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Individual Differences in Ultrasonic Vocalizations and Freezing Behavior During Fear Learning and Extinction in Female Rats

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By

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Abstract

Although many people experience traumatic events, only 10%-20% go on to develop post-traumatic stress disorder (PTSD). Women are particularly susceptible, with a prevalence rate double that of men, even when controlling for type of trauma exposure. This disparity suggests the existence of distinct neurobiological processes, particularly related to sex, that predispose some individuals to be more resistant to extinguishing learned fear. Similar differences exist in rodent fear conditioning and extinction, though female rodents are considerably understudied. We hypothesized that female rodents would exhibit individual differences in fear extinction similar to those that we have observed previously in males. The present study examined freezing behavior, plus both 22 kHz and 50 kHz ultrasonic vocalizations (USVs) of female Long Evans rats (N=14) during acquisition of fear conditioning and cued fear extinction. Similar to prior studies in males, rats were divided into extinction competent (EC) and extinction resistant (ER) phenotypes based on a median split of freezing behavior during the last ten tone presentations of the extinction trial. Similar to males, in females freezing behavior during fear learning did not differ between EC and ER groups, but there was a difference during cued fear extinction. During fear acquisition trials, all female rats produced 50 kHz USVs, which were emitted mostly during the unconditioned period prior to the tone-shock pairings. During fear acquisition, EC rats also emitted significantly more 50 kHz USVs than ER rats during the unconditioned freezing period and in total throughout the trial. Both number and duration of 50 kHz calls differed between EC and ER groups over time. Only about half the female rats exhibited “distress” USVs in the 22 kHz range, and only during tone-shock pairings during fear acquisition and tone presentations during extinction learning. There was no significant difference in number of 22 kHz USVs between ER and EC groups. These results suggest that like males, female rodents show individual differences in both freezing and vocalizations during fear learning and extinction, although males appear to vocalize more in the 22 kHz range while females show a distinct pattern and higher number of 50 kHz USVs.

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Introduction

Post-Traumatic Stress Disorder and the Fear Conditioning Model

It is an unfortunate reality that many people experience trauma. In fact, over 70% of adults worldwide will experience a traumatic event in their lifetime (Benjet et al., 2016). While most people who are exposed to trauma will return to normal functioning after a short period of emotional recovery, approximately 10%-20% of these individuals will develop post-traumatic stress disorder (PTSD) (Ross et al., 2017). PTSD is a chronic and often debilitating psychiatric disorder that can develop following exposure to death, threatened death, actual or threatened serious injury or sexual violence, either directly or indirectly. The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) identifies four primary categories of symptoms: intrusion symptoms (e.g. flashbacks, nightmares), avoidance of trauma-related stimuli, negative alterations in cognition and mood (e.g. inability to recall key features of the trauma, negative affect), and alterations in arousal and anxiety (e.g. hypervigilance, insomnia, risky behavior) (American Psychiatric Association, 2013). Further, about 50% of people diagnosed with PTSD experience comorbid depression, anxiety, or substance use disorders (Pietrzak et al., 2011).

One of the dominant neurobiological models used to explain PTSD is that of dysregulated fear learning and extinction. People readily learn to associate neutral stimuli with painful or dangerous stimuli, and will subsequently experience fear when the neutral stimulus is present (i.e. fear learning or conditioning). Rapid fear learning is highly adaptive in the appropriate context, with the purpose of promoting behaviors that help an individual to avoid situations that have proven to be harmful in the past (Steimer, 2002). However, once the neutral stimulus is continuously encountered on its own, with no negative consequences, individuals

should be able to learn that the stimulus is no longer predictive of danger. This process is referred to as extinction learning. Exposure therapy, a common PTSD treatment, follows the same principle (Fucich et al., 2016). Fear extinction is not forgetting or undoing the original fear memory, but rather forming a new memory, stronger than the old one, that the conditioned stimulus no longer predicts harm (Holmes & Singewald, 2013). This is where individual differences emerge; some individuals are resilient and can extinguish their fear responses quickly, while others continue to experience fear (Norrholm et al., 2011). Some support for this conceptualization of PTSD comes from fear conditioning studies in humans. During both conditioning and extinction trials, PTSD patients exhibit greater fear-potentiated startle (Jovanovic et al., 2010; Norrholm et al., 2011) and elevated skin conductance (Blechert et al., 2007; Orr et al., 2000; Peri et al., 2000), and take longer than controls to extinguish these responses.

A similar experimental Pavlovian learning paradigm has guided much of the preclinical rodent research in this field. A typical Pavlovian fear learning and extinction study, such as used in our studies, might go as follows: during the fear learning phase, also called acquisition, rodents are repeatedly exposed to some noxious stimulus, such as a foot shock, in conjunction with a neutral stimulus, such as a tone. The noxious stimulus is the unconditioned stimulus (US, foot shock) that normally induces a fear behavior, while the tone represents the conditioned stimulus (CS, tone). The rodents quickly begin to exhibit fearful behavior (e.g. freezing, 22 kHz ultrasonic vocalizations) in response to the tone-shock pairing. Then, after a short period (usually 24-48 hours), animals are exposed to the tone (CS) without any shock to assess cue-conditioned responses (i.e. how much fearful behavior is now elicited by the tone or CS alone). With repeated exposure to the tone sans foot shock, rodents will eventually stop producing fearful

behavior in response to the tone, demonstrating extinction learning. It is during this phase of the experiment that individual differences are usually observed, with some rodents exhibiting more cue-induced fearful behavior than others, and taking longer to extinguish these responses (Bush et al., 2007; Galatzer-Levy et al., 2013; Monfils et al., 2019; Sharko et al., 2017; Shumake et al., 2014). Based on these phenotypes, rodents can reliably be categorized as either extinction competent (EC) or extinction resistant (ER).

Fear is traditionally measured in rodents in terms of freezing behavior, defined as the cessation of all movement not necessary for respiration (Gruene et al., 2015; LeDoux, 1994; Sharko et al., 2017). High freezing during fear learning is thought to reflect a strong fear association, while low freezing is interpreted as poor fear learning. Conversely, during extinction trials with repeated presentations of the CS in a safe environment, rodents who exhibit reductions in freezing behavior are thought to have successfully created a new extinction memory that outcompetes the fear learning memory. Individuals that sustain freezing behavior (sometimes called “high freezers”) despite the lack of negative consequences during cue presentation are considered to have an impaired ability to extinguish learned fear (Holmes & Singewald, 2013).

A more recently recognized behavioral fear response is darting, in which a rodent rapidly runs forward, likely as an attempt to escape (Gruene et al., 2015). Darting behavior is noteworthy because it is essentially the opposite of freezing, yet potentially reflects the same type of sympathetic nervous system arousal. In studies where freezing is the only measure of fear learning, rats who exhibit darting behavior would be inaccurately categorized as having a learning deficit. Furthermore, perhaps quite consequentially, female rats appear to dart more and freeze less than males during fear conditioning (Gruene et al., 2015).

Another measure of fearful behavior is ultrasonic vocalizations (USVs). Rats emit a wide range of USVs, but they typically produce long (>300 ms) vocalizations around 22 kHz (ranging from 18-32 kHz) in distressing situations (e.g. receiving an electric shock, smelling a predator) (Portfors, 2007). See Figures 1 and 2 for examples. These and other rodent USVs are hypothesized to function as an index of affective state and/or as a social signal (Knutson et al., 2002; Litvin et al., 2007; Sangiamo et al., 2020). It is important to note that freezing and 22 kHz USVs do not always occur simultaneously, and some rats will exhibit one behavior but not the other, suggesting distinct neurobiological mechanisms (Shumake et al., 2014). These 22 kHz USVs, primarily associated with avoidance behavior in a number of social and non-social contexts, may provide information about the affective state of the rats that freezing does not (Brudzynski, 2001).

Adult and juvenile rats can produce another type of vocalization: the 50 kHz USV. These USVs are shorter (20-80 ms) and range from 32-70 kHz (Figures 1 and 2). Rats produce 50 kHz USVs in a variety of social situations, usually relating to a potential reward (e.g. juvenile play, initiation of mating, being tickled by a familiar human). They can also be induced pharmacologically, such as with amphetamine injections to the nucleus accumbens (NAcc) (Burgdorf et al., 2001), and with electrical stimulation to the ventral tegmental area (VTA) or lateral hypothalamus (LH) (Burgdorf et al., 2000). The NAcc, VTA, and LH are all components of the reward system, and activation of these brain regions is usually associated with approach behavior in rats (Knutson et al., 2002). Given the correlation between emission of 50 kHz USVs and approach behavior, these vocalizations are often deemed reflective of positive affect, and are not generally reported during exposure to fearful stimuli.

Neuroanatomy of Fear Learning and PTSD

These rodent studies have yielded results critical for understanding the neurobiological underpinnings of fear conditioning and extinction. The amygdala in particular has been implicated in emotional learning, especially fear. A key component of the limbic system, the amygdala is a collection of nuclei located deep in the anteromedial temporal lobe (A. McDonald, 2003). Though the amygdala can be divided into several different sub-regions, in the context of fear learning, the central (CeA), lateral, and basolateral (BLA) nuclei are most pertinent (LeDoux, 2000). Sensory input (i.e. tone) travels through the auditory association areas of the cortex and thalamus and projects to the lateral nuclei of the amygdala (McDonald, 1998). The lateral nucleus projects to the BLA, which modulates the information and then sends it to the CeA (LeDoux, 1994). The central nucleus projects directly to the brainstem and other brain regions, which produce both sympathetic nervous system arousal and fear behaviors.

