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Strain and sex differences in fear conditioning: 22 kHz ultrasonic vocalizations and freezing in rats

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Abstract

Strain and sex differences in fear conditioning were investigated in two commonly used laboratory rats: Sprague Dawleys and Long-Evans. Twenty-two kHz ultrasonic vocalization (USV) distress calls and freezing behavior were used to measure fear responses to contextual and auditory conditioned stimuli (CSs), which were previously paired with a footshock unconditioned stimulus (US). Both strain and sex had significant effects on USVs and freezing during training and subsequent context and tone tests. Overall, the male Sprague Dawley rats froze and emitted USVs more than the other groups. Additionally, levels of freezing and USVs were differentially influenced by the type of CS (context or tone). These results suggest that species-specific defense responses in laboratory rats are highly influenced by the strain and sex of the subject, and that these factors should be considered in future fear conditioning studies. **Keywords:** classical conditioning, learning, memory, amygdala, hippocampus.

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Introduction

Fear conditioning is widely used in neurobiological studies of learning and memory because of two major characteristics. Procedurally, there are only two stimuli involved, which are well-defined and can be precisely controlled, and the association between the conditioned stimulus (CS) and the aversive unconditioned stimulus (US) is both rapidly learned and long-lasting (Kim & Jung, 2006). Functionally, it occurs in nearly every animal group that has been studied (e.g. fruit flies, snails, fish, birds, rabbits, monkeys, rats, and humans) and the neural pathways for fear conditioning are very similar in all mammals (LeDoux, 1994). Thus, many of the findings from laboratory animals can be applied to humans with potential clinical implications to anxiety, phobias, posttraumatic stress disorders, and panic attacks (Fendt & Fanselow, 1999; Maren, 2001).

In laboratory settings, rodents are typically conditioned to associate discrete (such as tone and light) and non-discrete (such as context) CSs with an aversive US (such as footshock and loud noise) (Fanselow, 1984b). Freezing, a species-specific defensive response to a danger-eliciting stimulus (Bolles, 1970), is the most widely used measure of fear in rats and mice (Blanchard & Blanchard, 1969; Bolles & Fanselow, 1980; Chen, Kim, Thompson, & Tonegawa, 1996). This immobile crouching behavior is easy to measure, via human observation (e.g., Blanchard & Blanchard, 1969) and computer automation (e.g., Kim, Rison, & Fanselow, 1993), and does not require invasive procedures. More recently, 22-kHz ultrasonic vocalization (USV) calls have been adapted as an additional noninvasive fear measure in rats (e.g., Blanchard, Blanchard, Agullana, & Weiss, 1991; Lee, Choi, Brown, & Kim, 2001). Similarly to freezing behavior, USVs are a reflexive response to stressful and potentially hazardous situations (Brudzynski & Ociepa, 1992; Kim, Koo, Lee, & Han, 2005). Specifically, USVs have been observed in the presence of predators (Blanchard, Blanchard, Aguallana, & Weiss, 1991; Brudzynski, Bihari, Ociepa, & Fu, 1993), in response to painful or alarming stimuli (e.g. footshock and airpuff; Brudzynski & Holland, 2005), after intracerebral injection of the cholinergic agonist carbachol (Brudzynski, Ociepa, & Bihari, 1991), and post-ejaculation (Bruzdynski, 2005).

Generally, a single measure of fear, such as freezing, is assessed in fear conditioning studies. However, Antoniadis and McDonald (1999) found that multiple fear responses from the same animals have different learning parameters, with freezing, urination and locomotion showing fast acquisition rates while heart rate, USVs and defecation exhibiting slower rates of acquisition. These differences in fear measures suggest the possibility that different neural

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substrates are engaged during fear conditioning. Thus, the use of multiple measures of fear is likely to give a more complete assessment of fear conditioning and its underlying mechanisms.

