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Differential Effects of Training Intertrial Interval on Acquisition of Trace and Long-Delay Fear Conditioning in Rats

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Many factors govern conditioning effectiveness, including the intertrial interval (ITI) used during training. The present study systematically varied the training ITI during both trace and long-delay fear conditioning. Rats were trained using one of six different ITIs and subsequently tested for conditioning to the white noise conditioned stimulus (CS) and the training context. After trace conditioning, percent freezing to the CS was positively correlated with training ITI, whereas percent freezing to the context was negatively correlated with training ITI. In contrast, when rats were trained using a long-delay paradigm, freezing during the CS test session did not vary as a function of training ITI; rats exhibited robust freezing at all ITIs. The long-delay conditioned rats exhibited relatively low levels of freezing during the context test. Thus, trace is more sensitive than long-delay fear conditioning to variations in the training ITI. These data suggest that training ITI is an important variable to consider when evaluating age or treatment effects, where the optimal ITI may vary with advancing age or pharmacological treatment.

Keywords: trace conditioning, freezing, intertrial interval, cue, context

Pavlovian fear conditioning paradigms are ideal for studying the cellular and molecular mechanisms of rapid forms of learning and memory. Pairing a neutral or innocuous conditioned stimulus (CS) with an aversive unconditioned stimulus (US) results in the expression of conditioned fear to both the cue CS and the background training context (Fendt & Fanselow, 1999; LeDoux, 2000). The two most common variants of Pavlovian fear conditioning to a cue CS are the delay and trace paradigms. In delay conditioning, the CS and US are presented such that onset of the US occurs either during or immediately following termination of the CS whereas in trace conditioning, a stimulus-free trace interval is interposed between CS offset and US onset. The differences between delay and trace paradigms may seem trivial; however, the introduction of a trace interval results in recruitment of other brain structures that are not required for acquisition of a delay paradigm. For example, acquisition of delay fear conditioning to a tone or white noise CS requires intact amygdalar and brainstem structures but does not depend on hippocampus or other cortical structures (Fanselow & Poulos, 2005; LeDoux, 2000; Thompson, 2005). In contrast, acquisition and expression of trace fear conditioning requires an intact hippocampus and prefrontal cortex (Blum, Hebert, & Dash, 2006; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Quinn, Oommen, Morrison, & Fanselow, 2002; Runyan, Moore, & Dash, 2004). Higher cortical structures (such as hippocampus, prefrontal cortex, and perirhinal cortex) are likewise implicated in the acquisition and expression of fear to the training context (Anagnostaras, Gale, & Fanselow, 2001; Anagnostaras, Maren, & Fanselow, 1999; Bucci, Phillips, & Burwell, 2000; Burwell, Bucci, Sanborn, & Jutras, 2004; Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Runyan et al., 2004; Sacchetti, Lorenzini, Baldi, Tassoni, & Bucherelli, 1999).

How well an animal learns a Pavlovian conditioning paradigm (conditioning effectiveness) is governed by a number of factors, including the time between the offset of the US and onset of the CS of the next trial (termed the intertrial interval or ITI). As a general rule, cue conditioning is better when the training trials are temporally spaced (i.e., a long ITI) than when they are massed (i.e., a short ITI). The first study to evaluate the effect of trial spacing on acquisition of eyeblink conditioning demonstrated that human subjects conditioned better when trained using 3 trials per minute compared with 9 or 18 trials per minute (Calvin, 1939). Spence and Norris examined the effects of using one of four different ITIs (9, 15, 30, and 90 s) on acquisition of the human conditioned eyeblink response and found that the 9-second ITI group gave consistently fewer responses than the 90-second ITI group (Spence & Norris, 1950). Similarly, Prokasy and colleagues used three different ITIs (15, 45, and 135 s) and found in human subjects that the longest ITI was associated with better eyeblink conditioning (Prokasy, Grant, & Myers, 1958). These and other recent studies provide evidence for the benefits of spaced learning over massed learning (Brelsford & Theios, 1965; Fanselow & Tighe, 1988; Gibbon, Baldock, Locurto, Gold, & Terrace, 1977; Prokasy et al., 1958; Salafia, Mis, Terry, Bartosiak, & Daston, 1973; Spence & Norris, 1950).

Little to no work has been done evaluating the ITI function in Pavlovian fear conditioning and specifically in trace fear conditioning. One study used three different ITIs (15, 60, and 900 s) and found that rats trained in a delay paradigm exhibited an increase in

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freezing to the CS as a function of the training ITI, with maximal freezing at the longest ITI (Barela, 1999). Another group looked at the effects of six ITIs (15, 45, 75, 105, 165, and 225 seconds) on cue and context conditioning using an escape-from-fear paradigm (McAllister, McAllister, & Weldin, 1974). They found optimal cue and context conditioning when the animals were trained with longer ITIs than with short ITIs. However, others have reported qualitatively different effects of trial spacing on fear conditioning to a cue CS (McNally & Westbrook, 2006; Yeo, 1976). For example, Yeo (1976) trained rats on a conditioned suppression paradigm using one of four different ITIs (30, 60, 180, and 360 seconds) and reported an inverted U-shaped ITI function with optimal cue conditioning at the 60-second ITI. Likewise, McNally and Westbrook (2006) trained rats on a 2-trial delay fear conditioning paradigm using a short (2 min) or long (24 hr) ITI. During the ITI, however, all rats were removed from the training chamber and placed in their home cage. They found that rats trained using a 24 hr ITI exhibited poor freezing to the CS compared to rats trained using a 2-min ITI suggesting an inverse relationship between ITI and cue conditioning. Although the effects of ITI on acquisition of trace fear conditioning have not yet been explored, experiments using trace autoshaping suggest that pigeons trained with shorter ITIs failed to learn the CS–US relationship whereas pigeons trained with the longest ITIs successfully acquired the autoshaping response (Kaplan, 1984).

In the present study, we examined the effects of varying the training ITI on acquisition of trace and long-delay fear conditioning in adult rats. The two main purposes of this study were to determine if longer ITIs enhanced either or both cue and context fear conditioning, and if there were any differences in the ITI function between rats trained using trace and long-delay fear paradigms. Understanding the parameters that govern Pavlovian fear conditioning will aid in evaluating the effects of aging or drug studies on learning. The ITI that is optimal may vary with age of the animal or with pharmacological treatment. Portions of these data have been presented in abstract form (Detert & Moyer, 2004).