Lesioning the BLA produces deficits in ability to learn conditioned fear (Gale et al., 2004; Koo et al., 2004), and evidence from pharmacological studies suggest the BLA is also especially important for the formation of extinction memories (Sierra-Mercado et al., 2011; Zimmerman & Maren, 2010). Neuroimaging studies show that people with PTSD have greater amygdala volume (Kuo et al., 2012) and experience increased activation of the amygdala in response to fearful faces (Felmingham et al., 2010). Amygdala activation and symptom severity in PTSD are positively correlated (Shin et al., 2006), although some studies have failed to replicate these results.

The prefrontal cortex (PFC), broadly, is the brain region involved with executive functioning, decision making, memory consolidation, and other complex cognitive processes. The medial prefrontal cortex (mPFC) is a sub-region of the PFC implicated in emotional

processing, and includes the anterior cingulate cortex (ACC), infralimbic cortex (IL), and prelimbic cortex (PL). When the amygdala is activated by a fear-provoking stimulus, it sends inhibitory signals to the mPFC, briefly limiting its function. The mPFC is then capable of "deciding" whether the fear is warranted (or to what degree); if not, it inhibits the amygdala, reducing fear. Evidence suggests that in individuals with PTSD, this feedback loop may be dysregulated; the amygdala is hyperactive, while the mPFC is hypoactive (Ross et al., 2017; Shin et al., 2004).

In rodents, pharmacological inactivation of the IL impairs extinction learning and extinction recall (Sierra-Mercado et al., 2011), while inactivation of the PL results in reduced fear expression (i.e. freezing) during extinction learning, but not extinction recall (Corcoran & Quirk, 2007; Sierra-Mercado et al., 2011). This suggests that the PL is necessary only for the expression of fear, but not the acquisition of fear learning. Human fear conditioning studies utilizing functional magnetic resonance imaging (fMRI) show that activity in the ACC is heightened in response to conditioned stimuli during both fear acquisition and extinction (Lang et al., 2009). Furthermore, those with PTSD experience reduced mPFC activation in response to various stressful cues (Shin et al., 2006), have smaller ACC volumes (Rauch et al., 2003; Woodward et al., 2006), and that ACC gray matter volume is negatively correlated with symptom severity (Yamasue et al., 2003).

The hippocampus is another brain region critical for learning and memory. While the hippocampus is widely known for its role in the consolidation of explicit, episodic memories, it is also involved in the formation and retrieval of emotional memories. The hippocampus receives input from the PFC, especially during fear acquisition (Lang et al., 2009), and projects to the lateral and basal nuclei of the amygdala, where stimuli may be ascribed emotional significance

(LeDoux, 1994; Pitkänen et al., 2000; Xu et al., 2016). Neuroimaging studies of people with diagnosed PTSD often show reduced hippocampal volume (Bremner et al., 2003; Wignall et al., 2004) and less activation in the hippocampus during fear extinction compared to controls (VanElzakker, Dahlgren, Davis, Dubois, & Shin, 2014). One study found that not only do veterans with PTSD have smaller (by ~10%) hippocampi than veterans without PTSD, but so do their non-combat-exposed monozygotic twins, indicating that reduced hippocampal volume may be a risk factor for PTSD that precedes trauma exposure (Gilbertson et al., 2002).

In fear learning, the hippocampus seems to be particularly important for learning the context in which the conditioning takes place (LeDoux, 1994; Shalev et al., 2017; Xu et al., 2016). Fear extinction is also context dependent; normally, after extinction learning, rats will still exhibit a fear response if they encounter the conditioned stimulus (i.e. tone) in a different context than the one associated with extinction. Lesioning or inactivating the hippocampus in rodents impairs retrieval of the fear memory in a novel context, or in other words, disrupts the context-specific expression of fear extinction (Corcoran & Maren, 2001; Ji & Maren, 2005).

Sex Differences in PTSD and Fear Conditioning

Notable sex differences have been observed in the prevalence and symptomology of PTSD, as well as in both rodent and human fear learning paradigms. Women are twice as likely to develop PTSD compared to men (Ramikie & Ressler, 2018), though the probability varies considerably depending on the type of trauma. For example, after a physical assault, 2% of men will develop PTSD, versus 22% of women (Kessler et al., 1995). These differences may be explained by both social and biological factors. Despite men being exposed to more traumatic events than women overall (e.g. military combat, accidents, fights), women more frequently encounter interpersonal trauma, like sexual assault and domestic abuse. History of sexual

violence is the greatest risk factor for PTSD (Norris, 1992), and seeing as women are more likely to be victims of sexual violence, this may explain the disparity in prevalence. However, it seems that even when controlling for type of trauma, women have a higher risk of developing PTSD (Tolin & Foa, 2006), suggesting a distinctly female vulnerability (Ramikie & Ressler, 2018).

Furthermore, mounting evidence suggests that men and women with PTSD often present different symptoms. Women seem to experience more overall distress than men (Guina et al., 2016; Hourani et al., 2015), report worse impairment in concentration (Gay et al., 2020; King et al., 2013) and engage in both internal and external avoidance behaviors (Cao et al., 2019; Gay et al., 2020; Guina et al., 2016). Males tend to exhibit hypervigilance (Hourani et al., 2015; King et al., 2013), loss of interest and emotional numbing (Cao et al., 2019; Gay et al., 2020; King et al., 2013), and flashbacks (Cao et al., 2019; Gay et al., 2020). These diverging symptomologies, though not always consistent, may indicate sex-specific stress responses and etiologies.

In the few studies examining sex differences in fear responses in PTSD patients, the results are contradictory and thus largely inconclusive, although most studies demonstrate some type of significant sex difference. During fear acquisition, Inslicht et al. (2013) observed elevated skin conductance response (SCR) (i.e. enhanced fear learning) in women with PTSD compared to men with PTSD, while Shvil et al. (2014) saw greater SCR in men than women, both with and without PTSD. Shvil and colleagues also found that during the extinction recall phase of the experiment, men with PTSD exhibited greater SCR (i.e. impaired extinction recall) compared to women with PTSD and trauma-exposed controls of both sexes. When looking at fMRI data, men with PTSD had greater activation in the rostral dorsal ACC (a region often included as part of the mPFC) during extinction recall (Shvil et al., 2014). Felmingham et al. (2010) conducted an fMRI study comparing men and women with PTSD to trauma-exposed controls and non-trauma-

exposed controls while viewing fearful faces. Men with PTSD had greater hippocampal activation compared to women with PTSD. The brainstem, too, was differentially affected by fear stimuli; this hub of sympathetic nervous system arousal had enhanced activation in women who had been exposed to trauma, both with and without PTSD, while for men elevated brainstem activity occurred only in those with PTSD (Felmingham et al., 2010). Though the results of these studies are inconsistent, they demonstrate that sex differences are likely present in the prevalence, etiology and presenting symptoms of PTSD and warrant further investigation.

Fear conditioning studies comparing men and women without PTSD also show gender differences. During fear learning, although women report greater subjective fear or stress (Lonsdorf et al., 2015; Zorawski et al., 2005), they exhibit lower (Lonsdorf et al., 2015; Milad et al., 2006) or equal (Zorawski et al., 2005) SCR compared to men. These studies observed no significant sex differences during extinction learning (Lonsdorf et al., 2015; Milad et al., 2006; Zorawski et al., 2005).

Significant sex differences also exist in rodent fear conditioning studies, although similar to human studies, there is no consistent pattern of differences. Some studies report that females have enhanced fear extinction compared to males, while others report a reduction; these differences may be explained by variability in experimental paradigms, behavioral measures, animal strain, or sample size (Shansky, 2015). For instance, in studies measuring only freezing behavior, female rodents usually exhibit less freezing than males during cued fear learning (Baran et al., 2009; Gruene et al., 2015), which is interpreted as impaired fear learning. However, Colom-Lapetina et al. (2019) observed that during fear conditioning, female rats, in addition to freezing less, exhibited more darting than males. Females also performed a more diverse array of

active behaviors (e.g. climbing, head-shaking, diving) during a subsequent forced swim test, suggesting that each sex may utilize differential behavioral coping strategies when under stress.

Study Rationale

Nonetheless, prior studies have not consistently reported distinct extinction phenotypes in females, like are seen with PTSD in humans (Ramikie & Ressler, 2018; Shalev et al., 2017) and in male rodents (Bush et al., 2007; Galatzer-Levy et al., 2013; Sharko et al., 2017; Shumake et al., 2014). It may be the case that our previously established models (namely, only measuring freezing) are not sensitive to female-specific phenotypes of fearful behavior and the biological mechanisms behind them. Further, less than 2% of fear conditioning and extinction studies have examined female samples (Lebron-Milad & Milad, 2012), indicating that this body of literature is substantially lacking in sex-specific and female data.