In fear conditioning, it is also important to consider strain and sex differences. There is evidence of greater levels of freezing and less sensitivity to opioid antagonists by Sprague Dawleys relative to other strains (Helmstetter & Fanselow, 1987). Other evidence suggests that male rodents freeze more frequently and for longer durations than females to frightening stimuli (Archer, 1975). Closer examination of this sex difference in freezing has revealed that although males condition to a context CS more rapidly than females (i.e., freeze more given shorter pre-shock context exposure), if given enough time to explore the context before the shock, both sexes will freeze to a similar asymptote (Maren, De Oca, & Fanselow, 1994; Wiltgen, Sanders, Behne, & Fanselow, 2001). Gupta, Sen, Diepenhorst, Rudick and Maren (2001) also found that male rats exhibited significantly higher levels of contextual freezing than female rats. As a possible explanation, they suggested that estrogen in female rats exerts an inhibitory influence on both contextual fear conditioning and perforant path-granule cell LTP, a cellular process positively correlated with fear conditioning. Another study examined sex differences of three strains of rat (i.e., Wistar, Lewis, and Fischer) and found that Fischer male rats froze more than Fischer females in both context and tone tests, while the other two strains did not show sex differences in freezing to either context or tone CSs (Pryce, Lehmann, & Feldon, 1999). There are also sex differences in how stress influences subsequent learning; stress enhances aversive eyeblink conditioning in male rats but impairs it in female rats (Bangasser & Shors, 2007; Wood & Shors, 1998).

Based on these findings of strain and sex differences in freezing, the present study tested its generalizability to another non-invasive fear response measure, the USV. To do so, USV and freezing responses were simultaneously measured in male and female Long-Evans and Sprague Dawley rats during fear conditioning to tone and context CSs.

Method

Subjects

Forty-one experimentally naïve rats, comprising 10 male and 11 female Sprague Dawleys and 10 male and 10 female Long-Evans (initially weighing 250-300 gm; Charles River, Boston, MA), were individually housed in an Association for Assessment and Accreditation of

Laboratory Animal Care (AAALAC) accredited animal care facility located in the Department of Psychology, Yale University. The animals were given *ad libitum* access to food and water, except during behavioral testing, and handled daily for 7 days prior to the start of the experiment. Behavioral training and testing were conducted during the light phase of the 12 hour light:dark cycle (light on at 7 AM) and in strict compliance with the Yale University Institutional Animal Care and Use Committee guidelines.

Apparatus and Procedure

Two very distinct chambers (A and B), each equipped with speaker modules (Coulbourn Instruments, Allentown, PA) and located in a controlled acoustic room (Industrial Acoustics, New York, NY), served as the contexts for training and testing. Chamber A was rectangular (27 cm width X 28 cm length X 30.5 cm height), had front and back walls made of clear Plexiglas and side walls made of aluminum, had a grid floor consisting of 16 stainless steel bars (4.5 mm diameter) spaced 17.5 mm center-to-center and connected to a Coulbourn precision-regulated animal shocker, had the tone and light modules inserted in the upper right side wall, and was placed into a white isolation box (46 cm width X 53 cm length X 49 cm height). Chamber B was octagonal (26.5 cm diameter X 25 cm height), had all clear Plexiglas walls, had a grid floor consisting of 17 stainless steel bars (5 mm diameter) spaced 15 mm apart and wired to a Coulbourn precision-regulated animal shocker, had the tone and light modules placed in the upper rear wall, and was placed in a black isolation box. The overhead light in the isolation room was on for the sessions run in Chamber A and off for the sessions run in Chamber B; the walls of Chamber A were wiped with 5% ammonium hydroxide, and the walls of chamber B were wiped with 1% acetate.

On training day (Day 0), rats were placed in Chamber A. After a 1-minute baseline period, 10 presentations of a 10-second tone (2 kHz, 80 dB) that overlapped and coterminated with a 1-second footshock (1 mA) were given to the rats. The inter-trial interval (ITI) was 1 minute. Animals were removed 1 minute after the last shock and returned to their home cages. On context testing day (Day 1), rats were placed back in the trained context for 8 minutes without tone or footshock. On tone testing day (Day 2), animals were placed in the novel Chamber B for 1-minute baseline period followed by 8 minutes of continuous tone.

One week later, both the context and tone tests were re-administered (Days 7 and 8) following the same procedures described above.