Method

Subjects

Subjects were 185 adult male Sprague–Dawley rats (mean age = 3.35 ± 0.1 months, mean weight = 366 ± 3 g). Subjects were maintained on a 14 hr light–10 hr dark cycle and housed individually with free access to food and water. All procedures were conducted in accordance with the University of Wisconsin–Milwaukee animal care and use committee (ACUC) and NIH guidelines.

Apparatus

Fear conditioning chambers. Fear conditioning occurred in four identical Plexiglas and stainless steel chambers (28 × 20.5 × 21 cm; Med Associates, Fairfield, VT), each located in separate sound-attenuating boxes. The chambers had a standard grid floor consisting of 18 parallel steel rods (5 mm diameter and 12 mm spacing). The grid floor of each chamber was connected to a Model 700 Grason-Stadler shock generator (West Concord, MA) for delivery of a scrambled footshock US. Within each sound-attenuating box was a ventilation fan that produced a constant background noise of about 63 dB (measured by a sound level meter, A scale; model #33–2050, Realistic, Fort Worth, TX). Each sound-attenuating box was illuminated by an overhead white light (7.5 W) and contained a transparent Plexiglas front door that allowed recording of animal behavior by a remote GBC video camera (model #CCD-405; CCTV Corp., South Hackensack, NJ), which was connected to a VCR for data backup and off-line analysis. A PC computer running custom software (Med PC, Med Associates, Fairfield, VT) was used to control all stimulus presentations. For some experiments, each chamber contained a video camera connected to a PC computer running FreezeScan 1.0 software (Clever Sys, Inc., Reston, VA), which was used to control stimulus presentations and digital acquisition of the video data for offline analysis. In each case, data were hand-scored as described below. The fear conditioning chambers were wiped with a 5% ammonium hydroxide solution before each training session. During training, the room lights were left on for the entire session.

Auditory CS testing chambers. Four additional Plexiglas and stainless steel chambers (28 × 25 × 17.5 cm, Med Associates, Fairfield, VT) were used for the auditory cue test. These chambers were located in a different room from that used for conditioning. Each test chamber was housed in a separate sound-attenuating box, equipped with a ventilation fan providing a constant background noise of about 55 dB. The testing chambers were physically different from the conditioning chambers in that they had a slanted wall on one side, a curved wall on the other side, and the floor was made of Plexiglas (instead of stainless steel grid bars). In addition, the testing chambers were wiped with a 2% acetic acid solution before each test session, providing an olfactory stimulus different from that used during training. Each test chamber was illuminated by an overhead white light (7.5 W) and had a transparent Plexiglas front door that allowed recording of animal behavior via a remote video camera connected to either a VCR or a PC computer for data backup and off-line analysis. During testing, the room lights remained off for the entire session.

Procedure

Trace fear conditioning. Rats were handled daily for 5 to 7 days prior to training (the last three days also included acclimation to transportation) after which they were randomly separated into six groups based on the mean ITI used during conditioning (0.7, 2.2, 3.7, 5.2, 8.2, and 11.2 min). For conditioning, rats were placed in any of four identical training chambers (described above). After a 2-min baseline, each rat received one 10-trial session of trace fear conditioning (see Figure 1) using a white noise CS (15 s, 75–80 dB), a 30-s trace interval, and a scrambled footshock US (1 s, 1 mA). Within each ITI group, the ITIs were randomly selected within ± 20% of the mean ITI for that group. Immediately following the last conditioning trial, each rat was removed and returned to the colony room.

Since the amount of time rats spent in the training chamber varied as a function of training ITI, additional trace fear conditioning experiments were conducted to equate the amount of time between the shortest (0.7 min) and longest (11.2 min) ITI groups. These rats received training identical with the 0.7-min ITI group except that one group received training *immediately before* a 105-min context exposure and the other group received training

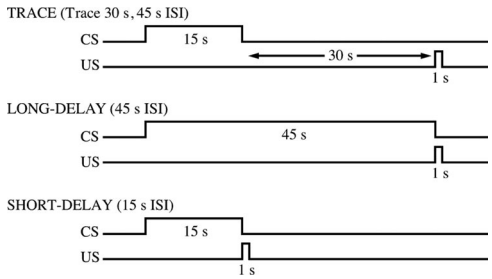

Figure 1. Fear-conditioning paradigms. For trace conditioning, a stimulus-free trace interval was inserted between offset of the 15-s white noise conditioned stimulus (CS) and onset of the 1-s footshock unconditioned stimulus (US). For long-delay conditioning, termination of a 45-s CS was immediately followed by onset of the US. For short-delay conditioning, termination of a 15-s CS was immediately followed by onset of the US. Notice that the 45-s duration of the interstimulus interval (ISI; measured from CS onset to US onset) is the same for both the trace and the long-delay fear conditioning paradigms whereas the CS duration is the same between the trace and short-delay paradigms.

immediately following a 105-min context exposure. Thus, the amount of time spent in the training chamber was identical (120 min) with that of rats trained using an 11.2-min ITI (120 min).

Long-delay fear conditioning. Rats were handled and separated into groups as described above for the trace fear study. Each rat received one 10-trial session of long-delay fear conditioning (see Figure 1) using a white noise CS (45 s, 75–80 dB) followed immediately by onset of a scrambled footshock US (1 s, 1 mA). Thus, the 45-s ISI matched that used during the trace fear conditioning study.

Short-delay fear conditioning. Rats were handled as described above for the trace and long-delay fear studies, except that only one ITI was used (0.7 min). Each rat received one 10-trial session of short-delay fear conditioning (see Figure 1) using a white noise CS (15 s, 75–80 dB) followed immediately by onset of a scrambled footshock US (1 s, 1 mA). Thus, the 15-s CS was the same as that used during the trace fear conditioning study.