The present study sought to fill this gap in the field by measuring both freezing and ultrasonic vocalizations (USVs) in a sample of female rats during a fear conditioning and extinction paradigm. Based on the reviewed literature and our previous findings, *we hypothesized that like males, female rats would exhibit individual differences in fear extinction responses, presenting as either extinction competent (EC) or extinction resistant (ER) phenotypes.*

Methods

Subjects

Subjects were 14 outbred female Long Evans rats, initially weighing 150-175g or >6 weeks old (Envigo, Indianapolis, IN). Rats were housed individually and handled daily for two weeks prior to behavioral testing. To assess estrous cycle phase, females received a vaginal smear every day, starting from the day after arrival until the day of euthanasia. On behavioral testing days, smears were done after testing to avoid additional stress. Smears were read

immediately after all smears were completed; they were later fixed with 95% EtOH, stained with hematoxylin and eosin, and cover slipped. Brains, hearts, and uteri were saved after euthanasia performed immediately after a generalization trial (data not shown). Wet mass of uteri was measured on the day of euthanasia, and dry mass was measured three days post-euthanasia to confirm the day of the estrous cycle at euthanasia.

Fear Conditioning and Extinction Procedures

Experimental procedures were adapted from the protocol used by Sharko, Fadel, Kaigler, and Wilson (2017) (see Figure 3). On day 1, for fear acquisition, rats were placed in a shock box (Context A; Med Associates, Inc.) within a sound-attenuating box containing a ventilation fan and a house light for 6.5 minutes. Unconditioned freezing and USVs were recorded for the first three minutes. Beginning at the third minute, animals were presented with a co-terminating tone-shock pairing every 60 seconds (tone: 2 kHz, 10 sec, 80 dB; shock: 1mA, 1 sec), for a total of three tone-shock pairings. On day 2, context recall was assessed by placing rats in the same box for 8 minutes, with no tones or shocks (data is not presented in this report). On day 3, for the cued extinction portion of the experiment, they were placed in round bottom Plexiglas bowls in a different sound attenuated chamber with aspen bedding and lemon scent (Context B). After three minutes in the novel Context B to assess unconditioned fear responses, females were presented with twenty conditioned tones (2 kHz, 10 sec, 80 dB) without shock at one-minute intervals, for a total testing period of 23 minutes. After 48 hours on day 5, the rats were returned to Context B to assess extinction recall using another 20 tone presentations with the same procedures as day 3 (data is not presented in this report). On day 8, to assess generalization of fear learning, rats were placed in a cylindrical glass bowl in a novel sound-attenuated chamber with a distinct floor and vanilla scent (Context C). One minute of unconditioned freezing and USVs was followed by ten

novel tones (3.5 kHz, 10 sec, 80 dB) at one-minute intervals (data not shown). Animals were smeared for estrous cycle assessment and euthanized immediately after the generalization test.

Freezing behavior in all trials was assessed in one minute bins using Freezescan software (CleverSys, Inc, Reston, VA) in all trials, and parameters were set to detect freezing as the absence of movement other than respiration. As described previously in Sharko, Fadel, Kaigler, and Wilson (2017), animals were divided into extinction resistant (ER, high freezers) and extinction competent (EC, low freezers) based on a median split of average freezing during the last 10 tone presentations of extinction learning.

Ultrasonic vocalizations were recorded using an ultrasonic microphone (full spectrum, USB port, 250,000 samples per second, and 16 bit resolution) with UltraVox XT software version 3.2.106/3.2.108 (Noldus Information Technologies, Leesburg, VA). During acquisition of conditioned fear and context exposure, microphones were placed below the animal. During extinction learning and extinction recall trials, microphones were oriented above the rats on one side of the experimental Plexiglas chamber. USVs in the 22 kHz range and 50 kHz range were manually labeled on a spectrogram (SFT length = 2048, Zero Padding = 1, Overlap = 90%) in the Analysis/Call Labeling tab, which provided quantitative information including call duration (msec), call start and stop time, peak frequency (frequency at maximum amplitude, Hz), and mean amplitude for each call. Data was then exported into Excel and divided into 60 second bins for analysis. The number of bouts, defined as the number of groups of successive calls separated by less than 320 seconds, vocalizations per bout, and latency to vocalize were also calculated (see Wöhr, Borta, & Schwarting, 2005).

Statistical Analyses

Due to time constraints, the present study only examined data from the fear acquisition trials and cued extinction learning trials. Freezing behavior during each trial was recorded as percent freezing per one minute time bin. High freezers (ER) and low freezers (EC) were compared in one minute bins over each trial using a one-way analysis of variance (ANOVA; ER versus EC freezing) with repeated measures (time) for freezing, number of USVs (22 and 50 kHz), and duration of USVs (22 and 50 kHz). Twenty-two kHz and 50 kHz USVs were analyzed separately for each trial. Specific differences between groups were assessed using Bonferroni post-hoc analysis.

For the 22 kHz and 50 kHz USV data, the total number of calls, total duration of calls, average duration per call, average peak frequency of calls, and mean amplitude of calls was also analyzed using two-way ANOVA to compare overall ER versus EC differences between fear learning and fear extinction trials. Specific differences between groups were assessed using Bonferroni post-hoc analysis.

Additionally, trials were divided into periods of unconditioned freezing (first three minutes of each trial with no tone) and conditioned freezing (remainder of trial during tone/shock or tone alone presentations) to assess the effects of cue conditioning on 22 kHz and 50 kHz USVs. The total number of calls and total duration of calls were analyzed using two-way ANOVA to compare overall ER versus EC differences between fear learning and fear extinction trials. Specific differences between groups were assessed using Bonferroni post-hoc analysis.

Results

Fear Learning

Freezing Behavior

As seen in Figure 4A, there was no significant difference in freezing behavior between ER and EC rats during fear learning. There was a significant effect of time ($F(2.282,27.38)=58.23$, $p<0.0001$), but no time x group interaction ($F(5,60)=0.73$, $p=0.61$). The effect of time was related to the low level of unconditioned freezing during the first 3 minutes, which increased with each tone-shock pairing.

22 kHz Ultrasonic Vocalizations

On the whole, the total number of 22 kHz USVs ("distress calls") during fear learning was very low, with some female rats not producing them at all. Among the ER rats, 43% vocalized in the 22 kHz range, compared to 71% of the EC rats. One ER rat was excluded from analyses as an outlier (number of 22 kHz calls >2 standard deviations from the mean of other rats). During fear acquisition, there was no significant difference in the total number of 22 kHz calls between ER and EC rats ($F(1,6)=0.86$, $p=0.39$). There was no significant effect of time ($F(1.15,6.92)=1.99$, $p=0.21$) or time x ER/EC interaction ($F(1.07,6.42)=0.66$, $p=0.46$) (Figure 5A). For total call duration, there was no significant effect of time ($F(1.77,10.60)=2.25$, $p=0.16$), extinction phenotype ($F(1,6)=1.08$, $p=0.34$), or time x ER/EC interaction ($F(1.36,8.17)=1.37$, $p=0.29$) (Figure 5C).

50 kHz Ultrasonic Vocalizations

The overall number of 50 kHz vocalizations was very high during fear learning, with 100% of rats producing 50 kHz USVs. As seen in Figure 5, EC rats emitted significantly more 50 kHz calls than ER rats in total throughout the trial, and had significantly longer total call

duration. Interestingly, females emitted significant 50 kHz USVs during the first 3 minutes in the new context, and these calls decreased during the tone-shock pairings. There was no significant difference in average call duration, average peak frequency, or mean amplitude between ER and EC rats (see below and Figure 8). To examine time effects, average number of 50 kHz USVs was analyzed using a two-way (phenotype x time) ANOVA with repeated measures. EC rats produced significantly more 50 kHz USVs during minutes 1, 2, 3, and 4, and in total throughout the trial (Figure 5B). There was a significant effect of extinction phenotype group ($F(1,12)=45.04$, $p<0.0001$), time ($F(1.68,20.18)=11.41$, $p=0.0008$), and time x ER/EC interaction ($F(5,60)=4.832$, $p=0.0009$). In addition, the total call duration of 50 kHz vocalizations was significantly higher for EC rats compared to ER rats during minutes 1, 2, and 3, and in total throughout the fear learning trial (Figure 5D). There was a significant effect of extinction phenotype ($F(1,12)=49.74$, $p<0.0001$), time ($F(2.06,24.74)=9.38$, $p=0.0009$), and time x ER/EC interaction ($F(5,60)=3.397$, $p=0.0091$) on 50 kHz call duration during fear learning.

Extinction Learning

Freezing Behavior

As seen in Figure 4B, ER rats froze significantly more than EC rats ($F(1,12)=8.125$, $p=0.0146$), and both groups displayed significant differences in freezing over time ($F(22,264)=18.12$, $p<0.0001$), although the time x ER/EC interaction was not significant ($F(22,264)=0.87$, $p=0.63$). This supports the notion that like males, females show distinct extinction phenotypes, since the last 10 tones of extinction learning were used to separate the ER and EC groups.

22 kHz Ultrasonic Vocalizations

Similar to what was observed during fear learning, very few rats vocalized in the 22 kHz range during extinction learning. Both ER and EC rats had only 43% vocalizers; of these, one ER rat was excluded as an outlier due to a very high number of calls, and the remaining vocalizers produced fewer than five 22 kHz USVs throughout the duration of the entire trial. A two-way RMANOVA did not show any significant effects of extinction phenotype ($F(1,12)=0.97$, $p=0.34$), time ($F(1.01,12.10)=0.96$), or time x ER/EC interaction ($F(22,264)=0.9998$, $p=0.46$) on the total number of 22 kHz calls during fear extinction (Figure 6A). There was no significant difference in total call duration, average call duration, average peak frequency, or mean amplitude between ER and EC rats (see below and Figure 7).

