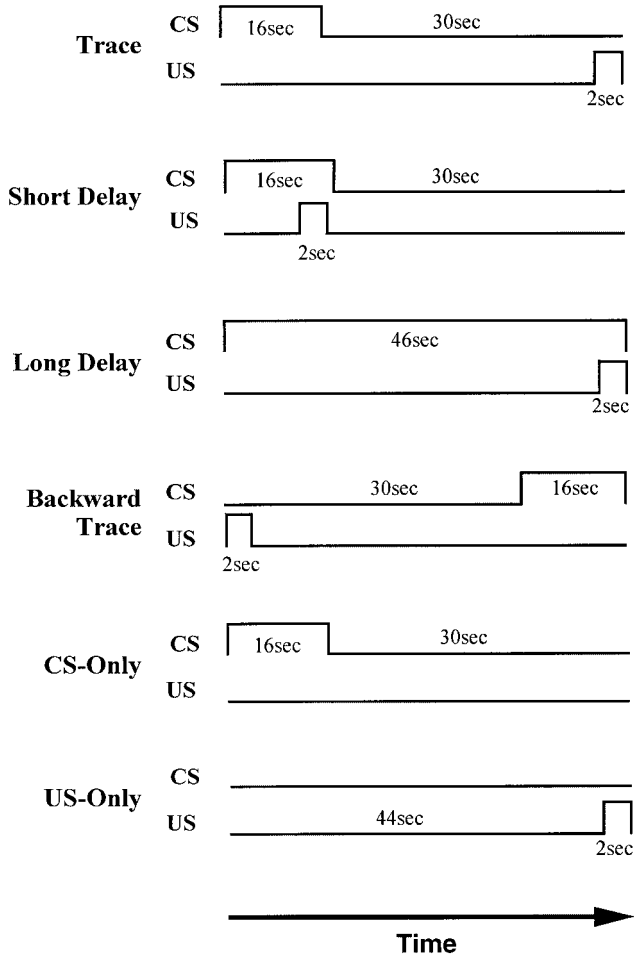


FIGURE 1. Schematic representation of the temporal relationships between conditional stimulus (CS) and unconditional stimulus (US) presentation for a single trial in each of the six training procedures used: trace, short delay, long delay, backward trace, CS-Only and US-Only.

(US-only) or footshock (CS-only) was eliminated. All tones were 2 kHz at 85 dB. All footshocks were 0.9 mA and 2 s in duration.

Animals were transported to the experimental room in a black plastic carrier (15 inches × 12 inches × 9 inches), separated into four equal compartments by an opaque black Plexiglas insert. The carrier and insert were rinsed with water, wiped with a 5% sodium hydroxide solution, and dried before placement of the rats for transport. Rats were placed into the conditioning chambers and, after a 192-s acclimation period, the first trial began. Ten training trials were delivered and were separated by 3.5 min for all groups except short delay, in which each trial was separated by 4 min in order to equate total training time for all groups. After the tenth trial, animals remained in the chamber for an additional 64 s before being transported back to their home cages.

One day later, one-half of the animals from each training condition received NMDA-induced lesions of the dorsal hippocampus, while the rest received sham surgery. After surgery, they were returned to their home cages. They remained in their home cages for 6 days without any further experimentation. The last 5 of the 6 days, they were individually handled in the vivarium for approximately 20 s/day.

On the seventh day after surgery, rats were again transported (in the same manner as above) to another experimental room and placed into the tone testing chambers, highly discriminable from the training chambers. After 192 s of acclimation, animals received 7 trials similar to the trace conditioning trials except that the shock was replaced by a 2-s stimulus-free interval. One-half of the lesion and sham LD conditioned animals received 7 trials similar to the LD conditioning trials except that the shock was eliminated. This was done so that half of the LD conditioned animals were tested similar to all other groups (i.e., with a 16-s tone) while the other half of the LD conditioned animals were tested using the same tone (46 s) that they received during conditioning. At 24 h after the tone test, animals were transported back to the original training context and placed in the chamber for 512 s of observation.

Histology

Upon completion of behavioral testing, animals were deeply anesthetized (75 mg/kg pentobarbital, i.p.) and were transcardially perfused with 0.9% saline, followed by 10% formalin. The brain was extracted and placed in 10% formalin. Two days before slicing, brains were transferred to a 10% formalin/30% sucrose solution. The brains were cut in 50-μm coronal sections using a cryostat at −16 ± 1°C. Every fourth slice (200 μm) was mounted on a microscope slide. Once dry, the sections were stained using thionin. Verification of lesion size and location was made on the stained slides by an observer blind to the treatment condition and behavior of the animal.

