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Impaired Trace and Contextual Fear Conditioning in Aged Rats

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Trace and contextual fear conditioning were evaluated in adult (3–6 months), early middle-aged (8–12 months), late middle-aged (16–20 months), and aged (24–33 months) Sprague–Dawley rats. After trace conditioning, aged animals exhibited significantly less freezing to the tone conditioned stimulus and training context. Levels of trace-cue and context conditioning were negatively correlated with age ($r = -0.56$ and -0.59, respectively) and positively correlated with each other ($r = +0.52$). Aged rats showed robust conditioning in short- and long-delay fear paradigms, suggesting that the trace interval, rather than the use of a long interstimulus interval, is responsible for the aging-related deficits in trace fear conditioning. The authors suggest that these aging-related conditioning deficits furnish useful indices of functional changes within hippocampus or perirhinal cortex.

Keywords: aging, trace fear conditioning, ultrasonic vocalizations, freezing, context

Aging is known to cause a variety of learning and memory deficits, especially on tasks that require intact medial temporal lobe (MTL) function (e.g., see Knuttinen, Gamelli, Weiss, Power, & Disterhoft, 2001; Moyer, Power, Thompson, & Disterhoft, 2000; Rapp & Amaral, 1991; Rosenzweig & Barnes, 2003; Schoenbaum, Nugent, Saddoris, & Gallagher, 2002; L. T. Thompson, Moyer, & Disterhoft, 1996). Within the MTLs, both the hippocampus and perirhinal cortex have been of considerable interest in relationship to both Alzheimer's disease and normal aging-related cognitive decline. Perirhinal cortex is one of the earliest and most severely damaged structures in Alzheimer's disease (Braak & Braak, 1991; deToledo-Morrell et al., 1997; Juottonen et al., 1998; Mitchell et al., 2002; Van Hoesen, Augustinack, Dierking, Redman, & Thangavel, 2000; Van Hoesen & Solodkin, 1994; Yilmazer-Hanke & Hanke, 1999). Because the perirhinal cortex furnishes one of the important direct and indirect inputs to the hippocampus (Burwell & Amaral, 1998; Pitkänen, Pikkarainen, Nurminen, & Ylinen, 2000; Shi & Cassell, 1999), one might expect some overlap with hippocampus in terms of the mnemonic functions or adaptive computations.

In the present study, we used Pavlovian fear conditioning to explore and quantify evidence of aging-related behavioral changes that might reflect hippocampal or perirhinal dysfunction. This approach makes use of the fact that certain Pavlovian conditioning paradigms have consistently been shown to be sensitive to the integrity of hippocampus and/or perirhinal cortex, whereas others are known not to depend on these MTL structures (Fanselow & Poulos, 2005; R. F. Thompson, 2005). Acquisition of *delay fear conditioning* to a tone or a white noise conditional stimulus (CS) requires intact amygdalar and brain stem structures but does not depend on either hippocampal or perirhinal cortical function (reviewed in Fanselow & Poulos, 2005; LeDoux, 2000). By contrast, acquisition and expression of *trace fear conditioning* to a tone CS requires an intact hippocampus (Fendt, Fanselow, & Koch, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Tseng, & Disterhoft, 2000; Quinn, Oommen, Morrison, & Fanselow, 2002). Trace cue conditioning is also hippocampus dependent when the conditional response (CR) is an anticipatory eyeblink (e.g., see Beylin et al., 2001; Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaff, Thompson, & Weisz, 1986; Tseng, Guan, Disterhoft, & Weiss, 2004; Weiss, Bouwmeester, Power, & Disterhoft, 1999). In addition, both perirhinal cortex and hippocampus have been implicated in acquisition or expression of contextual fear conditioning (Anagnostaras, Gale, & Fanselow, 2001; Anagnostaras, Maren, & Fanselow, 1999; Bucci, Phillips, & Burwell, 2000; Burwell, Bucci, Sanborn, & Jutras, 2004; Chowdhury, Quinn, & Fanselow, 2005; Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Sacchetti, Lorenzini, Baldi, Tassoni, & Bucherelli, 1999).

Several studies of aged rats have reported deficits in context conditioning but no deficits in delay conditioning to a tone cue (Houston, Stevenson, McNaughton, & Barnes, 1999; Oler & Markus, 1998; Stoehr & Wenk, 1995; Ward, Oler, & Markus, 1999). A study of senescence-accelerated mice similarly found no deficit in delay conditioning to a tone cue but impaired conditioning to the training context (Ohta et al., 2001). Age-related deficits have also been reported in a one-trial trace fear conditioning paradigm in C57BL/6J mice (Blank, Nijholt, Kye, Radulovic, & Spiess, 2003). In addition, recent studies of Fischer 344 and F1 hybrid Fischer–Brown Norway rats have found aging-related def-

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icits in trace fear conditioning, as measured by both freezing and heart rate (McEchron, Cheng, & Gilmartin, 2004; Villarreal, Dykes, & Barea-Rodriguez, 2004).

In considering aging-related deficits in trace conditioning, it is important to distinguish between the effects of introducing a trace interval and the use of a longer interstimulus interval (ISI) between CS and unconditional stimulus (US). In principle, a longer ISI could increase the task difficulty (see Beylin et al., 2001). To distinguish between these possibilities, the present study included a long-delay paradigm, with an ISI equal to that used for trace-cue conditioning. Age-related changes in the acquisition of trace and contextual fear conditioning were evaluated across the life span of the animal. We selected Sprague–Dawley rats because much is known about the pertinent cellular, systems, and behavioral neuroscience in these animals (e.g., see Choi & Brown, 2003; Drapeau et al., 2003; Faulkner & Brown, 1999; Frick, Magee, & Johnston, 2004; Jarrard, Davidson, & Bowring, 2004; Lee & Kim, 2004; Lindquist & Brown, 2004; Moyer & Brown, 1998, 2002; Rumpel, LeDoux, Zador, & Malinow, 2005).

Both freezing and 22-kHz ultrasonic vocalizations (USV; see Brudzynski, 2005; Choi & Brown, 2003; Lee, Choi, Brown, & Kim, 2001; Lindquist & Brown, 2004) have been measured as fear-related or defensive CRs in previous studies. However, in the present studies, the analysis of age-related changes was based solely on freezing because older animals failed to emit 22-kHz USV CRs. The results show that aging is associated with a sizable decline in both trace-cue conditioning and context conditioning, whereas both short- and long-delay cue conditioning remained intact.

Materials and Methods

Subjects

Thirty-six experimentally naïve male Sprague–Dawley rats (Charles River, Kingston, NY) were used in the present study. The rats were from four different age groups: *adult* (3–6 months, mean age = 4.5 ± 0.2 months.; $n = 8$), *early middle-aged* (8–12 months, mean age = 10.5 ± 0.6 months; $n = 8$), *late middle-aged* (16–20 months, mean age = 18.4 ± 0.8 months; $n = 8$), and *aged* (24–33 months, mean age = 28.0 ± 0.7 months; $n = 12$). Rats were housed individually on a 12-hr day–night cycle (lights on at 7 a.m.), with free access to food and water. Five to seven days before training, rats were transported in their home cages to a separate waiting room in the laboratory where they were individually removed and handled for approximately 1 min. Experiments were conducted during the light portion of the cycle and were in strict compliance with both National Institutes of Health and Yale Animal Resource Center guidelines.