50 kHz Ultrasonic Vocalizations

During extinction learning, 86% of the rats vocalized in the 50 kHz range, with 71% of ER rats and 100% of EC rats vocalizing in the high frequency range. One EC rat was excluded from analyses as an outlier. A two-way ANOVA demonstrated a significant effect of extinction phenotype group ($F(1,11)=10.33$, $p=0.0082$), time ($F(1.88,20.69)=4.39$, $p=0.0276$), and time x ER/EC interaction ($F(22,242)=2.227$, $p=0.0017$) on the total number of 50 kHz calls (Figure 6B) during fear extinction. The effect of time is due to a higher number of 50 kHz USVs during the first three minutes of the trial in a new environment prior to tone presentation. When examining total call duration, there is no significant group difference ($F(1,12)=3.32$, $p=0.09$), but there is an effect of time ($F(2.92,35.03)=4.163$, $p=0.0133$) and time x ER/EC interaction ($F(22,264)=1.923$, $p=0.0089$). There were no significant differences between groups in average duration, average peak frequency, or mean amplitude of calls (see below and Figure 8).

Comparison Between Fear Learning and Fear Extinction Trials

22 kHz Ultrasonic Vocalizations

Overall, likely due to the overall low number of 22 kHz calls, most of these results comparing trials and extinction phenotypes for 22 kHz calls were not significant. One ER rat was excluded from analyses as an outlier in the extinction learning trial. There was no significant difference in total number of 22 kHz calls between ER and EC rats ($F(1,23)=0.86$, $p=0.36$), no difference between fear learning and fear extinction trials ($F(1,23)=1.53$, $p=0.23$), and no group x trial interaction ($F(1,23)=0.78$, $p=0.38$) (see Figure 7). Total call duration was not significantly different between trials ($F(1,23)=2.67$, $p=0.12$), between ER/EC phenotypes ($F(1,23)=1.06$, $p=0.31$), nor was there a group x trial interaction ($F(1,23)=1.09$, $p=0.31$). There was no significant difference in average call duration between trials ($F(1,10)=1.32$, $p=0.28$), between ER/EC phenotypes ($F(1,10)=0.35$, $p=0.57$), nor was there a group x trial interaction ($F(1,10)=1.17$, $p=0.30$). Mean call amplitude was not significantly different between trials ($F(1,10)=2.59$, $p=0.14$), between ER/EC phenotypes ($F(1,10)=0.50$, $p=0.50$) and there was no significant group x trial interaction ($F(1,10)=0.29$, $p=0.60$). Only average peak frequency was significantly different between fear learning and fear extinction trials ($F(1,10)=5.11$, $p=0.047$), but there was no ER/EC main effect ($F(1,10)=1.53$, $p=0.44$), nor group x trial interaction ($F(1,10)=0.65$, $p=0.44$).

50 kHz Ultrasonic Vocalizations

As seen above, EC rats produced significantly more 50 kHz USVs during both fear learning and extinction learning compared to ER rats ($F(1,23)=31.96$, $p<0.0001$), but there was no overall difference between fear learning and extinction ($F(1,23)=0.13$, $p=0.72$) and no group x trial interaction ($F(1,23)=0.24$, $p=0.63$) (Figure 8A). Also demonstrated previously, EC rats had

significantly longer total 50 kHz call duration compared to ER rats ($F(1,23)=33.94$, $p<0.0001$), with no significant difference between trials ($F(1,23)=2.91$, $p=0.10$) and no group x trial interaction ($F(1,23)=1.45$, $p=0.24$) (Figure 8C). There were no significant differences between fear learning and extinction learning for average call duration ($F(1,22)=0.03$, $p=0.86$), average peak frequency ($F(1,22)=1.97$, $p=0.17$), or mean amplitude ($F(1,22)=0.26$, $p=0.61$). There were no significant differences between ER and EC rats for average call duration ($F(1,22)=0.06$, $p=0.80$), average peak frequency ($F(1,22)=0.40$, $p=0.53$), or mean amplitude ($F(1,22)=0.26$, $p=0.61$). Finally, there were no significant group x trial interactions for average call duration ($F(1,22)=0.006$, $p=0.94$), average peak frequency ($F(1,22)=0.43$, $p=0.52$), or mean amplitude ($F(1,22)=0.46$, $p=0.51$).

Distinguishing Between Periods of Unconditioned and Conditioned Freezing

Two-way ANOVA was used to compare ER versus EC group differences in USV production between fear learning and extinction trials when dividing the trials into periods of unconditioned and conditioned responses. The first three minutes of both fear learning and extinction learning trials do not contain any tones or shocks, as this time is meant to assess baseline (i.e. unconditioned) levels of freezing and USV production in a new context. By contrast, conditioned freezing begins once the first tone is played and continues for the remaining duration of the trial (minutes 3-6 in fear learning and minutes 3-23 in fear extinction).

22 kHz Ultrasonic Vocalizations

As seen in Figure 9, like in most of our analyses, there were no significant differences in total number of 22 kHz USVs between ER and EC groups ($F(1,24)=0.51$, $p=0.48$) or between fear learning and extinction ($F(1,24)=1.42$, $p=0.25$). For analysis of total call duration, one ER

rat was removed from extinction as an outlier. There was no significant difference in total call duration between groups ($F(1,23)=1.06$, $p=0.31$) or between trials ($F(1,23)=2.67$, $p=0.12$).

50 kHz Ultrasonic Vocalizations

During periods of unconditioned freezing, EC rats emitted significantly more 50 kHz calls than ER rats in both the fear learning and extinction learning trials ($F(1,24)=19.50$, $p=0.0002$) (Figure 10A), and had significantly longer total call duration than ER rats in both trials ($F(1,23)=16.90$, $p=0.0004$) (Figure 10C). This suggests females vocalize in the 50 kHz range during exposure to a novel context.

During conditioned freezing, EC rats had a significantly higher total number of 50 kHz USVs than ER rats ($F(1,23)=14.04$, $p=0.0011$) (Figure 10B). EC rats also produced significantly more vocalizations during extinction learning than they did during fear learning ($F(1,23)=14.04$, $p=0.0011$), and there was a significant group x trial interaction ($F(1,23)=9.56$, $p=0.0052$). When looking at total call duration during conditioned freezing, EC rats vocalized longer than ER rats during extinction learning ($F(1,23)=20.39$, $p=0.0002$). EC rats also vocalized longer during extinction than they did during fear learning ($F(1,23)=5.90$, $p=0.023$), and there was a significant group x trial interaction ($F(1,23)=4.81$, $p=0.039$) (Figure 10D).

Discussion

As hypothesized, female rats exhibited marked individual differences in freezing behavior during extinction learning, dividing into extinction competent (EC) and extinction resistant phenotypes (ER). These individual differences were not present during fear learning. These results are consistent with those from studies of male rats (Bush et al., 2007; Galatzer-Levy et al., 2013; Monfils et al., 2019; Sharko et al., 2017), suggesting that the neurobiological

mechanisms that control freezing behavior during fear extinction are similar in males and females.

However, though female rats appear to exhibit freezing behaviors like males during fear learning and extinction, surprisingly they do not vocalize like males. Prior research in male rats has demonstrated that freezing behavior and 22 kHz USVs are typically correlated with one another (Wöhr et al., 2005), and extinction competent rats exhibit a lower number of 22 kHz calls than extinction resistant rats (Kellis et al., 2018; Shumake et al., 2014). Studies of male rats rarely report USVs in the 50 kHz range in fear conditioning paradigms (Koo et al., 2004; Portfors, 2007; Wöhr et al., 2005). A rare example from Shumake et al. (2014) reported some 50 kHz calls in both males and females, noting that most occurred at the end of extinction learning. By contrast, the female rats in the present study produced a high number of 50 kHz USVs, especially during unconditioned freezing, and almost no 22 kHz USVs. Thus, these findings in female rats were unexpected and novel.

Some other studies have reported that female rats produce fewer 22 kHz USVs than males during fear conditioning and fear extinction (Schwarting, 2018; Shumake et al., 2014), though in a comparison of USVs between three different rat strains, Schwarting (2018) saw that female Long Evans rats, the strain used in the present study, produced the most 22 kHz calls. Nonetheless, Schwarting did not see any such inter-strain difference in freezing behavior in females, and concluded that there must be some difference in the physiological mechanisms underlying freezing behavior and USV production. Wöhr et al. (2005) also noted that their rats did not always produce 22 kHz USVs in concordance with freezing (e.g. when re-exposed to the fear conditioning context), positing that USV must be influenced by factors other than freezing.

This would be supported by our current findings, since ER and EC rats showed no difference in freezing during fear learning, but did show differences in USVs between these two groups.