Behavioral Measure

Freezing behavior, defined as behavioral immobility, except for movement necessary for respiration (e.g., Fanselow, 1980), was used as the dependent measure. Freezing has previously proved a reliable measure of learned fear. An observer, blind to the condition of the animal, scored each rat as either freezing or not freezing every 2 s for the duration of the tone test and every 8 s for the duration of the context test. We used this finer sampling procedure during the tone test to allow for observations of temporal specificity surrounding the tone presentations. Precise temporal specificity during the context test is less critical, and the procedure used has proven to be sufficient and reliable. These observations were transformed into a percentage of observations spent freezing score by adding the number of times the animal froze during a given period of time, dividing it by the total number of observations taken during that time, and multiplying that value by 100.

RESULTS AND DISCUSSION

Histology

Photomicrographs of coronal sections from representative lesion and sham animals are shown in Figure 2. The extent of the

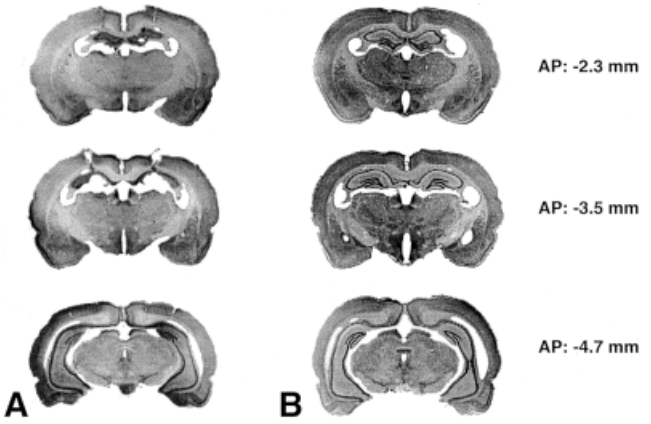


FIGURE 2. Photomicrographs of representative lesion (A) and sham (B) animals are presented. N-methyl-D-aspartate (NMDA)-induced damage was restricted to the dorsal region of the hippocampus.

lesions was similar to dorsal hippocampal NMDA-induced lesions previously produced in our laboratory (see Maren et al., 1997). Eight lesion animals were excluded from all analyses due to insufficient bilateral damage (n = 7) or extensive damage that included the ventral hippocampus (n = 1). For all statistical analyses, the number of animals in each condition is: LD-L lesion, 7; LD-L sham, 8; LD lesion, 6; LD sham, 8; SD lesion, 7; SD sham, 8; Trace lesion, 6; Trace sham, 8; Bkwd lesion, 7; Bkwd sham, 8; CS-Only lesion, 7; CS-Only sham, 8; US-Only lesion, 8; US-Only sham, 8.

Tone Test

Baseline freezing

Freezing was scored every 2 s across the 192-s acclimation period before the first tone presentation in a novel context. Figure 3A shows the mean (±SEM) percentage of observations spent freezing during these 96 observations for each condition. A one-way ANOVA reveals a significant main effect of condition [$F(13,90) = 2.37$, $P < 0.01$]. A priori planned comparisons ($P \leq 0.05$) between lesion and sham animals for each of the training procedures shows no statistically reliable differences related to dorsal hippocampectomy. Those training procedures that produce more freezing to the training context (see below) tend to produce more baseline freezing; there is a significant correlation between an animal's level of context freezing (training context) and its level of baseline freezing in the novel tone test context ($r = 0.38$, $P < 0.0001$). This indicates that there may have been some generalization between the training and tone test contexts.

Tone freezing

Freezing was scored every 2 s for the duration of each 16-s tone test presentation (8 observations per tone), except in the animals tested with the 46-s tone (LD-L), where there were 23 observations per tone. The average percentage of observations spent freezing during each tone was calculated. Responding across the first three tones was relatively stable for all conditions. Freezing to the tone gradually extinguished across tones 4–7 in many of the groups. Therefore, for all statistical analyses, the average of the first three tone test presentations was used.

Figure 3B shows the mean (±SEM) percentage of observations spent freezing during the three tones for all conditions. A one-way ANOVA reveals a significant main effect of condition [$F(13,90) = 6.36$, $P < 0.0001$]. A priori planned comparisons ($P \leq 0.05$) of all lesion animals to their corresponding sham controls reveals a reliable effect of dorsal hippocampectomy in the trace conditioned animals only ($P < 0.008$). The low level of residual responding (<20%) may result from spared tissue within the hippocampus. All other lesion animals do not differ significantly from their corresponding shams.