Conditioning and Testing Apparatus

Two different chambers were used—one for cue conditioning and testing context conditioning and a second for testing cue conditioning in a shifted context. The conditioning chamber (Habitest, Coulbourn Instrument, Allentown, PA) had aluminum side walls, Plexiglas front and back walls, an aluminum ceiling, and a standard grid floor that consisted of parallel steel rods (5 mm diameter and 15 mm spacing). A small animal shock generator (H13-16, Coulbourn Instruments) was used to generate a scrambled footshock. The interior dimensions of the conditioning chamber were 25 cm × 28 cm × 33 cm (length × width × height). Two circular lights (12 cm diameter) were used for illumination, and an odorant was added to the conditioning chamber (5 to 10 ml of 5% acetic acid added to the tray beneath the grid floor). Prior to placing an animal in the conditioning chamber (which was used for conditioning and context testing), the walls and grid floor were wiped with 5% acetic acid. After each use, the conditioning chamber was washed and wiped down with Windex glass cleaner.

The second chamber, which was used for testing CRs to the cue (in a shifted context, to reduce baseline freezing), was hexagonal in shape and made of clear Plexiglas, except for the floor, which was an aluminum plate with an array of holes drilled into it (providing a distinctive tactile cue). The interior dimensions were 32 cm × 34 cm × 50 cm (length × width × height). A small ventilating fan (4 cm in diameter) was attached to the ceiling. Prior to placing a rat in the cue-test chamber, fresh bedding was added to the bottom tray beneath the floor (providing a distinctive background odor different from the acetic acid used during conditioning), and the walls were wiped with Windex. For the tone-test session, both the chamber lights and the room lights were turned off (illumination inside the chamber was less than 1 lux). After each tone-test session, the chamber was washed and wiped down with Windex.

Both chambers were located within a 60 cm × 90 cm × 90 cm (length × width × height) sound-attenuating chamber equipped with a ventilating fan (3.5 in. [8.9 cm] diameter). An electrostatic loudspeaker (ES1, Tucker–Davis Technologies, Gainesville, FL), located near the upper center of the chamber ceiling, was used to deliver the tone CS. Intensity was measured with a digital sound level meter in C-weighting mode (Realistic, Model 33–2055, Forth Worth, TX). For each chamber, the digital sound level meter was located in the center of the chamber about 24 in. [61 cm] from the speaker. An infrared camera (CB-21, Circuit Specialists, Inc., Mesa, AZ) was used to record behavior. A heterodyne bat detector (Mini3, Noldus Information Technology, the Netherlands) recorded 22-kHz USVs (see Choi & Brown, 2003). A PC running custom software (TestPoint, Capital Equipment Corporation, Billerica, MA) controlled the delivery and timing of all stimuli during both conditioning and testing sessions.

Trace Fear Conditioning

Rats were transported to the waiting room (previously used for handling) in their home cages, after which they were transferred to the conditioning chamber in a small clear Plexiglas box (dimensions: 30 cm × 20 cm × 10 cm). Two minutes after they were placed in the conditioning chamber, each rat received one session of 10 trials (8.5-min intertrial interval [ITI]) of trace fear conditioning with a 15-s tone CS (4 kHz, 75 dB), a 30-s trace interval, and a 1-s footshock US (1 mA). Rats were removed from the conditioning cage within a minute of presentation of the last trace fear conditioning trial, transported back to their home cages in the waiting room, and then returned to the vivarium.

Tone and Context Testing

Twenty-four hours after trace-fear conditioning, rats received one tone-test session. For the tone test, rats were transported directly to the tone-test chamber in their home cages and placed in the tone-testing chamber, which was different from the conditioning chamber. Following a 2-min baseline period, rats were exposed to a 6-min tone CS (4 kHz, 75 dB) followed by a 4-min period in which the CS was not presented (post-CS period). Immediately upon completion of the tone test session, rats were returned to their home cages and transported back to the vivarium.

Twenty-four hours after the tone-test session, rats were tested for context conditioning. Animals were transported to the conditioning chamber in a manner identical with that used for conditioning except that the tone CS and footshock US were omitted. Rats were placed into the conditioning chamber for a 10-min context test, immediately after which they were returned to their home cage and transported back to the vivarium. To time lock information across testing sessions, we marked data with a transistor–

transistor logic (TTL) pulse within 10 s of introducing the rat into either chamber.

Short-Delay Fear Conditioning

A subset of 6 older rats (2 early middle-aged, mean age = 12.1 ± 0.03 months.; 2 late middle-aged, mean = 20.6 ± 0.1 months; 2 aged, mean = 28.5 ± 1.3 months.; mean age for all 6 older rats = 20.4 ± 3 months) that did not learn the trace fear paradigm (defined as a freezing level at least two standard deviations below the mean value in the adult rats) were subsequently trained (1 to 4 weeks later) in a delay fear paradigm, with the same training and testing chambers. The same 15-s CS was presented as used in trace conditioning, but the footshock US was presented immediately after termination of the CS (0-s trace interval). We refer to this as a *short-delay paradigm.* This stimulus arrangement was selected to represent an initial or limiting case for subsequent studies of trace-interval-dependent effects on conditioning. The mean ITI between CS–US presentations in the short-delay conditioning paradigm was approximately 3 min. All other procedures were as described for trace conditioning.

Long-Delay Fear Conditioning

To control for possible effects of training ISI on tone fear conditioning effectiveness, we trained an additional group of four experimentally naïve aged rats (mean age = 26.3 ± 1.7 months) in a long-delay fear paradigm. The only procedural change was that the tone CS lasted 46 s. It coterminated with the usual 1-s footshock US, resulting in the same 45-s ISI that was used in the trace paradigm. The ITI between paired CS–US presentations in this long-delay paradigm was the same as that used for trace fear conditioning (approximately 8.5 min).

Sensory Threshold Testing

To ensure that there were no age-related differences in footshock sensitivity, a subset of adult, late middle-aged, and aged rats were randomly selected and tested for sensitivity to the 1-s footshock US. For this test, rats were returned to the conditioning chamber 2–6 days after completion of all training and testing. For shock sensitivity, the 1-s footshock was gradually increased in intensity, starting from zero. An experimenter who was blind to the age of the animal and the stimulus intensity recorded the current needed to produce a forepaw flinch, a jump, and an audible vocalization. The sequence was repeated three times for each rat, and the values were averaged.

Baseline Activity Measures

To evaluate any age-related differences in gross baseline activity, the number of grid crossings was determined for each rat during the first minute of each training session (prior to receipt of any CS or US presentations). This was done by placing a grid of three vertical lines (evenly spaced) on the TV monitor while playing back the data from the video tape. A grid crossing was noted as the movement of both forepaws across any grid line. An experimenter who was blind to the age of the animal recorded the total number of grid crossings for each rat.

Data Analysis and Statistics

Freezing was defined as the absence of all body or head movement, except that required for respiration while in a stereotypic crouching posture (D. C. Blanchard & Blanchard, 1969, R. J. Blanchard & Blanchard, 1969). A PC running custom software (TestPoint, Capital Equipment Corporation, Billerica, MA) was used to obtain real-time measurements of freezing. A computer mouse was used as the input device to indicate freezing. The data from the mouse clicks were continuously digitized (at 100 Hz) via an analog-to-digital (A/D) board (Keithley Instruments, Cleveland, OH) and stored as a tab-delimited-text file. The TTL pulses were also recorded on videotape, indicating the start of the tone or context test session. The videotape recordings were time stamped so that the scoring of freezing data could be timed in relationship to the behavioral test session. USV calls were detected with an amplitude filter and were also fed into the PC. The data files were analyzed with a custom program (written and compiled in the C programming language) that provided latency to onset of freezing, the mean percentage of freezing, and the onsets and offsets of USV calls as functions of time.