A lesion study conducted by Koo et al. (2004) sheds some light on what kinds of neurobiological differences might account for the disparity between freezing and 22 kHz USVs. This team saw significantly reduced freezing behavior and reduced 22 kHz USVs during fear learning, context re-exposure, and fear extinction in male rats with bilateral neurotoxic lesions to the BLA and in those with electrolytic lesions to the CeA, compared to controls and rats with neurotoxic lesions to the CeA. Rats with neurotoxic CeA lesions also produced significantly fewer USVs than controls during context and extinction trials, though still more than the other two groups. Neurotoxic lesions destroy neuronal cell bodies but not axonal projections passing through the region; electrolytic lesions destroy both. Therefore, these results can be interpreted to suggest that conditioned fear (in terms of both freezing and 22 kHz calls) is modulated by BLA neurons that project through the CeA, not the CeA neurons themselves. Additionally, the fact that rats with neurotoxic CeA lesions vocalized less than controls during context and extinction trials may implicate CeA neurons, *in addition to* projections from the BLA passing through the CeA, in the production of 22 kHz USVs even when conditioned freezing behavior is unimpaired.

One possibility is that there are sex differences in the central nucleus of the amygdala; for example, female rats may have fewer CeA neurons or weaker outputs than males, resulting in reduced 22 kHz calls. It is well established that female rats exhibit greater hypothalamic-pituitary-adrenal (HPA) responses following stress compared to males (Shansky, 2020; Wilson et al., 2004), and prior research has demonstrated functional sex differences in the BLA (Blume et al., 2017) and medial amygdala (Wilson et al., 2002) of rodents. Exposure to chronic stress has also been shown to alter BLA activation in males and females in contrasting ways (Blume et al.,

2019). Furthermore, human neuroimaging studies of emotional memory show differential activation of left and right amygdalae in males and females (Shansky, 2020). Rat strain may also play a role, as Long Evans rats are sometimes reported to vocalize less than other strains (Shumake et al., 2014).

Another plausible explanation is that estrous cycle phase affected these results. Though estrous cycle data were collected, due to time constraints we were unable to analyze the data for this report. BLA activity is dependent upon estrous cycle phase and, critically, these activation patterns are disrupted by repeated stress exposure (Blume et al., 2019). Ability to learn and extinguish fear is also cycle-dependent, with female rats displaying enhanced fear learning during diestrus (in which estrogen levels are low) and slower extinction during proestrus (high estrogen) (Blume et al., 2019). In female rats in a low-estrogen phase, estradiol injections before extinction learning enhanced activity in the CeA during extinction, and improved extinction memory (i.e. reduced freezing during recall) (Maeng et al., 2017).

As for the 50 kHz USVs, it is highly unusual to observe so many 50 kHz calls during a fear conditioning study, although this may be due to the use of female rather than male subjects. Moreover, we found a significantly higher incidence of 50 kHz calls in extinction competent rats compared to extinction resistant rats. This might not be completely incongruent with the assumption that 50 kHz calls reflect positive affect, since these vocalizations may indicate reduced fear in EC rats (or, conversely, the absence of 50 kHz calls in ER rats may indicate enhanced fear). It may be that females generally respond more positively to a novel environment, especially given that most 50 kHz USVs were recorded during the first three minutes of unconditioned freezing in both fear learning and extinction, during the period when rats are first exposed to a novel context in the absence of any tone or shock. Schwarting (2018) also saw their

female rats produce 50 kHz USVs when placed in a new cage for the first time, though Long Evans rats produced by far the fewest USVs compared to other strains.

In conclusion, female rats exhibit individual differences in freezing behavior similar to males, with some rats presenting an extinction competent phenotype while others are extinction resistant. However, females appear to vocalize less in the 22 kHz range and more in the 50 kHz range compared to males, suggesting the presence of substantial sex differences in the neurobiological mechanisms behind USV production in fear learning and extinction. Moreover, our results support the notion that freezing behavior and USVs may be controlled by distinct brain processes. In addition, the number and duration of 50 kHz USVs differs significantly between ER and EC females, further indicating that individual differences in fear responses exist within the female population. Thus, individual differences in fear extinction are seen in both male and female rats, but there are clearly distinct sex differences between ER and EC populations, particularly with respect to USVs.

These results indicate that females differ from males in behavioral fear outputs, likely reflecting sex differences in neurobiology and endocrinology. Moreover, measuring freezing behavior alone does not seem sufficient to capture the full picture; future studies should consider other fear behaviors, such as ultrasonic vocalization and darting. Although qualitative video data was not coded for darting behavior in this study, it is possible this sample of females also exhibited some darting. In order to better understand risk factors for PTSD and develop more effective and targeted treatments, especially for women, substantially more research in females is still needed, particularly looking at individual differences within female populations.

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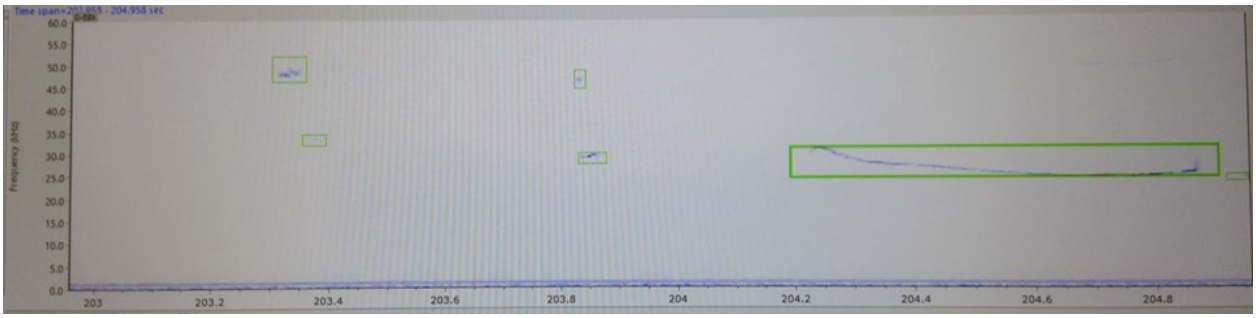

Figure 1. Examples of USV recordings on the spectrogram in UltraVox XT. These calls were recorded during fear learning. On the left half of the image, there are four 50 kHz USVs. The USVs closest to the top are at ~50 kHz, as is typically seen. The lower two calls are closer to ~32 kHz, but due to their short duration and prior literature establishing an accepted range of 32-70 kHz, they were categorized as 50 kHz calls. To the right is a 22 kHz USV; note its lower frequency and longer duration.

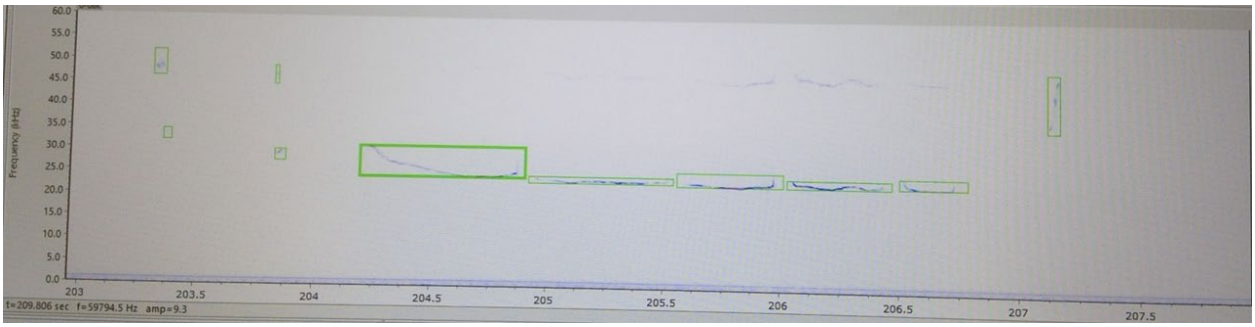

Figure 2. More examples of USV recordings on the spectrogram in UltraVox XT. The first five calls are the same as those in Figure 1. In this figure, a bout of five 22 kHz USVs is shown, followed by another 50 kHz USV.

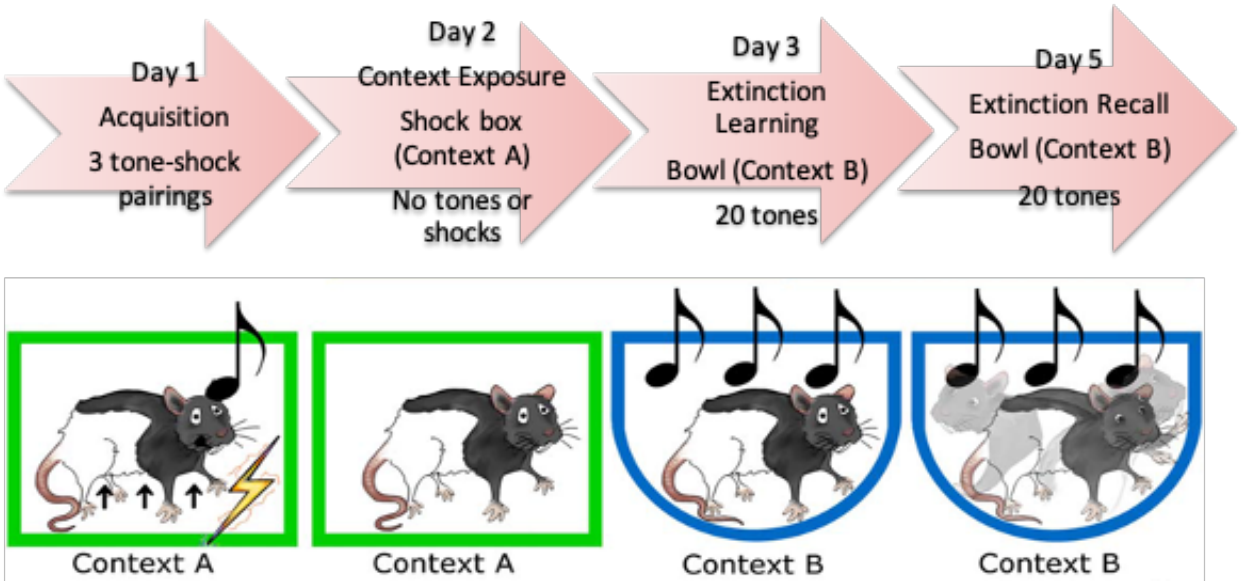

Figure 3. Diagram illustrates Pavlovian fear conditioning paradigm as used in the present study. Data from day 1 and day 3 are presented in this paper.