Behavioral Data Collection

The freezing data were collected by an IBM-PC equipped with the Coulbourn LabLinc Habitest Universal Linc System. Although the collection of the USV and freezing data were fully automated, each session was recorded for off-line video and audio analyses, if necessary, using an infrared light source and miniature video camera (CB-21; Circuit Specialists, Mesa, AZ).

The freezing behavior was assessed using a 24-cell infrared activity monitor mounted on top of each chamber. The monitor detects movement of emitted infrared (1300 nm) body heat images from the animals in the *x, y,* and *z* axes (cf., Koo, Han, & Kim, 2004; Lee & Kim, 1998). In brief, the total time of inactivity exhibited by each animal was measured using a computer program, and freezing was defined as continuous inactivity lasting at least 3 seconds. Any behavior that yielded an inactivity of < 3 seconds was recorded as general activity.

The USV data were collected using a Mini-3 heterodyne bat detector (Noldus Information Technology, Wageningen, The Netherlands) that transformed high-frequency (22 ± 5 kHz) vocalizations into the audible range. The output of the bat detector was fed through an audio amplitude filter (Noldus Information Technology), which filtered out signals falling below an amplitude range that was individually adjusted for each animal. The resulting signals were then sent to an IBM-PC equipped with Noldus UltraVox vocalization analysis software, which recorded the signals as USV onset times if the duration of a signal was ≥ 30 msec and as offset times if the onset of the ensuing episode was ≥ 40 msec prior. If the interval between onset times was less than 40 ms, then the two vocalizations were counted as a single USV episode (cf., Lee et al., 2001).

Statistics

Five animals were excluded due to technical malfunctions during training. The group sizes for final analyses were 8 Sprague Dawley (SD) males, 10 SD females, 9 Long-Evans (LE) males and 9 LE females. Freezing and USV data were analyzed with a two-way ANOVA (sex and strain). All *post hoc* analyses were run with Tukey's honestly significant difference (HSD).

Results

Freezing

Figure 1A presents the mean percentage freezing exhibited by SD male, SD female, LE male and LE female rats during the one minute baseline and the 10 ITIs separating the tone-shock pairings. All groups showed little freezing during the baseline period. However, they all rapidly came to freeze significantly during the postshock periods. A two-way ANOVA (strain and sex as factors), with time as a repeated measure, confirmed a main effect of time, F (10, 320) = 62.44, $p < .001$. There was no main effect of strain, F (1, 32) = 2.22, $p > .05$, no main effect of sex, F (1, 32) = 1.47, $p > .05$, but a significant interaction between sex and strain, F (1, 32) = 9.61, $p < .01$. *Post hoc* analyses with Tukey's HSD revealed that SD females froze significantly less than both the SD males and the LE females (ps < .05).

In the initial context test (Figure 2A), there was a significant main effect of strain: SD rats (73.18 ± 6.18, *M* ± *SEM*) froze more to the context than did the LE rats (49.62 ± 6.14), F (1, 32) = 7.31, $p < .05$. However, there was no main effect of sex, F (1, 32) = 2.42, $p > .05$, and no interaction between sex and strain, F (1, 32) = 1.33, $p > .05$. During the repeated context test one week later, once again there was a significant main effect of strain: SD rats (56.40 ± 5.96) froze more than LE rats (17.60 ± 5.92), F (1, 32) = 21.34, $p < .01$. In addition, males (47.28 ± 6.10) froze reliably more than females (26.72 ± 5.77), F (1, 32) = 6.00, $p < .05$. However, there was no interaction between sex and strain, F (1, 32) = 0.07, $p > .05$.

Freezing levels during initial and repeated tone tests are shown in Figure 2B. During the first tone test, there was a significant main effect of strain: SD rats (56.75 ± 5.64) froze more than LE rats (30.87 ± 5.61), F (1, 32) = 10.59, $p < .05$. However, there was no reliable main effect of sex, F (1, 32) = 0.04, $p > .05$, and no reliable interaction between sex and strain, F (1, 32) = 1.33, $p > .05$. During the repeated tone test, SDs rats (25.31 ± 5.08) again froze significantly more than LE rats (3.53 ±