Cue and context testing. Twenty-four hours following trace or delay fear conditioning, rats were placed into any of four auditory CS test chambers (described earlier). After a 2-min baseline period, a 6-min CS (75–80 dB) was presented followed by a 4-min post-CS period. The next day, rats were returned to the original training chamber for a 10-min context test. For all experiments, the order of cue and context testing was counterbalanced.

Data Analysis and Statistics

Data were stored on either VCR tape or as an mpeg file on a PC for off-line analysis. Freezing was defined as the absence of all movement except that required for respiration (Blanchard & Blanchard, 1969a, 1969b). Freezing was scored by an individual blind to the training ITI, and the total time spent freezing was recorded using a stopwatch and reported as mean percent time freezing.

Statistical analyses were performed using Statview (v 5.0; SAS Institute, Inc., Cary, NC). One- and two-way analyses of variance (ANOVAs) were used to evaluate ITI effects across trace and long-delay conditioning paradigms. Fisher's PLSD was used for post hoc comparisons. Averages are reported throughout as the mean ± *SEM*.

Results

Trace Fear Conditioning

CS test session. When placed into the novel test chamber, all trace fear conditioned rats exhibited little freezing during the baseline prior to presentation of the CS (Figure 2A), and there were no statistically significant differences between the training groups, $F(5, 42) = 0.39$, $p = .85$. However, presentation of the CS revealed a dramatic effect of training ITI on conditioned fear to the CS. Rats trained with the shortest ITI (0.7 min) exhibited very little freezing during the CS, and percent freezing increased as the ITI was increased such that CS freezing was maximal in rats trained with the longest ITI (11.2 min; see Figure 2B). A one-way ANOVA indicated that this effect of trial spacing during training was statistically significant, $F(5, 42) = 9.74$, $p < .01$, and post hoc analyses revealed that animals trained using the 3 shortest ITIs (0.7, 2.2, and 3.7 min) had CS freezing levels that were not significantly different from each other, but they were all different from the 8.2- and 11.2-min ITI groups ($p < .01$). Rats trained with a 5.2-min ITI exhibited intermediate freezing levels during the CS. Upon termination of the CS, all trace fear conditioned rats exhibited an increase in freezing behavior. Similar to the behavior observed during the CS, percent freezing during the period following CS offset (post-CS) increased as the training ITI increased, $F(5, 42) = 4.71$, $p < .01$ (see Figure 2C). Post hoc tests revealed that rats trained with the 8.2- and 11.2-min ITIs exhibited significantly more freezing than those trained using the shortest 3 ITIs ($p < .05$). Thus, spacing out the training trials resulted in significantly better acquisition of trace fear conditioning.

Context test session. Rats trained with the shortest ITI exhibited the highest levels of context freezing whereas rats trained with the longest ITI exhibited the lowest levels of freezing to the conditioning context (Figure 2D). Analysis of percent freezing revealed significant ITI-group differences, $F(5, 42) = 3.80$, $p < .01$. Post hoc analyses indicated that rats trained with the shortest ITI (0.7 min) froze significantly more during the context test than rats trained with a 5.2-, 8.2-, or 11.2-min ITI ($p < .01$). Also, rats trained with either a 2.2- or 3.7-min ITI froze more than rats trained with an 11.2-min ITI ($p < .05$). Thus, in the trace fear paradigm, massed training resulted in the highest levels of freezing to the conditioning context, and with further spacing between training trials, rats froze progressively less to the background context (Figure 2D).

Long-Delay Fear Conditioning

CS test session. As with the trace-conditioned rats, the long-delay fear conditioned rats exhibited negligible amounts of baseline freezing in a novel test chamber (Figure 3A). A one-way ANOVA indicated that there were no statistically significant differences between the six different ITI training groups, $F(5, 66) = 0.78$, $p = .57$. However, onset of the CS resulted in robust freezing in all groups of long-delay conditioned rats (Figure 3B). Analysis of percent freezing during the CS indicated that there was no statistically significant ITI-group effect, $F(5, 66) = 1.59$, $p = .18$.

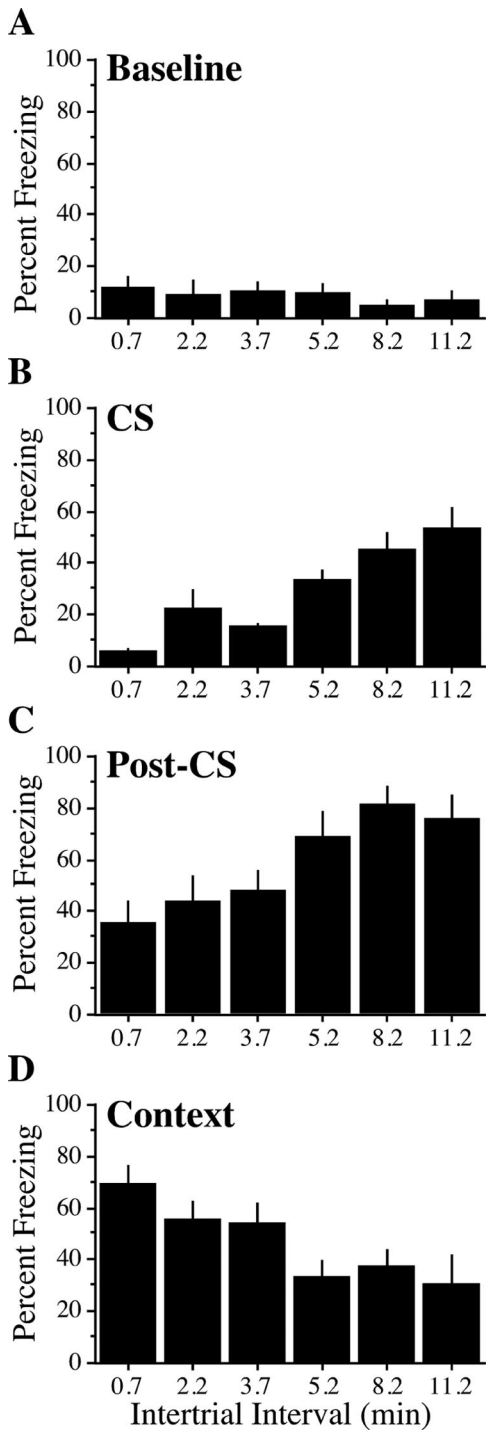

Figure 2. Effect of massed versus spaced training on conditioning effectiveness in trace fear conditioning. (A–C) Percent freezing in a novel testing chamber as a function of the intertrial interval (ITI; $n = 8$ rats per group) used during training. (A) Rats exhibited very little baseline freezing prior to onset of the conditioned stimulus (CS). Freezing during (B) and after (C) presentation of the CS increased as the training ITI was increased. At all ITIs, trace fear conditioned rats exhibited higher levels of freezing during the post-CS period than during presentation of the CS. (D) Freezing to the original training context was highest in rats trained with the shortest ITI (0.7 min). Notice also that context freezing continued to decrease with additional trial spacing.