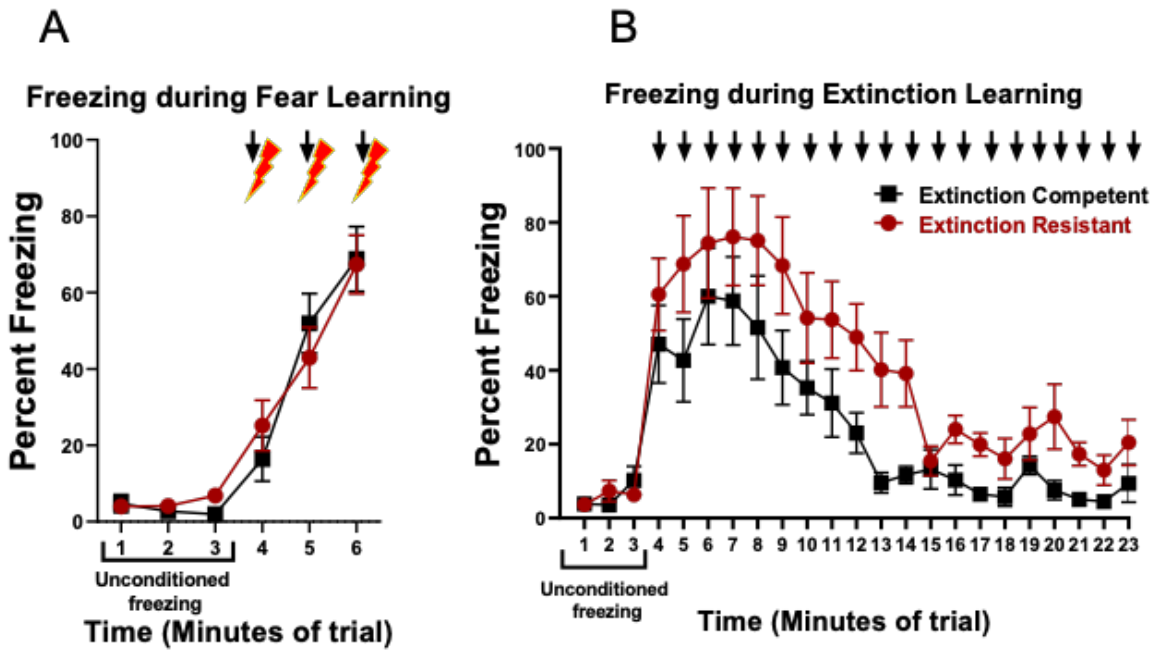

Figure 4. Individual differences in freezing behavior during fear learning and fear extinction. During fear learning, there is no significant difference in percent freezing between ER and EC rats (Panel A). Arrows denote tones and lightning bolts denote shocks. Panel B shows freezing behavior during fear extinction. Rats were divided into EC and ER groups based on a median split of freezing during the last ten tone presentations. ER rats froze significantly more than EC rats throughout extinction, and both groups showed reductions in freezing over time. Arrows denote tone presentations, without shock.

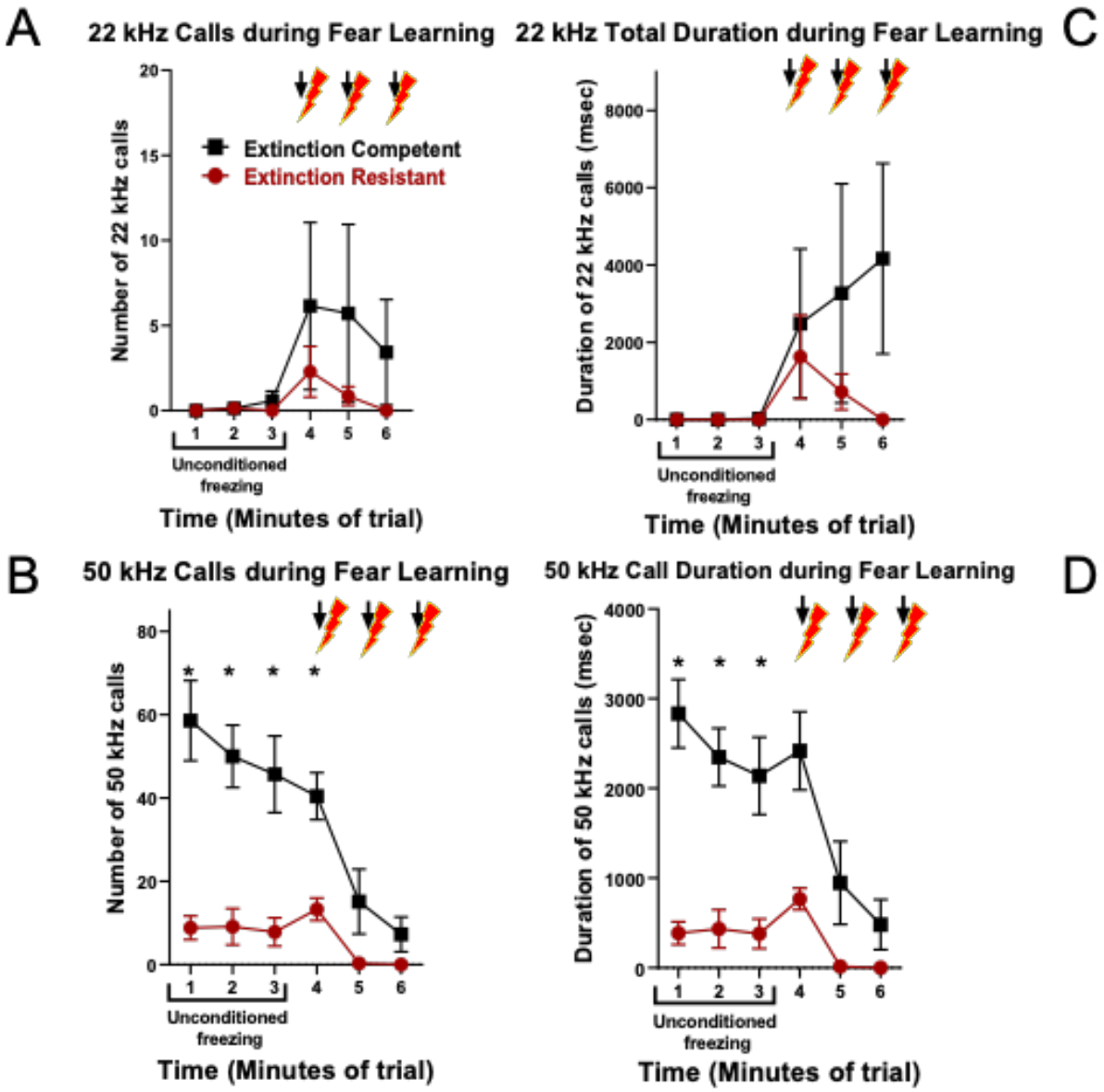

Figure 5. Individual differences in 22 and 50 kHz ultrasonic vocalizations during fear learning. There was no significant difference in the total number (Panel A) or total duration (Panel C) of 22 kHz calls between EC and ER rats during fear learning. Note the extremely low number of 22 kHz calls in Panel A. Panel B shows the total number of 50 kHz USVs during fear learning. EC rats produce significantly more 50 kHz USVs than ER rats during minutes 1, 2, 3, and 4, and in total throughout the trial. Panel D displays the total duration (msec) of 50 kHz calls during fear learning. EC rats had significantly longer 50 kHz call duration during minutes 1, 2, and 3, and in total throughout the trial. Arrows denote tones and lightning bolts denote shocks. * indicates $p<0.05$.