We used a repeated measures analysis of variance (ANOVA) to evaluate the time course of freezing and a one-way, between-groups ANOVA to assess age-group effects. Following a significant ($p < .05$) ANOVA, we used Fisher's protected least significant difference (PLSD) for post hoc comparisons. Averages are reported throughout as the mean ± the standard error of the mean.

Results

Trace Cue Conditioning

Figure 1A summarizes the effects of aging on trace fear conditioning during three periods of the tone-test session (pre-CS, CS, and post-CS periods). As indicated, there was negligible freezing ($M = 10.6 \pm 2.3\%$) in this novel context prior to the onset of the cue. A one-way ANOVA revealed that freezing during this 2-min baseline period did not differ across ages, $F(3, 28) = 0.31$, $p = .82$. The onset of the cue caused a sizable increase in freezing levels in all age groups (see Figure 1A), but there were significant differences in the levels of freezing among age groups, $F(3, 28) = 3.98$, $p < .02$. Aged rats froze significantly less than adult ($p < .005$) and early and late middle-aged ($p < .02$) rats. During the post-CS period (Figure 1A), there were also significant age-related differences, $F(3, 28) = 3.70$, $p < .025$. Again, aged rats froze significantly less than adult ($p < .005$), early ($p < .05$), and late ($p < .025$) middle-aged rats.

To evaluate the time course of freezing, we averaged the percentage of freezing for each minute of time throughout the tone test session (Figure 1B). The onset of the cue was accompanied by a sizable and immediate jump in the mean level of freezing. A repeated measures ANOVA of percentage of freezing during the 2-min baseline indicated no differences as a function of age group, $F(3, 28) = 0.31$, $p = .82$, or time sample, $F(1, 28) = 2.59$, $p = .12$, nor was there a time sample by age group interaction, $F(3, 28) = 0.69$, $p = .57$. Analysis of percentage of freezing during the 6-min exposure to the tone CS indicated a statistically significant main effect of age, $F(3, 28) = 3.98$, $p < .02$, but not of time sample, $F(5, 140) = 2.07$, $p = .07$. A statistically significant age by sample time interaction was also observed, $F(15, 140) = 1.84$, $p < .05$, reflecting the gradual decline in freezing in the middle-aged and aged rats.

Post hoc tests indicated that aged rats froze significantly less than adult, early, and late middle-aged rats for Minutes 3 and 4 ($p < .05$); aged rats froze significantly less than adult ($p < .005$) and early middle-aged rats for Minute 5 ($p < .01$); and aged rats froze significantly less than adult rats for Minute 6 ($p < .005$). With regard to trace conditioning, aged rats clearly show diminished freezing to the cue in comparison with levels characteristic of adult and middle-aged rats (see Figure 1).

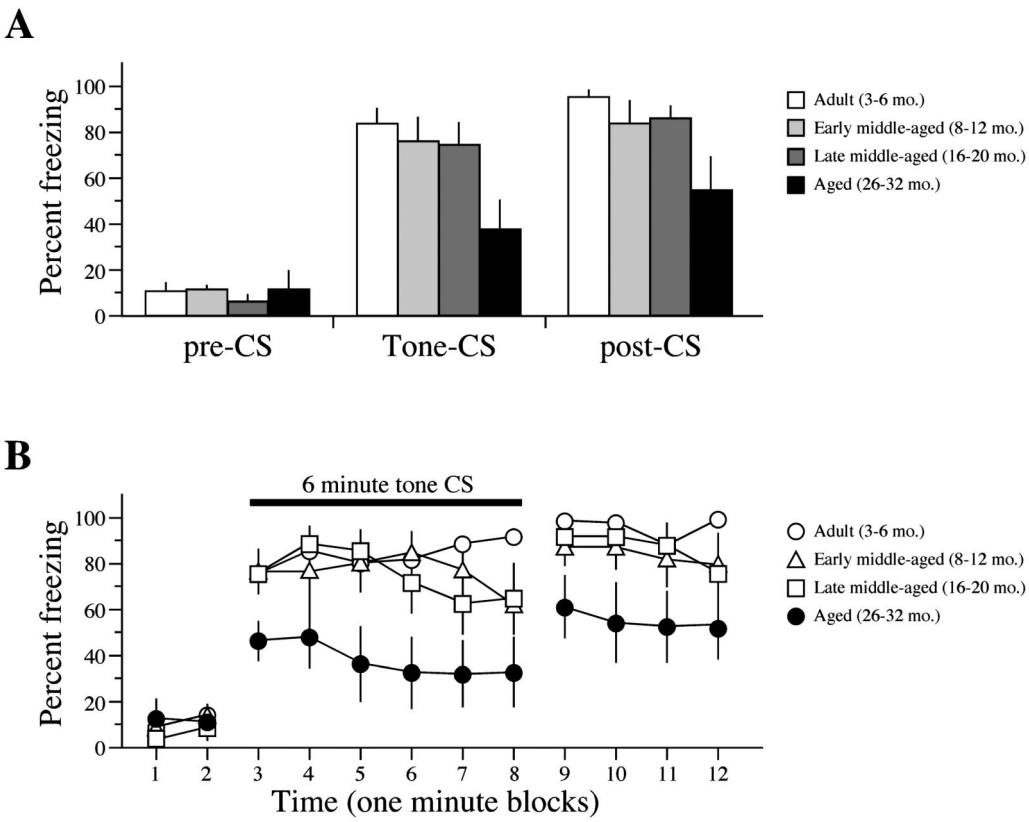

Figure 1. Aged rats were impaired in trace fear conditioning. (A) Mean percent freezing during the 20-min baseline (pre-conditional stimulus [CS]), the 6-min tone CS (tone-CS), and the 4-min period following CS offset (post-CS). Baseline freezing in a novel context was extremely low for all rats and did not vary with aging. Aged rats exhibited significantly less freezing during both the tone-CS and the post-CS period. (B) Time course of percent freezing throughout the test session as a function of aging. Aged rats exhibited consistently lower levels of freezing throughout the tone-CS and post-CS periods.

The offset of the cue was accompanied by an increase in freezing (Figure 1B). Analysis of mean percent freezing during the 4-min post-CS period revealed a statistically significant effect of age, $F(3, 28) = 3.70$, $p < .025$. There was no significant effect of sample time, $F(3, 84) = 1.49$, $p = .22$, and no significant Age × Sample Time interaction, $F(9, 84) = 0.51$, $p = .87$. Post hoc tests indicated that aged rats froze significantly less than adult ($p < .005$), early middle-aged ($p < .05$), and late middle-aged ($p < .02$) rats during Minutes 1 and 2. Aged rats froze significantly less than adult ($p < .05$) and late middle-aged rats ($p < .05$) during Minute 3. Aged rats also froze significantly less than adult rats ($p < .01$) during Minute 4 (see Figure 1B).

Context Conditioning

Aged rats showed the lowest mean levels of freezing to the conditioning context (see Figure 2). Analysis of percent freezing revealed significant age-group differences, $F(3, 28) = 8.06$, $p < .0005$. Aged rats froze significantly less than adult and early middle-aged rats ($p < .0005$). Late middle-aged rats froze significantly less than early middle-aged rats ($p < .05$). There were no significant differences in freezing levels between late middle-aged rats and either aged ($p = .06$) or adult ($p = .09$) rats.