We employed three control procedures (backward trace, CS-Only, US-Only) that were expected to produce low levels of freezing. Backward pairings of a CS and US typically produce less excitatory conditioning than do forward pairings (e.g., Siegel and Domjan, 1971), and we believed that the addition of a temporal interval between the backward paired US and CS would further minimize any excitatory conditioning to the CS that might occur. The CS-Only and US-Only procedures were used to control for nonassociative learning including sensitization and pseudoconditioning. To assess any potential contribution of such nonassociative factors, those sham groups that were expected to produce substantial levels of associative conditioning (LD-L, LD, SD, and trace) were collapsed and compared with each of the three sham control groups (Bkwd, CS-Only and US-Only) using post hoc comparisons with Bonferroni corrections [overall $P < 0.05$]. Each of the sham control groups show significantly less freezing compared with the collapsed sham associative conditioning groups.

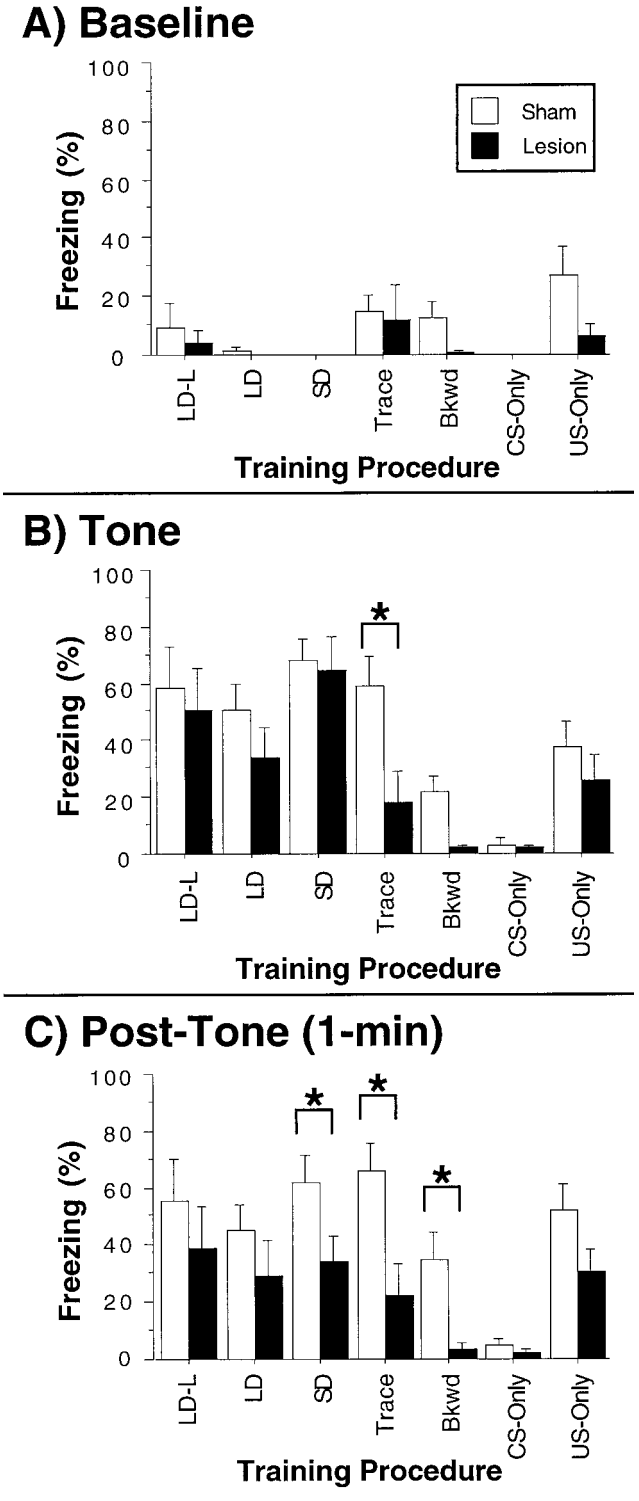
Post-tone freezing

Freezing was scored every 2 s during the 1-min period after each of the tone presentations. This period is important because it encompasses the time at which trace conditioned animals would have received footshock during training. The average percentage of observations spent freezing during the 1-min period after each tone was calculated. Because of the gradual extinction of responding during test tones 4–7 in many of the groups, the average of the 1-min periods after the first three tone presentations was used for all statistical analyses.