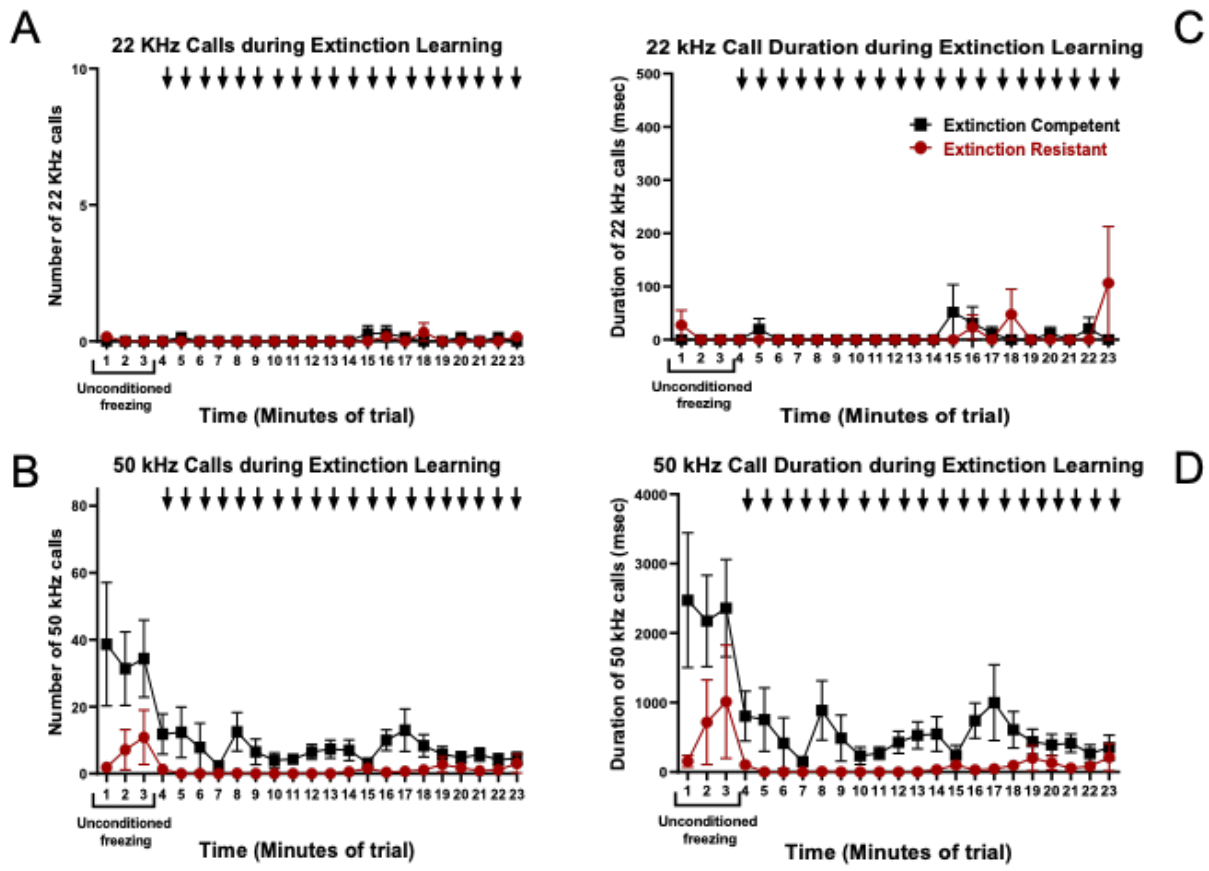

Figure 6. Individual differences in 22 and 50 kHz ultrasonic vocalizations during extinction learning. There was no significant difference in the total number (Panel A) or duration (Panel C) of 22 kHz calls between EC and ER rats during extinction learning. Note the very low level of 22 kHz calls during extinction learning. Panel B shows the total number of 50 kHz USVs during extinction learning. EC rats produced significantly more 50 kHz calls than ER rats, and there is a main effect of time and time x ER/EC group interaction. One EC outlier was excluded. Panel D displays the total duration (msec) of 50 kHz calls during extinction learning. There is a main effect of time and time x ER/EC group interaction. One EC outlier was excluded. Arrows denote tone presentations, without shocks.

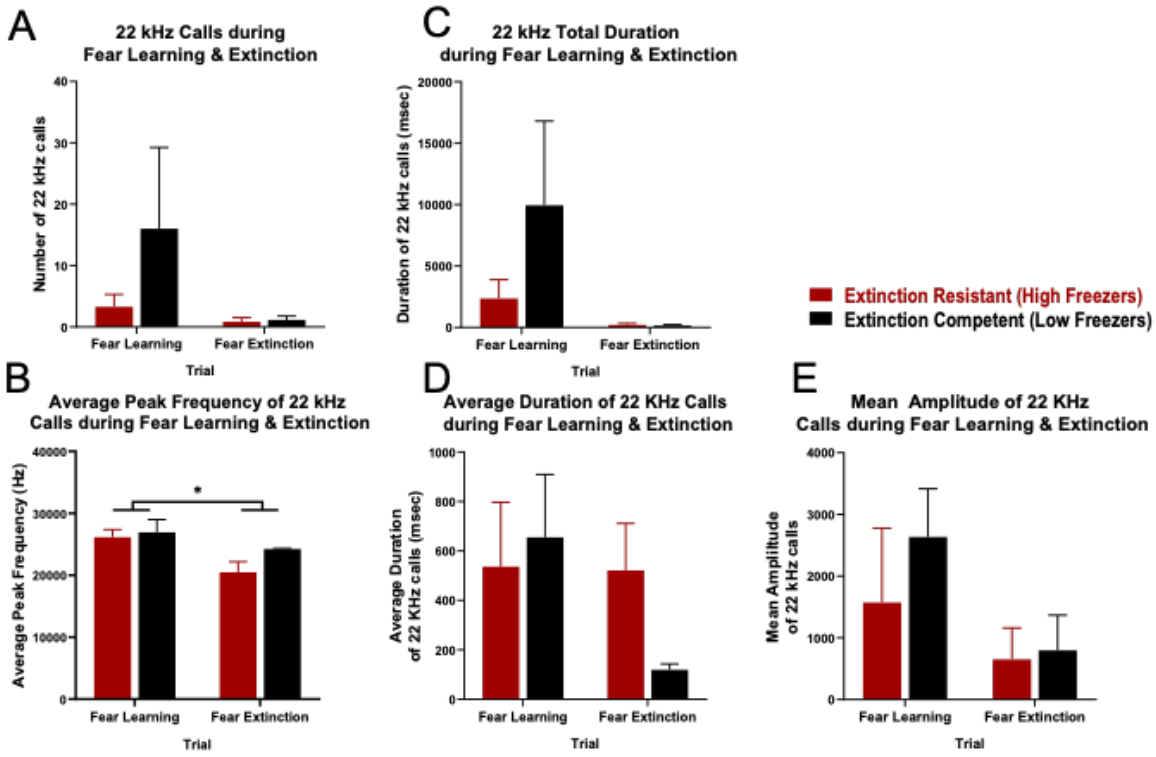

Figure 7. Individual differences in 22 kHz USVs compared between fear learning and extinction learning trials. There were no significant differences in 22 kHz total call number (Panel A), total call duration (Panel C), average call duration (Panel D), or mean call amplitude (Panel E) between groups or trials. There was a significant difference in average peak frequency between fear learning trials and extinction learning trials, though there was no ER/EC difference (Panel B). * indicates $p<0.05$.

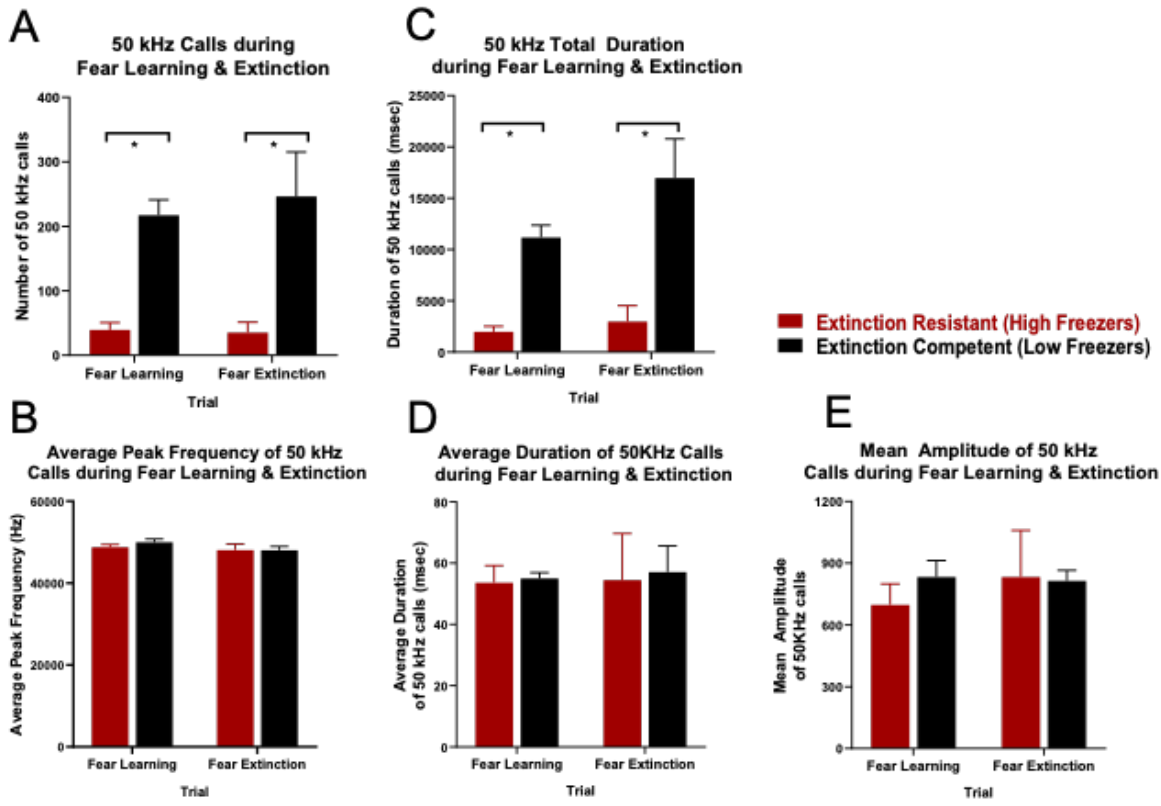

Figure 8. Individual differences in 50 kHz USVs compared between fear learning and extinction learning trials. EC rats emitted a significantly higher number of 50 kHz calls compared to ER rats during both fear learning and extinction (Panel A). EC rats also had significantly longer total 50 kHz call duration compared to ER rats in both trials (Panel C). There were no significant differences in average peak frequency (Panel B), average call duration (Panel D), or mean call amplitude (Panel E) between groups or trials. * indicates $p<0.05$.