Figure 2B shows for each age group the time course of freezing during the context test. The mean percent freezing increased during the first few minutes, peaking within 3 to 5 min. During the 10-min context exposure test, there were significant age-group differences, $F(3, 28) = 8.06$, $p < .0005$; significant effects of time block, $F(9, 252) = 6.96$, $p < .0001$; and a significant age group by time block interaction, $F(27, 252) = 1.90$, $p < .01$. Notice that the adult and early middle-aged rats maintained consistently high levels of freezing throughout the context (Figure 2B, open circles and triangles), whereas the late middle-aged and aged rats exhibited little freezing during the 1st min (Figure 2B, open squares and filled circles). The late middle-aged rats eventually achieved a reasonably high level of freezing, but it was not sustained during the last 2 min of the context exposure (open squares, Figure 2B). The aged rats showed an initial increase in percentage of freezing over the first 5 min, but they never achieved high levels of freezing during the context exposure.

Post hoc analyses revealed that aged rats consistently froze less than the adult and early middle-aged rats throughout the context

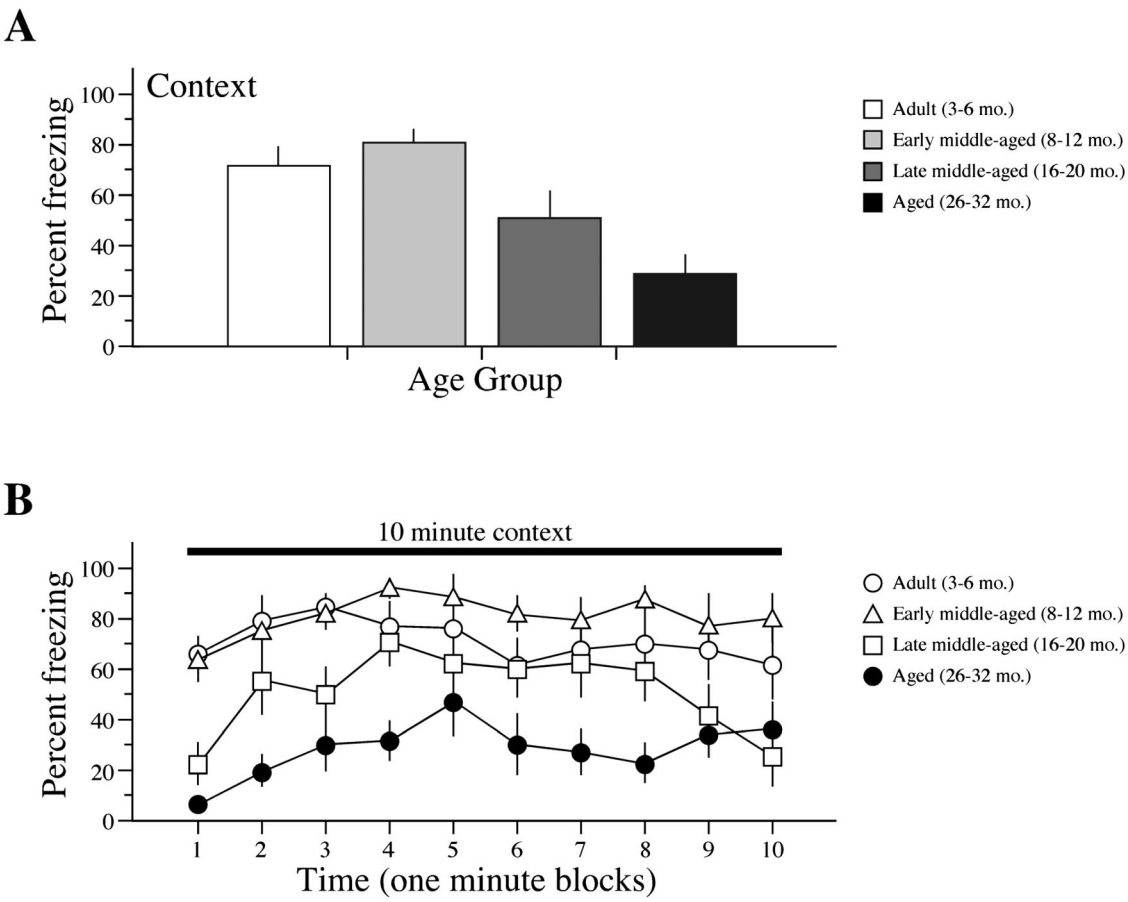

Figure 2. Aged rats were impaired in contextual fear conditioning. (A) Mean percentage of freezing during the 10-min context test session as a function of aging. (B) Time course of percentage of freezing throughout the context test session. Aged rats consistently spent less time freezing during the context exposure compared with adult and middle-aged rats. Notice that percentage of freezing in the late middle-aged rats was intermediate relative to the younger groups and the aged rats.

test (Minutes 1–4, $p < .001$; Minutes 6–10, $p < .05$). In addition, the aged rats froze less than the late middle-aged rats only during Minutes 2, 4, 7, and 8 of the context exposure ($p < .05$). In addition to spending significantly less time freezing during the context test, aged rats also required significantly more time to initiate freezing behavior relative to adult or middle-aged rats (see Table 1). This did not account for the mean deficits in percentage of freezing, however, because the aged rats still spent less time freezing than did the adult and early middle-aged rats, even after freezing behavior was initiated (see time series in Figure 2B).

Elicitation of Ultrasonic Vocalization Conditional Responses

Previous studies have shown that 22-kHz USV and freezing are positively correlated as fear-related CRs in adult Sprague–Dawley rats (Choi & Brown, 2003; Lee et al., 2001; Lindquist & Brown, 2004). Whereas rats from all age groups emitted 22-kHz USVs during the trace fear conditioning training session, none of the early middle-aged, late middle-aged, or aged rats emitted USVs at

Table 1
Aging-Related Changes in Latency to Onset of Freezing After Trace Fear Conditioning

	Onset latency in seconds ($M \pm SE$)	
Age group studied (n)	Tone test	Context test
Adult (8)	1.6 ± 0.9	5.4 ± 2.6
Early middle-aged (8)	1.6 ± 0.8	5.8 ± 2.4
Late middle-aged (8)	5.3 ± 1.9	21.2 ± 7.2
Aged (8)	7.3 ± 2.9	43.5 ± 13.7*

Note. Although latency to onset of freezing during the tone test showed a trend towards an increased latency in older rats, a one-way ANOVA indicated that this trend was not statistically significant, $F(3, 28) = 2.33$, $p = .10$. A one-way ANOVA of latency to onset of freezing during the context test indicated a significant effect of age, $F(3, 28) = 5.10$, $p < .01$, with aged rats having significantly longer latencies than adult and early middle-aged rats (*$p < .005$) but not late middle-aged rats ($p = .057$).

any time during the test sessions. Only adult (3–6 months) rats emitted USV CRs. During the tone test, 6 of the 8 adult rats emitted USV CRs. These 6 adult rats spent 44.1% of the time vocalizing during the tone CS presentation and 53.1% of the time vocalizing during the 4-min post-CS time period.

As shown in Figure 3, no USVs were emitted during the baseline period prior to tone CS onset. Following the CS onset, freezing levels increased faster than USV levels (see Figure 3). Half of the animals did not begin to emit 22-kHz USVs until 2 or more minutes after the CS onset. Among the 6 adult rats that emitted 22-kHz USV CRs, the mean latency to the onset of freezing (1.8 ± 1.2 s) was significantly shorter than the mean latency to the onset of USVs (132 ± 47 s), $t(5) = -2.70$, $p < .05$. After reaching peak levels, both CRs remained relatively stable throughout the CS and post-CS test sessions (see Figure 3).