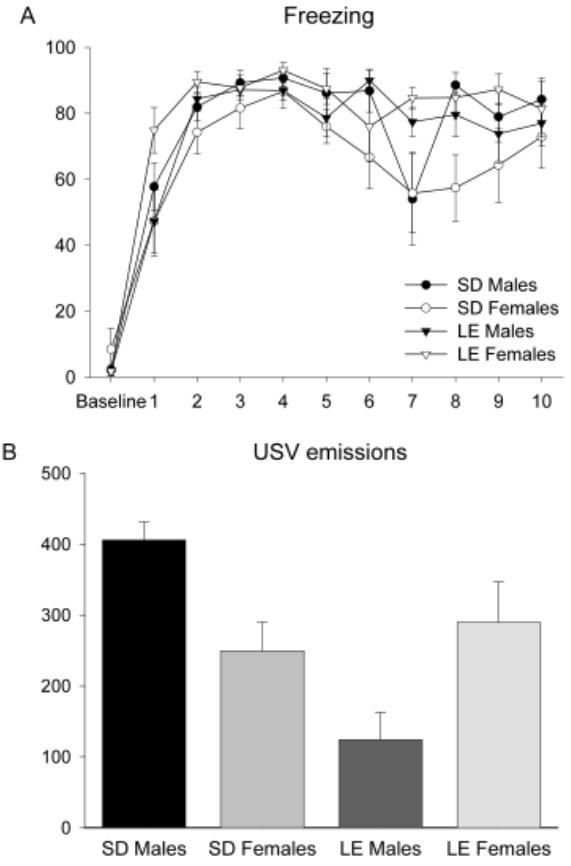

Figure 1. Fear conditioning in male and female Sprague Dawley (SD) and Long-Evans (LE) rats, as assessed by freezing and USV. A. The mean percentage (± *SEM*) of freezing during one minute baseline and 10 ITIs intervening 10 tone-shock pairings. B. The mean total duration (± *SEM*) of USV emitted during training.

5.05), F (1, 32) = 9.23 p < .01. Overall, females (22.20 ± 4.92) froze more than males (6.65 ± 5.21), F (1, 32) = 4.91, p < .05. There was no significant interaction between sex and strain, F (1, 32) = 3.59, p > .05.

To compare freezing trends to context and tone, freezing levels of animals were pooled during the initial context and tone tests (Figure 4). Overall, rats froze more to the context (61.30 ± 4.81) than to the tone (44.02 ± 4.46), t (70) = 2.63, p < .05.

Ultrasonic Vocalization

During training, there was a difference in total USV call duration among the groups (Figure 1B). An ANOVA revealed a significant main effect of strain: SD rats (327.56 ± 30.38) emitted USVs for longer durations than did the LE rats (206.98 ± 30.19), F (1, 32) = 7.93, p < .01. Specifically, SD males (405.58 ± 45.29) emitted more USVs than did LE males (124.43 ± 42.70, Tukey's HSD, p < .01). There was no main effect of sex, F (1, 32) = 0.01, p > .05. There was a significant interaction between sex and strain, F (1, 32) = 14.06, p < .01. LE females vocalized reliably more than LE males (p < .05), though there was an opposite trend among the SD rats (Tukey's HSD, p > .05).