Following offset of the CS, long-delay fear conditioned rats exhibited a pronounced decrease in freezing behavior (Figure 3C), and there were no statistically significant differences in post-CS freezing as a function of training ITI, $F(5, 66) = 1.73$, $p = .14$. Thus, trial spacing during training had little effect on conditioning effectiveness as measured by freezing behavior in rats trained with a long-delay paradigm.

Context test session. When returned to the original conditioning context, long-delay conditioned rats exhibited relatively low levels of freezing (Figure 3D). Analysis of percent freezing during the 10-min context test revealed that there was no statistically significant ITI-group effect, $F(5, 66) = 0.21$, $p = .96$. Thus, trial spacing had little effect on freezing to the conditioning context in rats trained using a long-delay paradigm.

Comparison of Trace and Long-Delay Fear Conditioning

Effects of training ITI on CS freezing. Figure 4 illustrates an overlay of percent freezing as a function of training ITI for both trace and long-delay fear conditioned rats. Notice the significantly greater freezing during the CS in long-delay compared with trace animals (Figure 4B). A two-way ANOVA of percent freezing during the CS indicated a statistically significant effect of training ITI, $F(5, 108) = 5.54$, $p < .001$, as well as a significant effect of training paradigm, $F(1, 108) = 66.26$, $p < .001$. However, the ITI by training paradigm interaction was not significant, $F(5, 108) = 1.85$, $p = .11$. Post hoc tests revealed that for rats trained using 0.7-, 2.2-, and 3.7-min ITIs, CS freezing was significantly higher in long-delay compared with trace conditioned rats ($p < .001$). In contrast, CS offset resulted in comparable levels of freezing in delay and trace conditioned rats (Figure 4C). Although there was an overall effect of training ITI, $F(5, 108) = 4.08$, $p < .01$, this was driven by two factors: (1) the relatively low levels of post-CS freezing in the 0.7-min ITI group and (2) the steady increase in post-CS freezing observed in the trace conditioned rats as a function of ITI (see Figure 4C). In addition, there was no significant effect of training paradigm, $F(1, 108) = 1.00$, $p = .32$ nor was there a significant ITI by training paradigm interaction, $F(5, 108) = 1.28$, $p = .28$.

Effects of training ITI on context freezing. Figure 4D shows an overlay of percent freezing to the conditioning context for trace and long-delay conditioned rats as a function of training ITI. A two-way ANOVA indicated a statistically significant effect of ITI, $F(5, 108) = 2.97$, $p < .05$, training paradigm, $F(1, 108) = 26.81$, $p < .001$, and a significant ITI by training paradigm interaction, $F(5, 108) = 2.33$, $p < .05$. Post hoc tests revealed that the trace conditioned rats exhibited significantly greater levels of freezing than long-delay conditioned animals, but only when trained with a 0.7-, 2.2-, and 3.7-min ITI ($p < .005$). Notice that trial spacing resulted in a progressive decrease in context freezing in trace but not long-delay fear conditioned rats.

Time course of freezing during the CS test. Figure 5 shows an overlay of percent freezing during the CS test session between rats trained using trace compared with the long-delay fear paradigm (11.2-min ITI). Notice that initially, presentation of the CS results in comparable levels of freezing, however, as the CS presentation continues, rats trained with long-delay show sustained freezing whereas rats trained with trace show decreased freezing. Following termination of the CS, trace conditioned rats exhibited a pro-

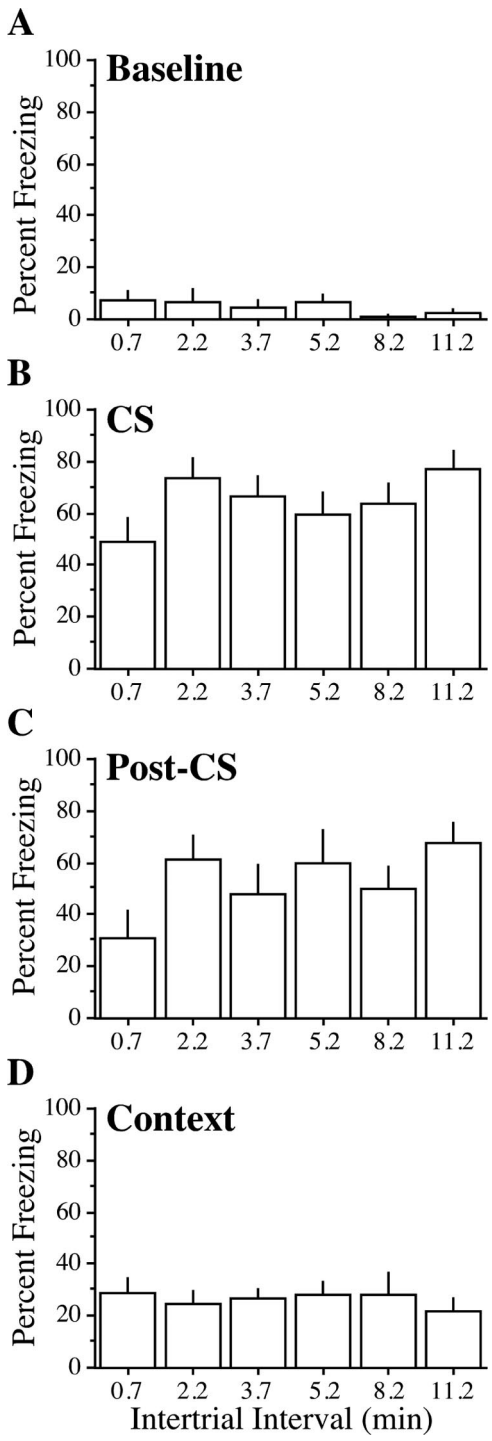

Figure 3. Effect of massed versus spaced training on conditioning effectiveness in long-delay fear conditioning. (A–C) Percent freezing in a novel testing chamber as a function of the intertrial interval (ITI; $n = 12$ rats per group) used during training. (A) Rats exhibited very little baseline freezing prior to onset of the conditioned stimulus (CS). The training ITI did not significantly influence the amount of freezing during (B) or after (C) presentation of the CS. Notice that at each ITI, CS freezing was greater than post-CS freezing. (D) Freezing to the original training context did not significantly vary with training ITI.