Figure 3C shows the mean (±SEM) percentage of observations spent freezing during the 1-min post-tone period for all conditions. A one-way analysis of variance (ANOVA) reveals a significant main effect of condition [$F(13,90) = 4.93$, $P < 0.0001$]. A priori planned comparisons ($P \leq 0.05$) between lesion and sham animals for each training condition show reliable deficits in the trace ($P < 0.005$), backward trace ($P < 0.04$), and short delay ($P = 0.05$) conditioned animals only.

An analysis of the sham control groups, similar to that performed on the tone data, was employed to assess potential nonassociative contributions to performance during the 1-min post-tone period. Those conditions expected to result in substantial associa-

tive conditioning (LD-L, LD, SD, and trace) were collapsed and compared with each of the three control groups (Bkwd, CS-Only and US-Only). Post hoc comparisons with Bonferroni corrections (overall $P < 0.05$) show that both Bkwd and CS-Only sham controls freeze significantly less than the collapsed delay and forward trace sham groups. However, US-Only controls do not differ significantly from the collapsed delay and forward trace groups. Thus, one might argue that this post-tone freezing is a pseudo-conditioned response resulting from simply receiving footshock previously. While this is possible in the present study, it seems unlikely. First, hippocampal lesions did not affect freezing in the US-Only animals during this period. Additionally, with slightly different conditioning and testing procedures, we have seen significantly higher tone and post-tone freezing in both delay and trace conditioned animals, compared with US-Only controls (J.J. Quinn and M.S. Fanselow, unpublished observations).



Overall Timecourse

Figure 4 shows the percentage of time spent freezing for both lesion and sham animals in each training condition across the entire timecourse of a trial. The data are averaged across the entire timecourse for each of the first three tone presentations. The averages of the three 16-s tones are plotted, except for the LD-L condition, where responding to the 46-s tones is broken into three blocks: first 16 s, middle 14 s, and last 16 s. The inter-trial interval periods are averaged across the time after each of the three tone presentations and broken into 20-s time blocks.

Context Freezing

The day after the tone test, rats were returned to their original training contexts. Freezing was scored every 8 s for a total of 512 s (64 observations per rat). Figure 5 shows the mean (±SEM) percentage of time spent freezing during the context test for all conditions. A one-way ANOVA shows a significant main effect of condition [$F(13,90) = 4.46$, $P < 0.0001$]. There is a trend toward a deficit in context freezing in all lesion animals compared with their corresponding sham controls. This is consistent with previous research indicating that post-training NMDA-induced lesions of the dorsal hippocampus attenuate contextual fear conditioning (Maren et al., 1997). However, a priori planned comparisons ($P \leq 0.05$) between lesion and sham animals for each training condition reveal that only the hippocampal lesion deficits in LD-L ($P < 0.001$), Trace ($P < 0.003$), and US-Only ($P < 0.004$) reached statistical significance, presumably because of the relatively

FIGURE 3. Mean (±SEM) percentage of observations spent freezing during the tone test for hippocampal lesion and sham animals across each of the seven training/testing procedures used. One-half of the animals trained with the long delay procedure were tested with the same 46-s tone that was used in training (LD-L), while the other half was tested with the same tone that all other groups were trained and tested with (LD). A: Baseline freezing during the first 192 s of the tone test, before presentation of the first tone. B: Tone freezing averaged across the first three tone presentations. Hippocampal lesioned trace conditioned rats show attenuated levels of freezing during the tone compared with their sham lesioned controls. Hippocampal lesions do not produce a deficit with any of the other training procedures. C: Post-tone freezing averaged across the 1-min periods after the first three tone presentations. Hippocampal lesions significantly attenuate freezing in short delay (SD), trace and backward trace (Bkwd) conditioned animals, relative to their sham lesioned controls. While there is a trend toward a hippocampal lesion deficit in the other groups as well, these deficits are not statistically reliable.