Short-Delay Conditioning

To control for the possibility that the deficits observed in aged rats following trace fear conditioning resulted from an inability of the aged rats to exhibit robust freezing behavior, we subsequently used a short-delay fear paradigm to train a subpopulation of 6 older rats (aged and middle-aged) that did not learn the trace fear conditioning task (see Method section). Figure 4A compares the mean percent freezing levels in these animals in the short-delay procedure (trace 0 s; black bars) with their performance in the trace procedure (trace 30 s; open bars). The older rats clearly showed robust cue conditioning in the short-delay paradigm (Figure 4A). The difference between the mean percent freezing in the short-delay and trace-conditioning procedures (81 ± 12% and 26 ± 4%, respectively) was statistically significant, $t(5) = -4.91$, $p < .005$, paired *t* test (see Figure 4). There were no significant differences in percent freezing during the pre-CS baseline period, $t(5) = -0.36$, $p = .74$, or the post-CS period, $t(5) = 0.04$, $p = .97$ (see Figure 4).

To visualize the time course of freezing across the training procedure, we plotted percent freezing as a function of 1-min blocks of time (Figure 4B). The mean percent freezing peaked within 2 min following both training procedures. After delay conditioning, the level of freezing remained relatively constant (at approximately 80%) during the CS presentation. By contrast, following trace conditioning, the level of freezing continuously declined after its initial peak (which reached approximately 40%). During the tone test the level of freezing increased after the CS offset in aged rats trained using trace but not delay conditioning. (Figure 4B). Analysis of percent freezing at each time point during the 6-min tone CS indicated that older rats froze significantly more following delay conditioning compared with trace conditioning: 1st minute, $t(5) = -3.87$, $p < .02$; 2nd minute, $t(5) = -4.31$, $p < .01$; 3rd minute, $t(5) = -2.96$, $p < .05$; 4th minute, $t(5) = -3.60$, $p < .02$; 5th minute, $t(5) = -3.44$, $p < .02$; and 6th minute, $t(5) = -6.92$, $p < .001$. The mean latency to onset of freezing to the CS during the test session was slightly longer after delay fear conditioning (delay = 5.9 ± 3.3 s; trace = 3.4 ± 2.5 s), but the difference was not statistically significant, $t(5) = -2.22$, $p = .08$.

Although the older rats performed well on short-delay conditioning, they failed to improve on the subsequent test of context conditioning (see Figure 5). The mean percentage of time spent freezing during the context test after delay conditioning (50% ± 14%) was not significantly different than the previous value following prior trace conditioning (45% ± 11%), $t(5) = -0.33$, $p = .75$ (see Figure 5A). No significant differences in percentage of freezing were observed during any 1-min block of time throughout the context test exposure: 1st minute, $t(5) = -1.38$, $p = .23$; 2nd minute, $t(5) = -0.23$, $p = .82$; 3rd minute, $t(5) = -0.54$, $p = .61$; 4th minute, $t(5) = 0.58$, $p = .59$; 5th minute, $t(5) = 0.49$, $p = .65$; 6th minute, $t(5) = 0.13$, $p = .90$; 7th minute, $t(5) = -0.08$, $p = .94$; 8th minute, $t(5) = -0.03$, $p = .98$; 9th minute, $t(5) = -0.75$, $p = .49$; and 10th minute, $t(5) = -2.01$, $p = .10$. No statistically significant effect of training paradigm was found on the mean latency to the onset of freezing on exposure to the conditioning context, $t(5) = 2.00$, $p = .10$. In summary, the older rats did not benefit

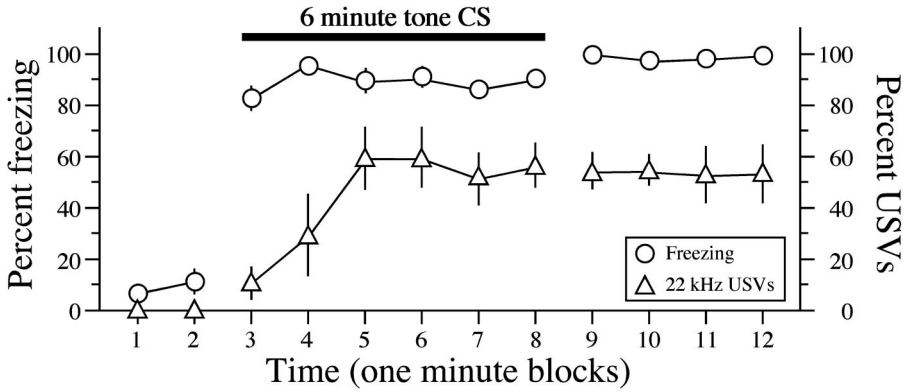

Figure 3. Adult rats emitted 22-kHz ultrasonic vocalizations (USVs) after trace fear conditioning. Time course of percent freezing (left ordinate) compared with time course of percentage of time spent emitting 22-kHz ultrasonic vocalizations (right ordinate) during the tone test session for adult rats. Only data from the 6 adult rats that emitted USVs during the test session are shown. Notice that the rats did not emit 22-kHz USVs until the conditional stimulus (CS) was presented. Although robust freezing was observed shortly after CS onset, emission of USVs did not appear until 1 or 2 min following CS onset (see also Table 1).

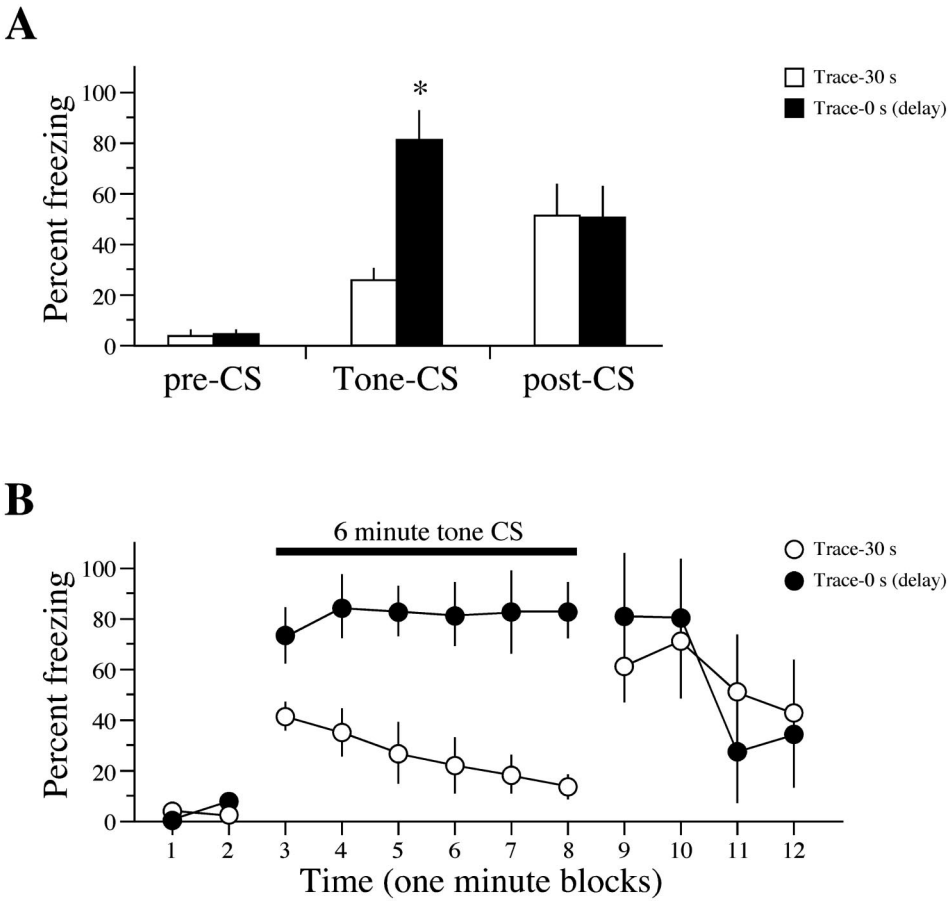

Figure 4. Older rats impaired in trace fear readily learned short-delay fear conditioning. (A) Mean percent freezing during the conditional stimulus (CS) test session. A subset of 6 middle-aged and aged rats that did poorly on the trace fear paradigm were subsequently trained on a short-delay fear paradigm (see Method section). Although these older rats exhibited little freezing to the tone CS after trace fear conditioning (open bars), they froze >80% of the time during the CS test following a subsequent short-delay conditioning (solid bars) session. No differences in mean baseline or post-CS freezing were observed. (B) Time course of percent freezing during the tone CS test session. Notice that older rats trained on the short-delay paradigm exhibited robust freezing throughout the CS presentation.

from an additional training session in terms of context conditioning, but they showed robust cue conditioning when switched to a short-delay paradigm (see Figures 4 and 5).