nounced increase in freezing, which was sustained throughout the post-CS period (see Figure 5). In contrast, the long-delay conditioned rats initially exhibit an increase in freezing followed by a rapid and steady decrease in freezing during the post-CS period following CS offset. Thus, the trace conditioned rats exhibit higher levels of freezing following CS offset whereas delay conditioned rats exhibit higher levels of freezing during the CS presentation. This was true for all groups of rats, including those trained at the shortest ITI (compare Figure 2B with 2C and Figure 3B with 3C).

Impact of CS duration on trace and delay fear conditioning. To evaluate whether the use of a long CS enhanced cue conditioning in the long-delay fear conditioned rats compared with the trace conditioned rats, an additional set of experiments was performed using a delay paradigm with a 15-s CS (short-delay paradigm). These experiments were conducted using a 0.7-min ITI to minimize potential ceiling effects and to allow us to see subtle differences between the long- and short-delay fear conditioned rats. When placed into a novel context, no differences in baseline freezing were observed between the three groups, $F(2, 26) = 0.41$, $p = .82$. However, there was a statistically significant effect of training condition on freezing during the CS, $F(2, 26) = 7.21$, $p < .005$. Post hoc analyses revealed that both the short- and long-delay fear conditioned rats had significantly higher levels of CS freezing than the trace conditioned rats (percent freezing: short-delay 53.3% ($n = 4$), long-delay 50.3% ($n = 14$), trace 12.3% ($n = 11$); $p < .05$). There were no differences in post-CS freezing between the three groups, $F(2, 26) = 0.32$, $p = .73$. A statistically significant effect of training condition was also observed on freezing during the context test, $F(2, 26) = 11.05$, $p < .001$, with post hoc analyses indicating that the trace conditioned rats exhibited significantly greater levels of context fear compared with both the short- and long-delay rats (percent freezing to the context: short-delay 29.9%, long-delay 26.8%, trace 67.7%; $p < .01$). Thus, regardless of whether the CS duration or the ISI was held constant, delay conditioned rats exhibited better CS and poorer context conditioning compared with the trace fear conditioned rats.

Effects of Context Exposure on Trace Fear Conditioning Using a Short ITI

Effects on CS freezing. Because rats trained using a 0.7-min ITI spent considerably less time in the training chamber (15 min) than rats trained using an 11.2-min ITI (120 min), additional experiments were conducted in which a group of rats trained using a 0.7-min ITI spent the same amount of time in the training chamber as rats in the 11.2-min ITI group. These rats were trace fear conditioned using a 0.7-min ITI either *before* (context extinction or 0.7/ctx) or *after* (context preexposure or ctx/0.7) a 105-min context exposure. These data were compared to rats trained using either a 0.7-min or an 11.2-min ITI (i.e., the original dataset from Figure 2 combined with additional cohorts that were trained with each batch of context exposure animals, see Figure 6). When placed in a novel test chamber, all animals exhibited low levels of baseline freezing, and there were no significant group differences, $F(3, 71) = 1.94$, $p = .13$. In contrast, a one-way ANOVA indicated a statistically significant effect of training condition on freezing during the CS, $F(3, 71) = 11.37$, $p < .001$. Post hoc tests revealed that rats in the 11.2-min ITI group froze significantly more during the CS than rats in the other 3 groups ($p < .05$; Figure