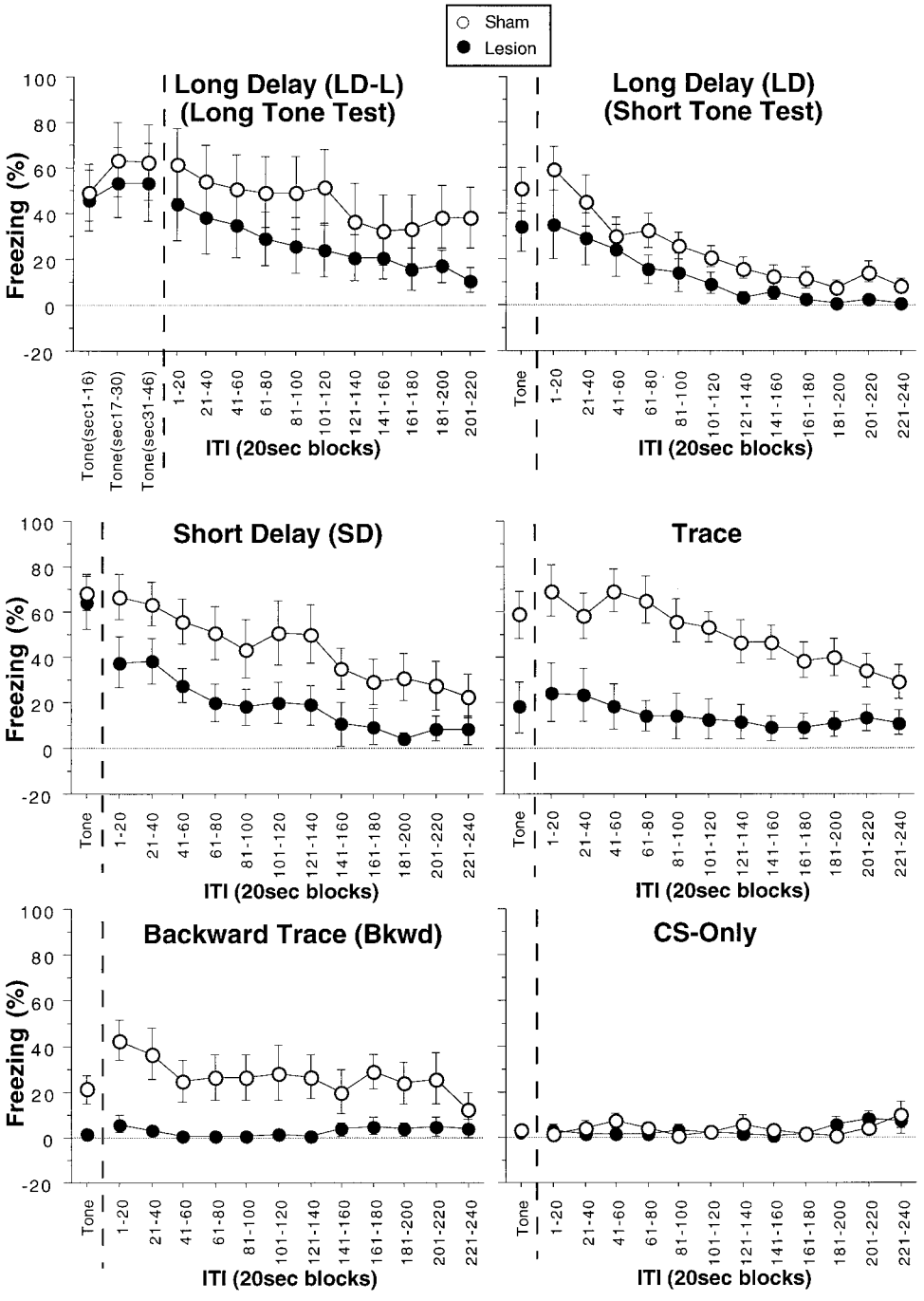


FIGURE 4. The overall timecourses averaged across the first three tone test trials and intertrial intervals (ITIs) for each of the training/testing procedures are shown. It does not appear that timing processes significantly contribute to the level of freezing in any of the training/testing conditions.

low levels of context freezing in sham animals. These low levels of context freezing are not entirely unexpected given that the tone may have "overshadowed" the training context in acquiring associative strength (Pavlov, 1927). However, we would have expected higher levels of context freezing in the backward animals since the tone did not become a good predictor of shock in this procedure.

GENERAL DISCUSSION

The present findings indicate that the hippocampus is necessary for trace fear conditioning to an auditory CS. During the tone CS, excitotoxic lesions of the dorsal hippocampus produced a deficit in trace, but not delay, fear conditioning. This is in accord with