Long-Delay Conditioning

To determine whether the age-related deficits in trace fear conditioning were specific to the trace paradigm or merely the result of using a long ISI, we trained experimentally naïve aged rats using a long-delay fear paradigm in which the ISI (45 s) was the same as that used during trace conditioning. The results were compared with those from aged rats that received trace fear conditioning (Figure 1B, solid circles). Figure 6 illustrates that the inability of aged rats to learn trace fear conditioning is not due to the use of a long (45-s) ISI. Instead, the impairment in trace conditioning appears to reflect the 30-s trace interval.

Aged rats trained in a long-delay paradigm exhibited robust cue-elicited freezing (89% ± 3%) when tested in a novel context (Figure 6A). There were no significant group differences in mean baseline freezing, $F(1, 10) = 0.25$, $p = .63$, or mean post-CS freezing, $F(1, 10) = 0.21$, $p = .66$. Note that the aged rats trained in the trace paradigm exhibited consistently lower levels of freezing throughout the CS presentation compared with aged rats trained in the long-delay paradigm (Figure 6B). Analysis of mean percent freezing throughout the 6-min CS presentation revealed a statistically significant main effect of training paradigm, $F(1, 10) = 7.21$, $p < .05$, but no effect of time, $F(5, 50) = 1.11$, $p = .37$. The training paradigm by time-block interaction also was not statistically significant, $F(5, 50) = 1.36$, $p = .26$. Following the CS offset, the aged rats that were trained in the long-delay and trace paradigms, respectively, decreased and increased freezing (Figure 6B; compare Blocks 3 through 8 with Blocks 9 through 12).

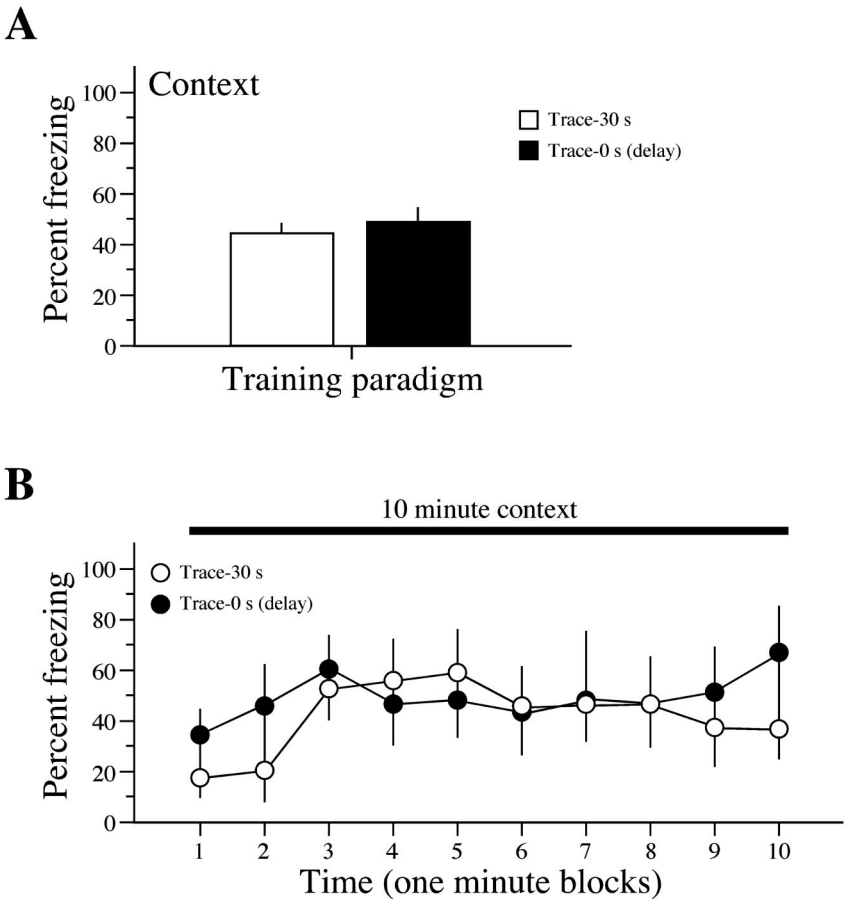

Figure 5. Older rats remained impaired to the context after subsequent delay fear conditioning. (A) Mean percent freezing during the context test after trace or short-delay fear conditioning. (B) Time course of percent freezing throughout the context test for older rats initially trained in a trace (open circles) and then a short-delay (solid circles) fear conditioning paradigm. Notice that these older rats exhibited similar mean levels of freezing to the background training context following either trace or short-delay fear conditioning.

Sensitivity to the Footshock Unconditioned Stimulus

Sensitivity to the footshock US was directly evaluated by comparing the amount of current required to elicit each of the following: a flinch, the raising of one forepaw, and the emission of an audible vocalization (see Method section). Table 2 shows that there were no statistically significant differences in footshock sensitivity with advancing age. Together with the fact that aged rats learned both a short- and a long-delay fear paradigm, these data suggest that the observed aging-related deficits in trace and contextual fear conditioning did not result from impaired processing of the footshock US.

Gross Baseline Activity

Gross baseline activity was determined by comparing the number of grid crossings during the first minute each rat spent in the conditioning chamber prior to any CS or US presentation (see Method section). Table 3 shows that there were no statistically significant differences in gross baseline activity, $F(5, 28) = 2.08$, $p = .13$. Together with the fact that aged rats learned both a short- and a long-delay fear paradigm, these data suggest that the observed aging-related deficits in trace and contextual fear conditioning did not result from abnormally high levels of baseline activity.

Discussion

Overall, the results show that aged Sprague–Dawley rats are significantly impaired in both trace cue and contextual fear conditioning but that they readily learned both a short- and a long-delay cue conditioning task. The fact that aged animals were impaired on trace but not long-delay conditioning shows that the trace interval (30 s), versus the long ISI (45 s) associated with trace conditioning, caused the impairment. The tasks on which aged rats are impaired or unimpaired correspond, respectively, to those that rely on or do not rely on the hippocampus and/or perirhinal cortex. We suggest that trace-cue and context conditioning are sensitive indices of aging-related changes in hippocampal and/or perirhinal