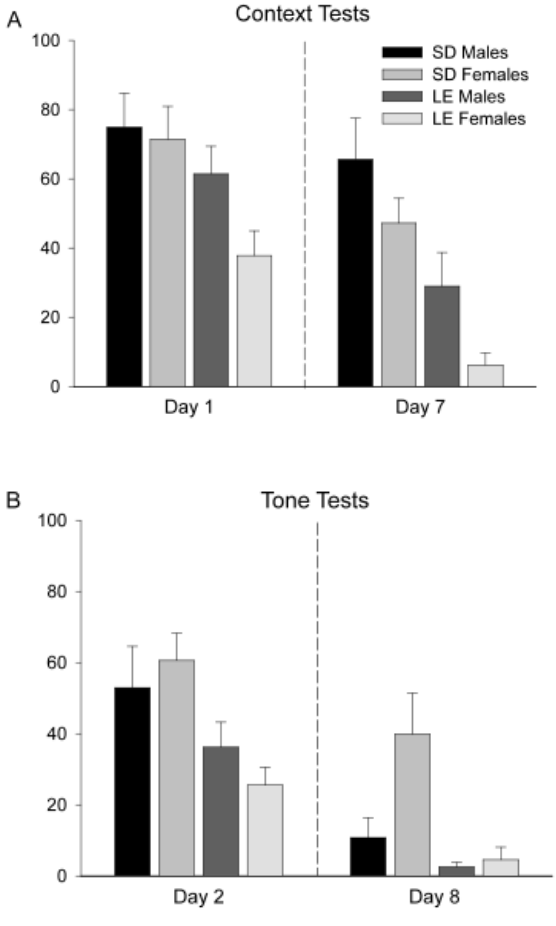

Figure 2. Conditioned freezing in SD male, SD female, LE male and LE female rats. A. The mean percentage (± *SEM*) of freezing during initial (Day 1) and repeated (Day 7) context tests. B. The mean percentage (± *SEM*) of freezing during initial (Day 2) and repeated (Day 8) tone tests.

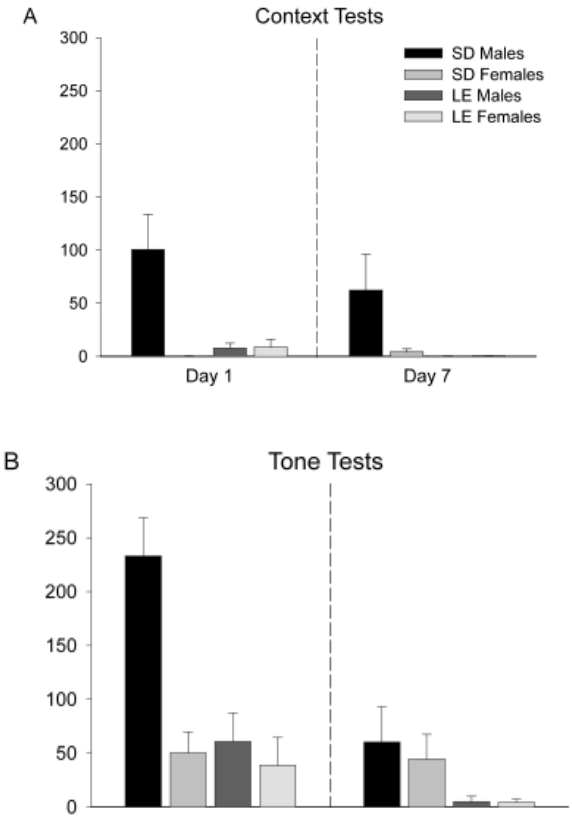

Figure 3. Conditioned USV in SD male, SD female, LE male and LE female rats. A. The mean total duration (± *SEM*) of USV during initial (Day 1) and repeated (Day 7) context tests. B. The mean total duration (± *SEM*) of USV during initial (Day 2) and repeated (Day 8) tone tests.

Figure 3A presents the mean durations of USVs emitted by each of the four groups during both initial and repeated context tests. During the initial test, there was a significant main effect of sex, with males (54.22 ± 11.09) vocalizing more than females (4.29 ± 10.48), F (1, 32) = 10.71, p < .01, and a main effect of strain, with SD rats (50.39 ± 10.82) vocalizing more than LE rats (8.12 ± 10.76), F (1, 32) = 7.68, p < .01. There was also a significant interaction between sex and strain, F (1, 32) = 11.04, p < .01. Post-hoc analyses revealed reliably more vocalization by the SD males than all three other groups (Tukey's HSD, ps < .01). Results for the repeated context test were similar, though with lower overall USV durations. There was a significant main effect of strain: SD rats (33.23 ± 10.68) vocalized more than LE rats (0.21 ± 10.62), F (1, 32) = 5.90, p < .05. However, there was no main effect of sex, F (1, 32) = 3.68, p > .05, and no interaction between sex and strain, F (1, 32) = 3.73, p > .05.