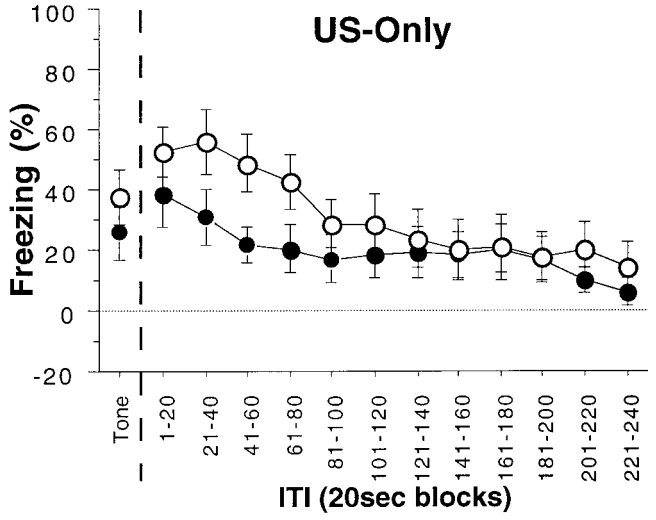


FIGURE 4. (Continued)

previous research in rats (e.g., McEchron et al., 1998), rabbits (e.g., McEchron et al., 2000) and humans (e.g., Clark and Squire, 1998). In the control groups (Bkwd, CS-Only, and US-Only), sham animals showed considerably less freezing during the tone than the delay and trace conditioned sham animals, indicating that the levels of freezing observed in these latter groups were the result of associative fear conditioning to the tone. However, there were higher levels of freezing in the Bkwd and US-Only shams, compared with the CS-Only sham group. This suggests that while there may have been some conditioning to the backward trace CS, it is possible that this effect is due to pseudo-conditioning that resulted from receiving footshock during training. The elevated level of tone freezing in the US-Only animals suggests that pseudo-conditioning may have contributed to, but cannot fully account for, the levels of conditioning in the delay and forward trace groups.

The period of time immediately after the tone is critical in the analysis of trace conditioning. We chose to analyze a 1-min period after tone offset because it fully surrounds the time at which trace conditioned animals received footshock during training (28–30 s). As Figure 3C indicates, there was persistence in freezing during this period in all groups (except CS-Only). Lesions of the dorsal hippocampus attenuated responding during this period across all of these groups, although only trace, SD and Bkwd showed statistically reliable deficits. The lesion deficit might be expected in the trace conditioned animals, since this period is more analogous to the time at which "adaptive" hippocampal-dependent eyeblinks occur in trace eyeblink conditioning (i.e., around the time of the expected US).

However, the lesion deficits observed in the Bkwd and SD conditioned animals were quite surprising. Why should the Bkwd conditioned animals, which were expected to show virtually no conditioning to the CS, actually show an increase in freezing after tone offset? In addition, why should the freezing observed in delay conditioned animals persist long after tone offset? Finally, why might this freezing after tone offset be hippocampal dependent in the SD and Bkwd conditioned animals? There are several possible explanations for these rather surprising findings. First, it is conceivable that the backward trace pairing, in essence, acts as a very long forward trace procedure. That is, during training each tone (except the last) is followed 3.5 min later by a footshock (3.5-min "trace interval"). Each "trial", then, is separated by a very brief 28-s interval. While this explanation is plausible, 3.5 min is a very long trace interval. Also, given this long forward trace scenario, these rats receive an "unsignaled" initial shock, followed by 9 trials of forward trace conditioning with a very long (3.5-min) trace interval and a very short ITI, and then an additional tone that is never paired with shock. All these factors would be expected to reduce any forward conditioning. Still, although this explanation might be capable of explaining why there is excitatory responding associated with the CS in the backward trace conditioned animals, it does not explain the persistence in freezing after tone offset that is observed in the delay conditioning groups.

There is another possible explanation for the persistence in post-tone freezing in the trace, backward trace, and delay conditioned groups that can be arrived at from the assumption that the tone and context not only become associated with shock during training, but also become associated with one another (i.e., context–CS associations; see Holland and Bouton, 1999). During the tone test, which is done in a different context, presentation of the tone that was used during conditioning may retrieve the memory of the training context. There is support for this interpretation in Rudy and O'Reilly's (2001) tests of their conjunctive representation theory, where presentation of individual elements contained in the original training context appears to result in retrieval of the entire memory representation of the context as a whole. In our study, the tone may act as an "element" of the training context. Therefore,