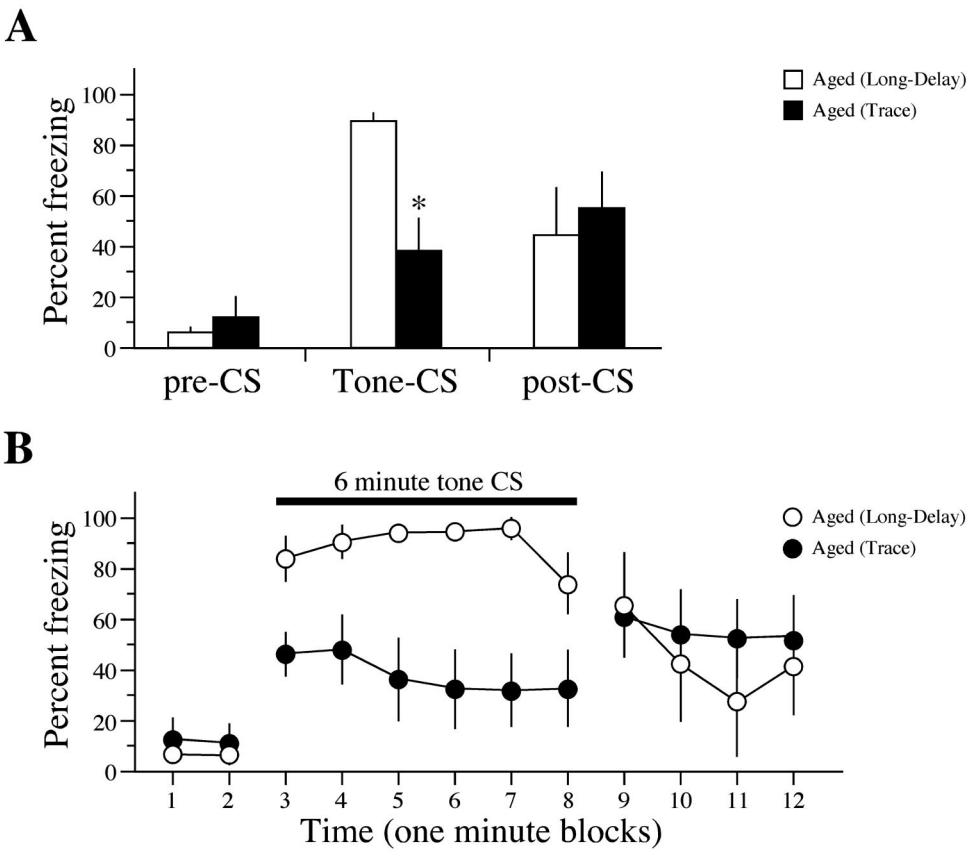

Figure 6. Aged rats were not impaired in long-delay fear conditioning. (A) Mean percent freezing, during the conditional stimulus (CS) test session, of aged rats trained in either a trace ($n = 8$; mean age = 28.8 ± 0.4 months) or a long-delay ($n = 4$; mean age = 26.3 ± 1.7 months) fear conditioning paradigm. The long-delay paradigm was selected to match the 45-s ISI used during trace conditioning (see Method section). (B) Time course of percent freezing throughout the entire CS test session. Notice that the aged rats exhibited robust freezing during the CS presentation following long-delay but not trace fear conditioning. No differences in either baseline freezing or post-CS freezing were observed.

function, with the important caveat that experimental data are currently insufficient to specify the relative importance or exact roles of these two structures in trace-cue and context conditioning.

As more is understood about the relative contributions of these two structures, variations in contextual and cue fear conditioning protocols should prove valuable for dissociating aging-related changes in hippocampal versus perirhinal function. Some functional dissociation might be achieved through the use of more complex cues, variations in the duration of the trace interval, context discrimination tests, and analysis of explicitly configural learning, as well as through better understanding the roles of these MTL structures in "occasion setting" (Blank et al., 2003; Bucci et al., 2000; Bucci, Saddoris, & Burwell, 2002; Burwell, Bucci, Sanborn, & Jutras, 2004; Burwell, Saddoris, Bucci, & Wiig, 2004; Fanselow, 2000; Lindquist et al., 2004; Maren, 2001; McEchron et al., 2004; Rudy, Barrientos, & O'Reilly, 2002; Sacchetti, Baldi, Lorenzini, & Bucherelli, 2002; Sacchetti et al., 1999; Villarreal et al., 2004). Further development of these fear conditioning protocols may also be useful for evaluating neurotherapeutic effects on aging-related cognitive decline in laboratory animals. The practical significance of these animal studies is enhanced by parallel research on Pavlovian fear conditioning in humans (e.g., Cheng, Knight, Smith, Stein, & Helmstetter, 2003; Knight, Cheng, Smith, Stein, & Helmstetter, 2004; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Olsson & Phelps, 2004).

Impairments in Trace-Cue and Context Conditioning

The experiments evaluated four different age groups to analyze conditioning changes across the adult life span of these animals. Compared with the younger animals, aged rats were profoundly impaired in trace fear conditioning (see Figure 1). Compared with adult rats, there were no detectable impairments in trace-cue conditioning in either of the middle-aged groups, suggesting that the behavioral deficits observed in aged rats emerge after 20 months of age. The aging deficits did not result from sensory or motor impairments because aged rats that were switched to a short-delay fear paradigm exhibited robust freezing to the CS (see Figure 4). Furthermore, aged rats trained using a long-delay paradigm (with the same 45-s ISI used in the trace fear paradigm) exhibited robust

Table 2
Footshock Sensitivity Is Not Altered by Aging

Age group studied (*n*)	Amount of current in milliamperes (Mean ± *SE*)		
	Flinch	Raise paw	Vocalize[a]
Adult (3)	0.13 ± .01	0.22 ± .02	0.25 ± .01
Late middle-aged (3)	0.11 ± .01	0.17 ± .03	0.36 ± .06
Aged (3)	0.11 ± .01	0.15 ± .01	0.29 ± .06

Note. Data are the average of three independent measurements for each of the rats. Amount of current in milliamperes required to produce each of the responses was determined by an individual unaware of the age of the rat. One-way analysis of variance indicated that there were no age-related differences in current required for rats to flinch $F(2, 6) = 0.96$, $p = .44$, raise a forepaw $F(2, 6) = 1.96$, $p = .22$, or vocalize $F(2, 6) = 1.24$, $p = .36$.

[a] Vocalize refers to elicitation of an audible vocalization that could be heard with the human ear — not to be confused with a 22 kHz ultrasonic vocalization. See Method section for experimental details.

freezing to the CS, suggesting that the aging deficits were specific to the trace paradigm. In addition, no sensory deficits were found with regard to sensitivity to the shock US (see Table 2).

An aging-related decline in trace-cue conditioning has also been observed in mice and other strains of rats. A recent study in aged mice demonstrated significantly lower freezing after a one-trial trace fear conditioning session, an effect that could be reversed by down-regulation of hippocampal SK3 channels (Blank et al., 2003). Aged female Fischer–Brown Norway rats were also reported to show decreased trace fear conditioning, as measured by freezing and heart rate (McEchron et al., 2004). In addition, aged male F344 rats were found to be significantly impaired in trace fear conditioning (Villarreal et al., 2004). In both of these studies of aged rats, there was no deficit in short-delay conditioning. Our data extend these studies to Sprague–Dawley rats and include two groups of middle-aged rats. The results show that aged rats readily learn a long-delay fear paradigm (see Figure 6), indicating that aging-related deficits in trace fear conditioning are due to the presence of a long trace interval and are not the result of using a longer ISI, which could increase the difficulty of the task (see Beylin et al., 2001).

The aged rats were also profoundly impaired in subsequent testing for memory of the training context, in agreement with studies of mice and other strains of rats. Following delay cue conditioning, 24-month-old male F344 rats were reported to show significantly less freezing to the conditioning context than 3- or 9-month-old rats (Stoehr & Wenk, 1995). Other studies of F344 rats have suggested deficits in consolidation of contextual fear conditioning in aged rats (Houston et al., 1999; Oler & Markus, 1998). Similarly, senescence-accelerated mice of 4 or 8 months were significantly impaired in conditioning to the background context but not to the tone cue in delay fear conditioning (Ohta et al., 2001).