USV durations during tone tests are presented in Figure 3B. In the initial tone test, males (146.83 ± 19.45) vocalized more than females (44.38 ± 18.39), F (1, 32) = 14.64, p < .01, SD rats (141.50 ± 18.99) vocalized

significantly more than LE rats (49.72 ± 18.87), F (1, 32) = 11.75, $p < .01$, and there was an interaction between sex and strain, F (1, 32) = 9.00, $p < .01$. As in the initial context test, these effects are due to SD males, who vocalized significantly more than all three other groups (Tukey's HSD, ps < .01). During the repeated tone test, SD rats (52.30 ± 13.93) vocalized more than LE rats (4.60 ± 13.84), F (1, 32) = 5.90, $p < .05$. There was no main effect of sex, F (1, 32) = 0.17, p > .05, and no sex by strain interaction, F (1, 32) = 0.14, p > .05. *Post-hoc* analysis found no differences between the four groups.

Vocalization levels of animals were pooled during the initial context and tone tests to compare USV trends to different cues (Figure 4). Overall, rats vocalized more to the tone (90.53 ± 18.16) than to the context (26.46 ± 9.91), t (70) = 3.10, p < .01.

Discussion

We evaluated fear conditioning in male and female SD and LE rats. Two frequently employed non-invasive fear responses, freezing and 22-kHz USV, were simultaneously monitored in the same rats during context and tone tests. Overall, SD rats froze and emitted USVs more than LE rats during both initial and repeated context and tone tests; across strains, males tended to freeze and vocalize more than females, except freezing during the repeated tone test. These results contrast with evidence that the effects of sex on fear conditioning are consistent between both contextual and discrete CSs within strains (Pryce et al., 1999). One influence on sex differences in contextual freezing may be the acquisition of contextual fear. Specifically, evidence suggests that males freeze more to context after one shock pairing, but males and females freeze comparably given three shock pairings (Maren et al., 1994). A similar finding has been reported in mice: males freeze more to context after shorter preexposure intervals (< 60 sec; Wiltgen et al., 2001). In the present study, rats were exposed to the context for one minute followed by 10 CS-US pairings

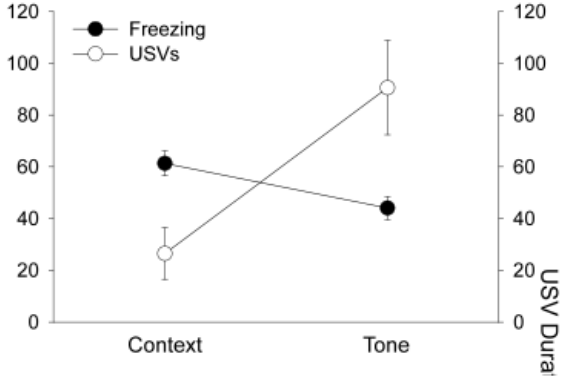

Figure 4. Overall mean (± *SEM*) percentage of freezing and total duration of USV during initial context and tone tests pooled across animals.

(1 min ITI) producing robust fear conditioning; this may explain why sex differences were not observed in the initial context test within the SD strain. Surprisingly, the females did not seem to extinguish their fear response as quickly as the males during the tone test, because they froze longer when tested one week later. Sex differences in contextual fear conditioning and in hippocampal long-term potentiation have been correlated, suggesting a neural basis for such differences (Maren et al., 1994). Based on our findings, it is likely that another neural variation between males and females underlies the observed difference in auditory fear conditioning. It does seem likely that gonadal hormones play a key role in these differences, whether by acting early on a developing brain, or by modulating behavior throughout adulthood. For instance, limbic regions subserving different aspects of fear conditioning (e.g.. contextual vs. discrete; immediate vs. long-term recall) seem to be distinctly susceptible to sex hormones (Toufexis, 2007). Certainly, more research is needed to determine the neural correlates of these sex differences along with the underlying hormonal or genetic influences.