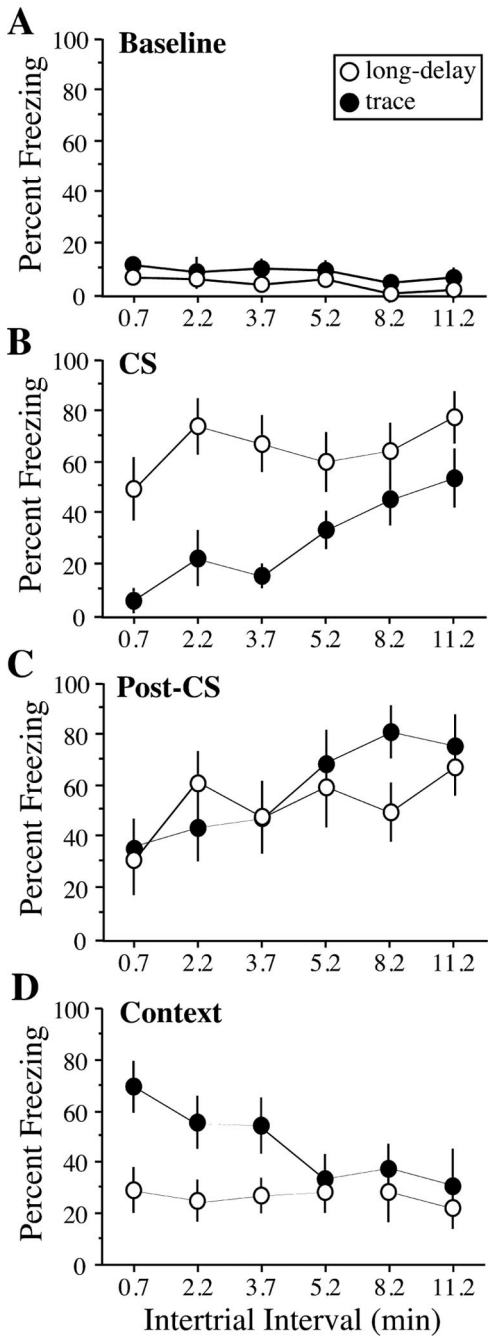

Figure 4. Conditioning effectiveness as a function of training intertrial interval (ITI) in trace and long-delay conditioned rats. For comparison, data from trace (solid circles, $n = 8$ per group) and long-delay (open circles; $n = 12$ per group) conditioned rats are superimposed on each graph. (A–C) Percent freezing in a novel testing chamber as a function of the intertrial interval (ITI) used during training. Notice that during presentation of the CS (B), the differences between long-delay and trace conditioned rats were most pronounced at the shorter ITIs (i.e., 0.7, 2.2, 3.7, and 5.2 min) and that post-CS freezing (C) was comparable between trace and long-delay conditioned rats. (D) Overlay of percent freezing during the context test in trace and long-delay conditioned rats as a function of ITI. Differences between the trace and long-delay conditioned rats were greatest at the three shortest training ITIs (i.e., 0.7, 2.2, and 3.7 min).

6B). In addition, rats in the context preexposure group froze significantly more during the CS than rats in the context extinction group ($p < .05$; Figure 6B). Both context exposure groups displayed CS freezing levels that were not significantly different from rats trained in the typical manner using a 0.7-min ITI. Similar results were observed when freezing was analyzed following CS offset (post-CS). A one-way ANOVA indicated a statistically significant effect of training condition on post-CS freezing, $F(3, 71) = 15.90$, $p < .001$ (see Figure 6C). Post hoc tests revealed that rats trained using an 11.2-min ITI froze significantly more following CS offset than all three groups of rats trained with a 0.7-min ITI ($p < .001$). Likewise, rats in the context preexposure group displayed significantly more post-CS freezing than rats in the context extinction group ($p < .05$), and neither group differed significantly from rats trained in the typical manner using a 0.7-min ITI (Figure 6C). Thus, regardless of the amount of time spent in the training chamber, rats that received trace fear conditioning using a 0.7-min ITI displayed less CS and post-CS freezing compared with rats trained using an 11.2-min ITI.

Effects on context freezing. Figure 6D illustrates the effects of context exposure on rats trained using a 0.7-min ITI compared with rats trained in the standard manner using either a 0.7- or an 11.2-min ITI. A one-way ANOVA indicated a statistically significant effect of training condition on freezing to the background context, $F(3, 71) = 7.29$, $p < .001$. Post hoc analyses revealed that rats trained using a 0.7-min ITI spent significantly more time freezing during the context test than rats in the 11.2-min ITI group ($p < .01$) as well as rats in the context extinction group (0.7/ctx; $p < .001$) but not rats in the context preexposure group. Notice that rats in the context preexposure group showed significantly greater context freezing than rats in the context extinction group ($p < .005$). In other words, when controlling for the amount of time spent in the training chamber, context freezing is significantly lower when the context exposure follows training but not when it precedes training.

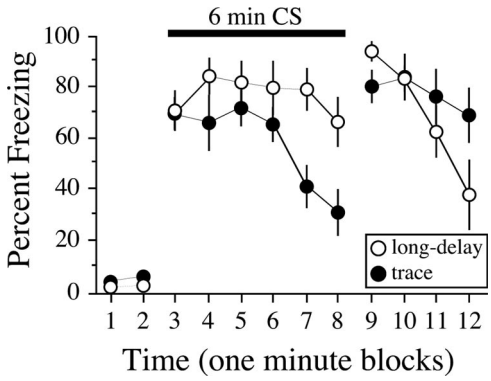

Figure 5. Enhanced freezing following conditioned stimulus (CS) offset in rats trained using trace compared with long-delay fear conditioning. In animals trained using an 11.2 min intertrial interval (ITI), the long-delay fear conditioning group froze more to the CS than did rats from the trace fear conditioning group. Notice that upon termination of the CS, trace conditioned rats exhibited a pronounced increase in freezing that decayed slowly whereas long-delay conditioned rats exhibited a transient increase followed by a rapid decrease in freezing. Although only data from the 11.2-min ITI groups are shown, these patterns were observed for all groups of rats, independent of training ITI.