Differences Between Trace and Long-Delay Conditioning

The results clearly demonstrate that aged Sprague–Dawley rats are selectively impaired in trace but not in long-delay fear conditioning. Previous studies that demonstrated aging deficits in trace fear conditioning used either a short-trace (1-s trace interval; 6-s ISI) or a short-delay (15-s ISI) paradigm to control for the inability of aged rats to learn the trace paradigm (McEchron et al., 2004; Villarreal et al., 2004). These findings left open the possibility that the aging deficits were not the result of using a trace paradigm per se but rather a result of the use of a long ISI (25 s and 45 s in the McEchron et al., 2004, and Villarreal et al., 2004, studies, respectively). This possibility was eliminated by our observation that aged rats trained in a long-delay paradigm (45-s ISI) exhibited robust freezing to the tone CS during the subsequent test session (Figures 4 and 6). Therefore, it is the 30-s trace interval rather than the 45-s ISI that caused the impaired learning in the aged rats (see Figures 4 and 6). In previous delay-conditioning studies of aging, the longest ISI was 35 s (Ward et al., 1999), but ISIs have more commonly ranged from 5 s to 30 s (Houston et al., 1999; Oler & Markus, 1998; Stoehr & Wenk, 1995; Villarreal et al., 2004). Here, we increased the ISI to 45 s and found robust freezing in aged rats (Figures 4 and 6).

Recent data from mice suggest that pre- and posttraining hippocampal lesions have no effect on delay but disrupt trace fear conditioning in mice—but only with trace intervals greater than 3 s (Chowdhury et al., 2005; Misane et al., 2005). Although similar lesion studies have not yet been conducted in rats, these data from mice suggest that aged rats should be able to learn trace fear conditioning but only when very short trace intervals are used (perhaps 1–3 s). Consistent with these findings, a recent report disclosed that aged Fischer–Brown Norway rats are impaired in trace fear conditioning with a 20-s, but not with a 1-s, trace interval (McEchron et al., 2004). We conclude that aging-related sensitivity to trace conditioning is not absolute but depends on the length of the trace interval. One possibility is that other brain systems can sustain short trace conditioning. We also need to consider that the effect of aging-related MTL dysfunction could be graded but become increasingly evident, ultimately causing failure to learn as the trace interval is increased.

Age-Related Heterogeneity in Conditioning

The results suggest that aged rats constitute a heterogeneous group with respect to conditioning impairments. The use of four different age groups allowed finer temporal resolution than is typical of aging studies in rats. Five of the 8 aged animals were categorized as impaired, meaning that the mean percentage of

Table 3
Gross Baseline Activity Is Unaltered During Aging

Age group (*n*)	Number of grid crossings
Adult (8)	14.1 ± 1.8
Early middle-aged (8)	11.9 ± 1.8
Late middle-aged (8)	14.9 ± 1.8
Aged (8)	18.0 ± 1.7

Note. Baseline activity was determined during the first minute each rat spent in the conditioning chamber prior to the first training trial. Scores are the number of times each rat crossed a grid line on the video monitor (see Method section). There were no statistically significant differences in the number of grid-line crossings as a function of age, $F(3, 28 = 2.08$, $p = .13)$.

freezing was more than two standard deviations below the mean of the adult rats (see Method section). However, the other 3 aged rats exhibited freezing levels comparable to those of adult rats. The coefficient of variation (standard deviation divided by the mean) of freezing to the cue was 0.9 ± 0.2 in aged rats, as compared with 0.2 ± 0.1 for adult rats, 0.2 ± 0.1 for the early middle-aged rats, and 0.3 ± 0.1 for the late middle-aged rats. To some extent, a ceiling effect could be contributing to this aging-related increase in the coefficient of variation, resulting from the high freezing levels that are characteristic of adult and middle-aged rats. However, others have also reported heterogeneity in trace-cue conditioning in aged rats and rabbits (e.g., see McEchron et al., 2004; L. T. Thompson et al., 1996).

Because a larger pool of case studies could have significance for cognitive aging, it is therefore worthwhile summarizing some statistics regarding individual animals. Two of the 8 early middle-aged rats were also classified as impaired in trace fear conditioning. Both impaired rats in this group were 1 year old (mean age = 12.1 months), suggesting that heterogeneity in learning ability may begin to emerge around this age. Two of the eight late-middle-aged rats were also classified as impaired. Both animals were approximately 20 months of age (mean age = 20.6 months). Freezing in the middle-aged groups of rats was lower than in adults (see Figure 1), but the differences were not statistically significant. The youngest of the aged rats used in the present study was 26 months old.

A similar age-related increase in heterogeneity was evident in context conditioning. The coefficient of variation of freezing to the context was 1.0 ± 0.3 in aged rats, as compared with 0.3 ± 0.1 for adult rats, 0.2 ± 0.1 for the early middle-aged rats, and 0.6 ± 0.1 for the late middle-aged rats. The results of both trace-cue and context testing are consistent with the possibility that age-related group differences in conditioning emerge from relatively abrupt changes in individual animals relative to the sampling intervals used in the present study. In terms of understanding the neurobiological mechanisms underlying aging-related cognitive decline, the distinction between impaired and unimpaired may prove to be more valuable than correlations with the age of the animal (see Nicholson, Yoshida, Berry, Gallagher, & Geinisman, 2004; Rapp, Deroche, Mao, & Burwell, 2002; Tombaugh, Rowe, & Rose, 2005).

Freezing and Ultrasonic Vocalizations as Conditional Responses in Aging Studies

The present study measured both freezing and USV as CRs for fear conditioning. Levels of freezing during the context and trace-cue test were negatively correlated with age ($r = -.59$ and $-.56$, respectively) and positively correlated with each other ($r = .52$). In contrast to freezing, only adult rats elicited USV CRs (see Figure 3). One interpretation of the failure to vocalize in older animals is that the circuitry responsible for generation of conditioned 22-kHz USVs has a higher activation threshold and possibly somewhat different requirements than the circuitry responsible for the expression of freezing.

Although emission of USVs has been shown to be closely related to freezing in adult rats as fear-related CRs (Choi & Brown, 2003; Lee et al., 2001; Lindquist & Brown, 2004), the motor circuits responsible for producing freezing (Bandler, Depaulis, & Vergnes, 1985) appear to be separable from those controlling USV (Brudzynski, 1994; Brudzynski & Barnabi, 1996). Notably, although amygdala-lesioned rats do not exhibit freezing or USV as CRs following fear conditioning, these animals are still capable of exhibiting freezing and USV as URs after ejaculation (Choi & Brown, 2003). The present results suggest that USV may not be an ideal dependent measure for aging studies. This is particularly true when the goal is to observe an aging-related deficit in as few training trials and sessions as possible, a circumstance that could be pertinent to rapid screening of cognitive enhancers.

Conclusions

Aged Sprague–Dawley rats are significantly impaired in trace and contextual fear conditioning. The aging-related deficits in trace conditioning did not result from sensorimotor deficit or a general fear-conditioning deficit because the aged rats readily learned both a short- and a long-delay task. In addition, aged rats appeared to be normally sensitive to the footshock US. Furthermore, there were no significant group differences in gross activity levels measured just before conditioning. The results demonstrate that the trace interval, rather than the use of a long ISI, is responsible for the aging-related deficits in trace fear conditioning. Whereas we used a 30-s trace interval, other findings suggest that short trace intervals (1–3 s) are not a sensitive measure of aging-related MTL dysfunction. These data add to the growing body of evidence suggesting that trace and context fear conditioning may be useful for studying the neurobiology of aging.

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