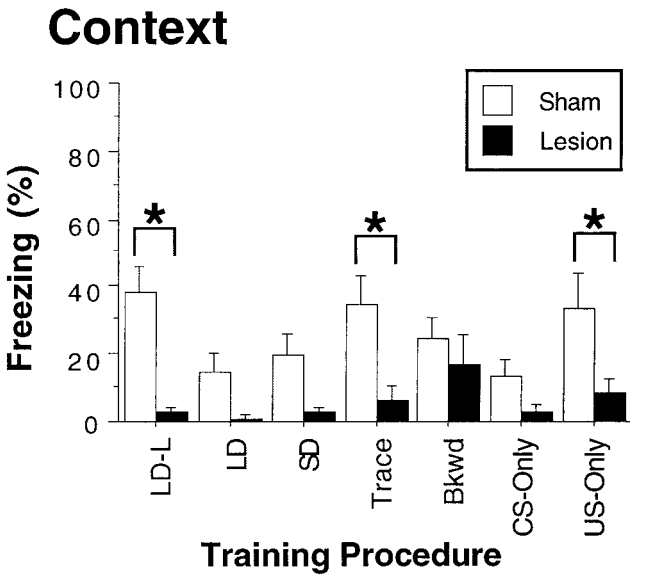


FIGURE 5. Mean (±SEM) percentage of observations spent freezing during the 8-min context test in both lesion and sham animals across the seven training/testing procedures. There are significant differences between lesion and sham animals in the LD-L, trace, and unconditional stimulus (US)-Only groups only.

presentation of the tone in another context may promote a fear response elicited by the memory of the training context. Based on our knowledge of the critical role for the hippocampus in contextual fear conditioning, we might expect freezing promoted by this tone association with the training context to be hippocampal dependent. In addition, this presumed association between the context and tone during acquisition may provide a mechanism by which the trace-conditioned tone becomes associated, after 28 s, with footshock. Since the tone and context are presumably associated, it is possible that the context is capable of maintaining a memory of the tone throughout the trace interval so that this tone memory can then be associated with footshock when it occurs.

Alternatively, it is possible that an element contained in a training session may be capable of retrieving a memory of the entire conditioning episode or event. The difference between the episodic and contextual interpretations is that the episodic memory contains information about the time and events contained in training, as well as the place where training occurs. Squire (e.g., 1992) and others have asserted that such episodic memory is dependent on the hippocampus, particularly during the retrieval of such memories (Eldridge et al., 2000). Therefore, according to this interpretation of the post-tone freezing data, a hippocampal lesion deficit might be expected.

In the present data, there appears to be no indication of a timed freezing response across any of the training conditions. Many investigators have suggested that animals have the ability to encode the temporal relationships between stimuli and some have suggested that it is these relationships which form the basis for Pavlovian conditioning (e.g., Church, 1978; Gallistel, 1990). For instance, applying a temporal hypothesis to the present data, one would expect to observe a peak in responding around 28-30 s after tone offset in trace conditioned animals, and an increase in freezing at the end of the ITI in backward trace conditioned animals. However, this is not what we observed (Fig. 4). Figure 4 shows the data collapsed into 20-s time bins, but we also see a similar pattern when looking at the data in smaller time bins (i.e., no peaks in responding during the ITI). There are at least two reasons why a timed freezing response may not have been observed in these data. First, it is possible that ten acquisition trials are too few for a precise temporal pattern to evolve (as is true in eyeblink conditioning). In contrast, it is possible that such a precise temporal pattern in the freezing response would never evolve, even after extensive training. Ethologically, it may not be advantageous to "time" the freezing response to a threatening stimulus. In many predators of rodents, movement serves as a releasing stimulus for attack. Thus, it is imperative to the animal's survival that it respond immediately upon detection of the threat and continue with that response until the threat becomes less imminent (see Fanselow and Lester, 1988). However, the lack of a temporal pattern in the freezing response does not preclude the existence of temporal encoding of the stimulus relationships, it simply suggests that these temporal relationships are not the sole basis for Pavlovian fear conditioning.

While the present data are in accord with other findings showing that delay conditioning to a tone CS does not depend on the hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992), Maren et al. (1997) reported that lesions similar to those used in the present study produced a hippocampal deficit in delay conditioning. There is an important difference between the Maren et al. study and the one presented here. During the tone test, Maren et al. used a single, prolonged exposure to tone (very different from the tone used during training) and assessed freezing throughout. The present study, on the other hand, used multiple, discrete presentations of the tone (same as the tone used during training). While we did not find a hippocampal lesion deficit during the tone in any of the delay conditioned animals, we did find a significant deficit in the short delay lesioned animals during the 1-min period after the tone. Since Maren et al. (1997) used a long tone test procedure that encompassed the time period during which we found a post-tone deficit, the deficits observed across the two studies may be due to a similar process. In support of this, Maren et al. (1997) did not observe a hippocampal deficit during the first 10 s of the tone test (period comparable to their training tone). The authors suggested that the deficit might reflect a failure to generalize from the short training tone to the longer test tone. While that is certainly still a possibility in their study, it is not a possible explanation for the lesion deficit that we have observed here in our SD animals, since our training and testing tones were identical.