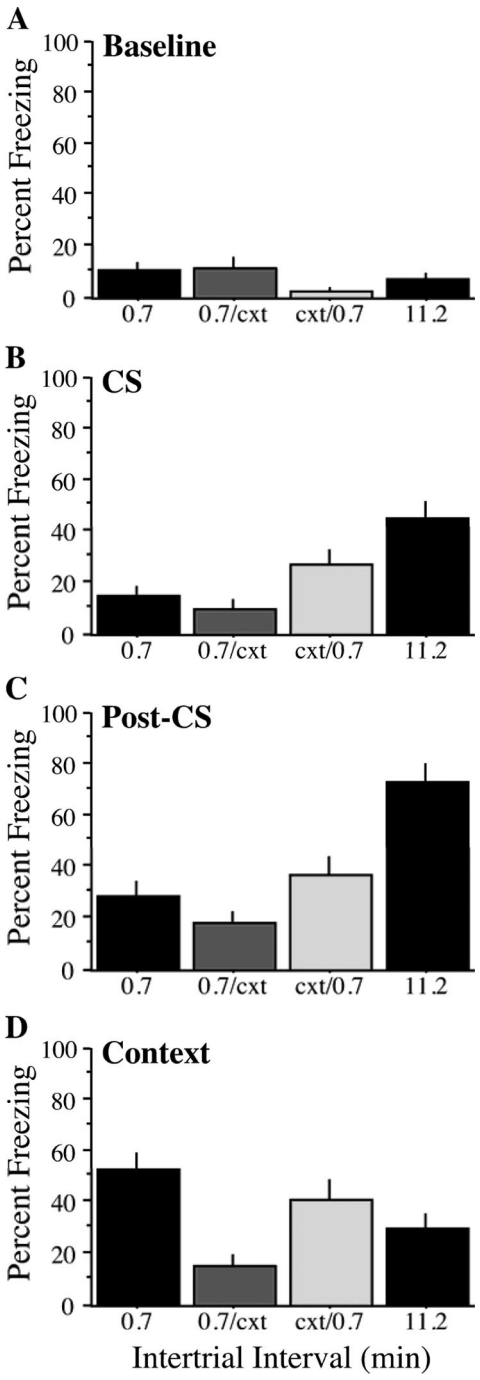

Figure 6. Effects of varying the training chamber exposure on conditioned stimulus (CS) and context freezing in trace fear conditioned rats trained using a short (0.7 min) intertrial interval (ITI). Graphs illustrate the percentage of time spent freezing during either the CS test session (A–C) or the context test session (D). Solid black bars indicate the freezing levels of animals trained in the typical manner using either a short (0.7 min; $n = 23$) or long (11.2 min; $n = 20$) ITI. Gray shaded bars indicate the freezing levels of rats trained using a 0.7 min ITI but who were either preexposed to the training chamber for 105 min prior to conditioning (cxt/0.7; light gray bars; $n = 15$) or who remained in the chamber for 105 min immediately after conditioning (0.7/ctx; dark gray bars; $n = 17$). Thus, these rats spent the same amount of time in the training chamber as rats trained using an 11.2 min ITI.

Discussion

The results of the present experiments demonstrate that trial spacing governs conditioning effectiveness to the cue in trace fear conditioning. Although trial spacing resulted in better cue conditioning in trace fear conditioned rats, the opposite effect was observed during the context test—freezing to the background training context was higher in rats trained using a massed versus spaced trace conditioning paradigm. In contrast, trial spacing did not significantly affect conditioning effectiveness to the cue in long-delay fear conditioning—rats exhibited robust freezing to the cue at all training ITIs.

Effects of Trial Spacing on Trace and Long-Delay Cue Conditioning

The current experiments evaluated trace and long-delay fear conditioning effectiveness using 6 different ITIs covering a broad time range (from 0.7 to 11.2 min). Following trace fear conditioning, rats trained with the shortest ITI (0.7 min) exhibited the lowest levels of freezing during the CS test session compared to animals trained using longer ITIs (see Figures 2B and 2C). In fact, relatively poor trace cue conditioning was observed in all 3 groups trained using an ITI less than or equal to 3.7 min. When the training ITI was increased to 5.2 min, trace cue conditioning was significantly enhanced, and conditioning effectiveness continued to improve when animals were trained using even longer ITIs (Figures 2B and 2C). Although there have been no prior studies evaluating the effect of training ITI on trace fear conditioning, the present data are consistent with those from previous classical eyeblink (Calvin, 1939; Prokasy et al., 1958; Spence & Norris, 1950) and delay fear conditioning (Barela, 1999) studies, which demonstrated enhanced learning with longer ITIs. The trace conditioning data are also consistent with results found by Kaplan (1984), who found increased conditioning with longer ITIs with trace autoshaping in pigeons. In fact, at the shortest ITIs the birds avoided the CS, possibly because of inhibitory backward conditioning (Ewing, Larew, & Wagner, 1985).

In contrast to the trace fear conditioning data, varying the training ITI in a long-delay fear paradigm had little impact on conditioning effectiveness to the cue (see Figure 3). This could have been the result of using 10 conditioning trials, which resulted in robust learning at all training ITIs. Indeed, Barela (1999) used only three training trials and observed a trial spacing effect during the CS test. It should be noted, however, that although there was no statistically significant effect of training ITI on freezing during the CS test session in long-delay conditioned rats, those trained using the shortest ITI did exhibit somewhat lower freezing levels to the CS compared with those trained at the longest ITI (Figure 3B). It is possible that even lower levels of freezing would be observed if a mean ITI of less than 0.7 min was used. We chose 0.7 min (44 s) as the mean for the shortest training ITI because it was approximately the duration of a single CS–US conditioning trial. Use of shorter duration ITIs during training might have resulted in lower levels of freezing to the cue, but shorter ITIs were not used because of the potential for inhibitory backward conditioning to occur (Siegel & Domjan, 1971).

It is also possible that our use of a relatively long CS (45 s) contributed to the enhanced CS freezing observed in the long-

delay conditioned rats, particularly those trained using the shorter ITIs (see Figure 4B). This is unlikely because rats trained in a short-delay paradigm (using a 15-s CS and a 0.7-min ITI) exhibited freezing behavior that was significantly greater than the trace and nearly identical to the long-delay fear conditioned rats. If anything, freezing to the CS was slightly greater in short- compared with long-delay conditioned rats (see Results), which would be consistent with previous reports suggesting that the ISI curve for delay fear conditioning rapidly peaks at around 10 s and gradually declines with longer ISIs (e.g., see Yeo, 1974).

Differences in Freezing Behavior Between Trace and Long-Delay Conditioned Rats

The behavior of rats during the CS test session varied as a function of training paradigm. At all training ITIs, the mean percentage of time spent freezing in the trace conditioned animals was greater during the interval following CS offset than during the CS (compare Figures 2B and 2C). In contrast, long-delay conditioned animals exhibited less freezing during the interval following CS offset compared with freezing during the CS (compare Figures 3B and 3C). These differences in freezing behavior during the CS test probably reflect differences in what the animal learns about the CS in each paradigm. In trace conditioning the CS offset signals delivery of the US (e.g., 30-s later), whereas in delay conditioning offset of the CS signals the end of the training trial and the beginning of the ITI. Figure 5 illustrates the robust and continuous freezing after CS offset in trace conditioned rats compared with the rapid decrease in freezing in the long-delay conditioned rats. Similar data have been observed using the fear-potentiated startle response in rats (Burman & Gewirtz, 2004). In their study, rats trained using a short-delay (4-s CS; 3.5-s ISI) paradigm exhibited a maximal startle response during the CS whereas rats trained using a long-trace (4-s CS; 16-s ISI) paradigm exhibited maximal startle 3 s following CS offset. This suggests that the present findings in trace and long-delay conditioned rats are generalizable among different dependent measures of fear.