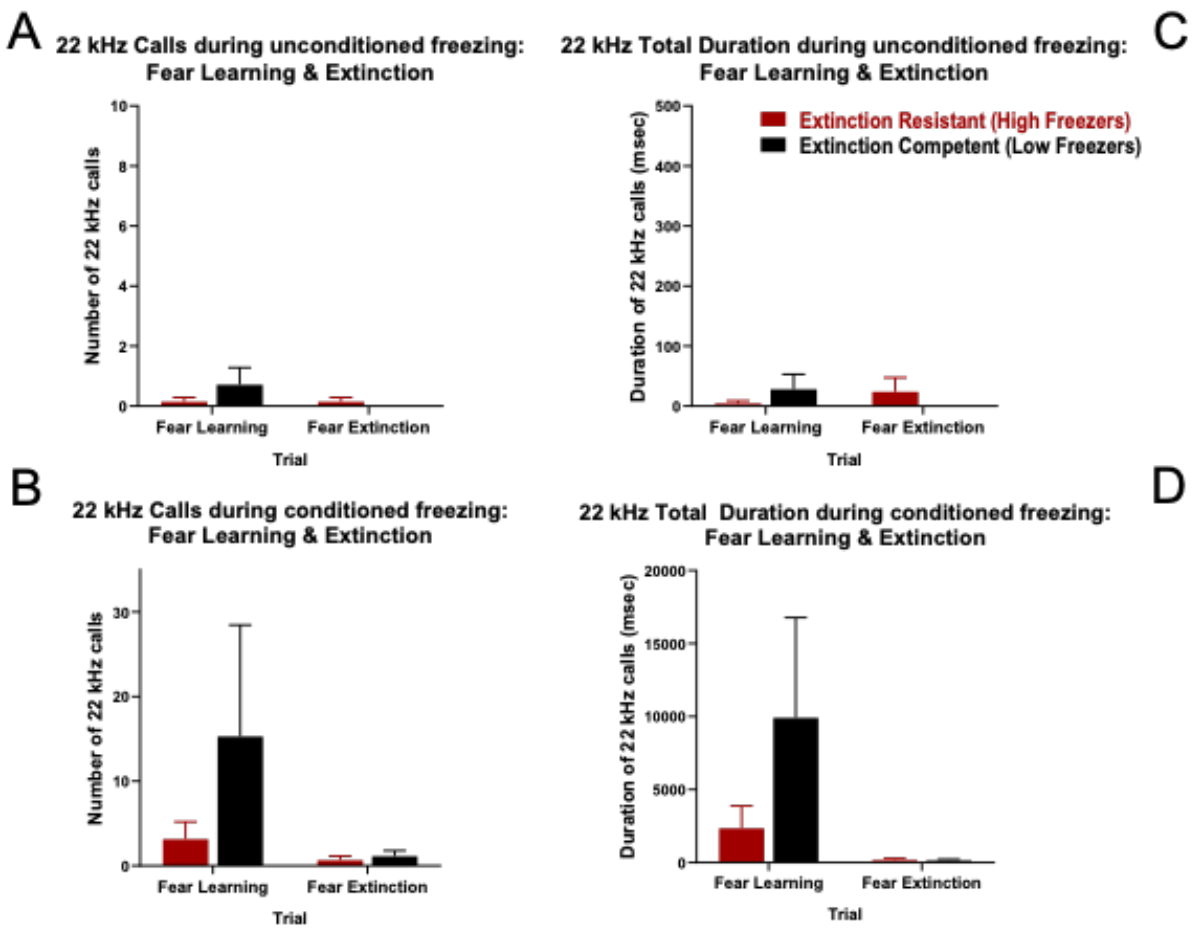

Figure 9. Individual differences in 22 kHz USVs compared between fear learning and extinction trials when divided into periods of unconditioned freezing (first three minutes of each trial with no tone) and conditioned freezing (remainder of trial during tone/shock or tone alone presentations). There were no significant differences in total number of 22 kHz calls (Panels A and B) nor total call duration (Panels C and D) between groups, trials, or group x trial interaction in both periods of unconditioned vs. conditioned freezing. Despite n.s. results, note the difference in total duration between unconditioned and conditioned freezing (Panels C and D).

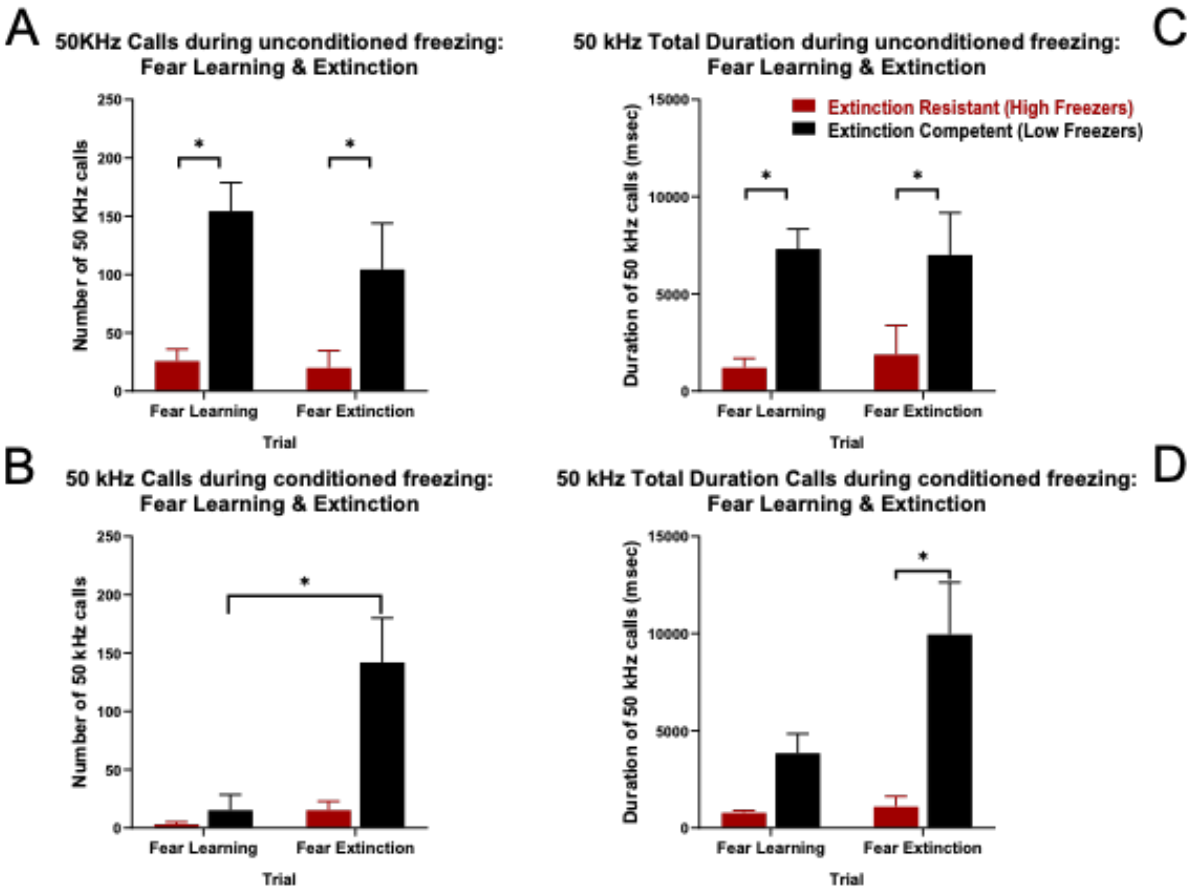

Figure 10. Individual differences in 50 kHz USVs compared between fear learning and extinction trials when divided into periods of unconditioned freezing (first three minutes of each trial with no tone) and conditioned freezing (remainder of trial during tone/shock or tone alone presentations). During unconditioned freezing, EC rats emitted significantly more 50 kHz calls than ER rats in both fear learning and extinction (Panel A) and had significantly longer total call duration in both trials (Panel C). During periods of conditioned freezing, EC rats produced a significantly higher number of 50 kHz calls compared to ER rats, and EC rats made more calls during extinction learning than they did during fear learning, and there is a significant group x trial interaction (Panel B). EC rats exhibited significantly longer call duration than ER rats in extinction learning trials (Panel D). * indicates $p<0.05$.