The present data also suggest potential sex differences in extinction of conditioned fear. As measured in both freezing and USV responses, the male rats showed much faster extinction of fear responses to the tone than to the context: the level of reduced fear responses from the first to the second tone tests was much greater than the reduced level of fear responses from the first to the second context tests. In contrast, the female rats showed similar extinction rates to both the tone and the context. At this point, little is known about sex differences in fear extinction both on behavioral and neural levels. The hippocampus was shown to play an important role in contextual fear extinction (Corcoran, Desmond, Frey, & Maren, 2005). It is plausible then that the hippocampus contributes to the differential extinction rate to the context seen between the male and female rats, as the hippocampus is suggested to be important for the sex differences in the acquisition of contextual fear conditioning (Maren et al., 1994). The prefrontal cortex is also considered to play an important role in fear extinction (Sortes-Bayon, Bush, & LeDoux, 2004). A recent study showed that there is a sex difference in prefrontal cortical involvement in fear conditioning (Stark et al., 2006). Thus, the prefrontal cortex may also contribute to the sex differences in the rate of fear extinction. Together, our data identify sex differences in conditioned fear extinction and suggest the presence of a potential sex dependent neural mechanism for the fear extinction process.

Three possibilities can account for strain and sex differences in fear conditioning. The first possibility is that SD rats display more fear expression than LE rats. If true, this difference should generalize across other fear measures. Consistent with this view, Helmstetter and Fanselow (1987) found that the opioid antagonist naloxone blocked conditioned analgesia in LE (but not

SD) rats. The second possibility is that SD rats learn fear conditioning faster (and perhaps stronger) than LE rats. In support, SD rats emitted more USVs during training than LE rats, although there was no difference in postshock freezing (possibly due to a ceiling effect). The third possibility is a difference in sensitivity to footshock pain. For example, if SD rats are more sensitive to footshocks than LE rats, then SD rats would probably produce greater amounts of fearful behaviors to the conditioned stimulus. To quantify pain sensitivity, we measured latency to resume post-shock freezing during training (Fanselow, 1984a). Neither strain nor sex affected latency to resume freezing, nor were any individual groups (e.g. SD males) significantly different from one another (data not shown). In a separate study from our lab (Kosten, Lee & Kim, 2006), SD female rats responded at lower shock intensity levels compared to SD male rats when a series of footshocks with incremental intensity was administered. If the shock sensitivity is directly related to the amount of conditioned fear responses, then SD female rats should display more conditioned fear responses compared to SD male rats. However, our previous and current data showed that SD female rats showed either less or equivalent, but not more, conditioned fear responses than those of the SD male rats. Thus, we conclude that the strain and sex effects observed in the context and tone tests reflect group differences in the production of fear responses and/or learning, but not pain sensitivity.

In addition to strain and sex variations in fear behavior, the overall amount of freezing and vocalizing differed between the context and tone tests. Rats vocalized more to the tone than to the context, and froze more to the context than to the tone. A hypothesized explanation for this result is that USVs are a defensive response to nearby predators (e.g. discrete stimuli in a lab setting), and freezing is a defensive response to distant predators (e.g. context; Bolles & Fanselow, 1980; Borszcz, 1995). Rats often use multiple defensive mechanisms simultaneously, but the degree to which they use them depends on the nature of the fearful stimulus. Others have concluded that USVs represent a state of anxiety rather than fear, based on evidence that vocalizations are more prevalent during inter-trial intervals of fear conditioning sessions than following the onset of the CS (Jelen, Soltysik, & Zagrodzka, 2003). While amount of freezing may represent differences in intensities of the two CSs (Santos, Gárgaro, Oliveira, Masson & Brandão, 2005), it is possible that USVs are representing an alternate gradient, such as anxiety level. However, it would be surprising if rats were less anxious when presented with a CS perceived as more intense (context). Accordingly, we think that the most likely explanation for this difference is not emotional state or intensity of the CS, but the type of perceived threat as represented by the two stimuli.

Bolles and Fanselow (1980) have identified that defensive behavior is both strain- and species-specific. Our results suggest that fear conditioning studies should also take into consideration that strain and sex of rats, as well as the type of fear response observed to different CSs, can influence both behavioral and neurobiological results. For example, if one studies fear conditioning only by measuring USV, our evidence suggests that male Sprague Dawley rats would be ideal subjects. In studies that require strong (or few trials to reach asymptote) or weak fear conditioning (or many trials and preclude a ceiling effect), SD rats and LE rats, respectively, would be ideal. More research is needed to determine the underlying mechanisms of these differences in fear responses. This question is particularly relevant to the sex effect on fear conditioning in rats, because such explanations may inform the nature of sex differences across a broad range of species.

Author note

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