All previous investigations of the role of the hippocampus in trace fear conditioning have used pre-training hippocampal manipulations. However, such manipulations do not allow one to distinguish between effects on acquisition, consolidation, and/or expression. The present findings indicate a critical role for the hippocampus in the consolidation and/or expression of trace fear conditioning to an auditory CS, since all rats had an intact hippocampus at the time of acquisition. This study did not attempt to distinguish between consolidation and expression. We suspect that this attenuation of trace fear conditioning by hippocampal lesions is actually a deficit in consolidation and not expression based on such findings in trace eyeblink conditioning (Kim et al., 1995) and contextual fear conditioning (Kim and Fanselow, 1992; Anagnostaras et al., 1999). In both tasks, hippocampal lesions made shortly after training (1-day) severely attenuated conditioned responding, while lesions produced long after training (28–50 days) had no effect on such responding. This is generally thought to indicate that at short training-to-lesion intervals, the memory has not yet been consolidated in extrahippocampal structures (presumably neocortex). At longer training-to-lesion intervals, the memory has been fully consolidated and therefore the hippocampus is no longer necessary. If the hippocampus were required for expression, lesions should produce a deficit at all training-to-lesion intervals.

Recently, Squire and colleagues (e.g., Clark and Squire, 1998; 1999; Manns et al., 2000) have begun exploiting human trace eyeblink conditioning to investigate a role for "awareness" of the stimulus contingencies in memory formation. Clark and Squire (1998) showed that amnesic patients with damage to the hippocampal system were unable to acquire trace eyeblink conditioning and remained unaware of the relationship between the tone CS and air-puff US, yet delay eyeblink conditioning was acquired normally. In addition, all subjects were distracted from the eyeblink conditioning session by simultaneously watching a silent movie. After conditioning, normal volunteers were divided into two groups based on their performance on items in a questionnaire

related to knowledge of the stimulus relationships. Those deemed "unaware" of the stimulus relationships also showed a deficit in the acquisition of trace conditioning, compared with those that were "aware" of the contingencies. Yet both groups of volunteers performed equally well on delay eyeblink conditioning.

It is conceivable that this declarative knowledge of the stimulus contingencies serves to bridge the temporal gap between the CS and US in trace conditioning. Explicit maintenance of an active representation of the CS during the trace interval may serve to bridge the temporal gap between the CS and US and allow them to become associated. Our data, along with the data of Kim et al. (1995), suggest that the role of the hippocampus in trace conditioning cannot be solely to acquire declarative knowledge of the stimulus contingencies during memory formation. Since post-training hippocampal lesions also produce a dramatic deficit in trace conditioning, a mnemonic role for the hippocampus is indicated. Thus, if declarative knowledge of the stimulus contingencies is important during the acquisition of trace conditioning, it appears that such knowledge also may be necessary for the consolidation of trace conditioning after acquisition.

We employed several delay conditioning groups in the present design in order to control for potential CS duration and ISI effects during acquisition and testing. There exists an extensive behavioral literature indicating relatively subtle effects of CS duration manipulations, while differences in the ISI can have drastic effects on conditioning (e.g., Kamin, 1965). In addition, the inter-trial interval (ITI) can play an important role in conditioning (e.g., Kaplan, 1984), and therefore should be kept constant across the experimental (e.g., trace) and control (e.g., delay) groups within an experiment. Finally, the total time spent in the conditioning context should be held constant since this will have an effect on context conditioning and differential levels of context conditioning may result in differential responding to discrete CS through stimulus competition (see Rescorla and Wagner, 1972). Thus, in order to control for all these factors within a single control group, one could argue that the most appropriate delay conditioning comparison group in investigations of trace conditioning is one in which the ISI is equivalent to that used in the trace conditioning procedure. Unfortunately, this delay conditioning control (our long delay conditioning procedure) is rarely employed in such investigations. In addition, one must choose an appropriate means of testing conditioned responding after acquisition. We chose to test our long delay conditioned animals with either the 46-s tone that they were trained with (LD-L) or the 16-s tone which was equivalent to the tone that the trace conditioned animals were trained and tested with (LD). Although there does not appear to be a large difference in these groups of animals, we propose that the short 16-s tone is superior for testing because it entirely equates testing conditions used in the trace conditioned animals.

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