Effects of Trial Spacing on Conditioning to the Background Context

While trial spacing improved conditioning to the CS in the trace paradigm, the opposite effect was observed during the context test—freezing to the background context decreased (Figure 2D). In fact, rats trained using the shortest ITI (0.7 min) exhibited the highest levels of context freezing and the lowest level of CS freezing. In contrast, rats trained at the longest ITI (11.2 min) exhibited the lowest levels of context freezing and the highest levels of CS freezing, possibly due to context extinction. In trace conditioning, the animal must learn to distinguish between the context as the trace interval and the context as the ITI (Mowrer & Lamoreaux, 1951). The only difference between the two is that the CS precedes and the US follows the trace interval such that the trace interval is reinforced whereas the ITI is not reinforced by the US (Chowdhury, Quinn, & Fanselow, 2005). As a result, trace conditioning using an ITI that is much longer than the trace interval enables the animal to discriminate between the two, resulting in better conditioning to the CS and less to the context. According to the Rescorla-Wagner model (Rescorla & Wagner, 1972; Wagner & Rescorla, 1972), with longer ITIs, animals freeze less to the context than to the discrete CS because the associative strength of the context will have extinguished during the long nonreinforced times between trials. As a result, the CS acquires associative strength and animals freeze more to the CS. With short ITIs, the context acquires more associative strength because the association of the context with the US will not have been extinguished. This then leaves the CS with less associative strength, resulting in decreased freezing to the CS. The relatively low levels of freezing to the background context in conjunction with high levels of freezing to the CS, after long-delay fear conditioning (Figure 3D), are also consistent with the Rescorla-Wagner model. In addition, ambiguities in discrete CS conditioning or a decrease in the salience of the discrete CS may also account for the increase in background context freezing in rats trained using shorter ITIs. For example, several studies have demonstrated that increasing the duration of the trace interval can result in a decrease in CS with a concomitant increase in context conditioning (e.g., see Marlin, 1981; Wilkinson, Humby, Robbins, & Everitt, 1995). In other words, conditioning to the background context should decrease when training paradigms are altered to increase the salience of a discrete CS. In our experiments this was accomplished by increasing the ITI in the trace conditioned rats or by switching to a long-delay paradigm. Indeed, the amount of context freezing of the long-delay conditioned rats (at any ITI) was comparable to that observed in trace conditioned rats trained using the longest ITIs (see Figure 4D). Our CS and context freezing data are also consistent with other theoretical models (e.g., see Gibbon & Balsam, 1981; Miller & Matzel, 1988; Miller & Schachtman, 1985; Solomon & Corbit, 1974; Wagner, 1981).

Other studies have looked at the effect of training ITI on context conditioning. For example, when rats were trained using 3-, 16-, or 60-s ITIs, freezing levels were highest in the 60-s group (Fanselow & Tighe, 1988). Their data suggest that when the US is presented without a discrete cue (conditioning to a foreground context), massed presentations of the US reduce context conditioning. These results are not inconsistent with our trace fear conditioning data. Our shortest ITI (44 s or 0.7 min) was similar to their longest ITI (60 s), and both resulted in high levels of context freezing. Had Fanselow and Tighe used longer ITIs, they would likely have observed lower levels of freezing to the context. Had we used shorter ITIs, their data suggest that we would not only have observed poor CS but we also would have observed relatively poor context conditioning. Taken together, it appears that the ITI-function for context fear (*as measured using freezing as the dependent variable*) may follow an inverted-U function that peaks rapidly at short ITIs and tapers more gradually with longer ITIs (see also Barela, 1999; Williams, Frame, & LoLordo, 1991).

Furthermore, the effects of training ITI on freezing to the background context in the trace conditioned animals did not merely result from differences in the amount of time the two groups spent in the training chamber. When rats received a prolonged context exposure *after* training with a short ITI, subsequent context freezing was significantly lower than rats trained in the typical manner with a short ITI (see Figure 6D). This effect is consistent with other reports in the literature and is likely the result of extinction during the 105-min posttraining context exposure (e.g., Blanchard, Dielman, & Blanchard, 1968). In contrast, context freezing in the context preexposure group (i.e., rats that

received the 105-min context exposure *before* training) was not significantly different from rats trained in the typical manner using a short ITI (Figure 6D). However, there was some evidence of latent inhibition in the context preexposure group, which showed freezing levels between those of the short and long ITI trained rats. Previous studies have also reported latent inhibition to the context, but the most profound effects typically involve multiple daily exposures (e.g., Kiernan & Westbrook, 1993) or an overnight exposure prior to conditioning (von Hertzen & Giese, 2005). Other factors, such as ambiguity of the discrete CS, context extinction, and the total amount of time spent in the conditioning chamber may all contribute to our observed differences in context conditioning as a function of training ITI.

Implications of the Current Research

Pavlovian conditioning paradigms have been used to study the neurobiology of learning and memory (Davis, Walker, & Myers, 2003; Knight, Cheng, Smith, Stein, & Helmstetter, 2004; Maren, 2001; Pare, Quirk, & Ledoux, 2004). Subtle changes in the temporal relationship between the CS and US alter the requirements of various brain regions for normal acquisition. For example, hippocampal lesions impair acquisition of trace but not delay conditioning paradigms (McEchron et al., 1998; Moyer, Deyo, & Disterhoft, 1990; Quinn et al., 2002; Weiss, Bouwmeester, Power, & Disterhoft, 1999). Furthermore, trace conditioning is often more sensitive than delay conditioning for detecting aging-related learning impairments. In fear conditioning studies, aged rats are impaired in trace (McEchron, Cheng, & Gilmartin, 2004; Moyer & Brown, 2006) but not delay conditioning paradigms (Houston, Stevenson, McNaughton, & Barnes, 1999; Moyer & Brown, 2006; Oler & Markus, 1998). Although many factors contribute to impaired acquisition of trace conditioning with aging, it is possible that the ITI-function changes with age. Our observations in adult animals suggest that the training ITI exerts a powerful influence on acquisition of trace fear conditioning. In addition, studies have demonstrated aging-related deficits in acquisition of trace fear conditioning not only when relatively short (~3 min) ITIs were used (McEchron et al., 2004; Villarreal, Dykes, & Barea-Rodriguez, 2004), but also when relatively long (~8 min) ITIs were used (Moyer & Brown, 2006). These data in conjunction with the present study suggest that in trace fear conditioning the ITI-function may be shifted to the right in aging animals. If this is true, then the paradigms used to test aging and potential drug effects may need to be altered to account for the potential impact of training ITI.

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