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Sex differences in fear expression and persistence in an animal model of Post-Traumatic Stress Disorder

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ABSTRACT

Post-Traumatic Stress Disorder (PTSD) is a complex psychiatric condition arising from traumatic experiences, marked by abnormal fear memories. Despite women are twice as likely as men to develop PTSD, the biological mechanisms underlying this disparity remain inadequately explored, particularly in preclinical studies involving female subjects.

Previous research shows that female rats exhibit active fear responses, while males display passive behaviors. Additionally, sex differences in ultrasonic vocalizations (USVs) during fear conditioning have been observed, indicating varying emotional responses.

Here, we validated a traumatic stress model consisting of footshock exposure paired with social isolation – originally developed in male rats – on females for the first time, focusing on sex differences in fear memory expression, retention and extinction. Our findings reveal that only during trauma exposure, males predominantly exhibited passive responses, whereas females demonstrated more active responses, despite both sexes emitting similar numbers of alarm USVs. Females also showed lower levels of freezing and USV emissions throughout extinction sessions and displayed a higher extinction rate compared to males. Notably, only males displayed a conditioned fear response when triggered by a single mild stressor.

These findings highlight sex differences in trauma responses and fear memory processes. The study emphasizes the importance of incorporating 22-kHz USV evaluations along with other behavioral metrics for a comprehensive understanding of fear memory. This research contributes to the existing literature on traumatic stress models as well as underscores the necessity of including female subjects in preclinical studies to better inform treatment and prevention strategies tailored to both sexes.

Introduction

Post-Traumatic Stress Disorder (PTSD) is a chronic psychiatric disease of significant prevalence and morbidity, and it is triggered by a traumatic experience leaving lasting alterations of its cognitive elaboration (Javidi and Yadollahie, 2012; Yehuda et al., 2015). Alterations of the fear memory trace are characterized by over-consolidation, excessive recall, and impaired extinction (Desmedt et al., 2015; Finsterwald et al., 2015; Morena et al., 2023; Trezza and Campolongo, 2013). PTSD therapy generally consists of a pharmacological or psychological approach (e.g., prolonged exposure, cognitive-behavioral therapy, eye movement desensitization and reprocessing, cognitive-behavioral therapy for healing CBT-H) or a combination of both (Bisson et al., 2021; Florido et al., 2023; Marchetta et al., 2023). However, there is still no one-size-fits-all solution. Many patients face significant challenges in overcoming and extinguishing the traumatic experience, leading to high drop-out rates and relapses of the therapeutic interventions (Alexander, 2012; Imel et al., 2013; Raut et al., 2022). Relapses generally occur because re-exposure to a cue-related trauma, may revoke or even intensify the fear response, thus inducing a re-activation of the previously extinguished traumatic memory (Alberini and LeDoux, 2013; Lancaster et al., 2020; Parvez et al., 2006).

Stress response can vary significantly among individuals according

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Nomenclature

Abbreviations

CBT-H	Cognitive-Behavioral Therapy for Healing
CS	Conditioned stimulus
ERT	Extinction retention test
FMRT	Fear memory reactivation test
PTSD	Post-Traumatic Stress Disorder
US	Unconditioned stimulus
USV	Ultrasonic vocalization

Glossary

FS	Rats exposed to trauma
No-FS	Rats not exposed to trauma

to different factors, including biological sex (Mancini et al., 2023; McEwen and Stellar, 1993). It has been extensively demonstrated that women are twice as likely to develop stress related disorders (e.g., PTSD) than men (Bangasser and Valentino, 2014; Breslau, 2009). In PTSD sex differences occur in many aspects: different clinical manifestations, comorbidities, treatment responses, and different retention in care (Hiscox et al., 2023). Although evidence suggests that these differences are influenced by genetic and epigenetic factors and sexual dimorphism in neurocircuitry of fear (Ramikie and Ressler, 2018), their biological underpinnings remain unclear and controversial, mainly because the majority of preclinical studies have primarily focused on male animals (Lebron-Milad and Milad, 2012; Shansky, 2015).

Pavlovian fear conditioning is an experimental paradigm widely used to understand the biological and behavioral mechanisms of fear memory processes (Kim and Richardson, 2007). Briefly, rodents learn the association between a neutral stimulus (conditioned stimulus, CS; e.g., tone or context) and an aversive one (unconditioned stimulus, US; e.g., footshock). Subsequently, after repeated exposure to the CS without the presence of the US, the conditioned response gradually decreases, thus indicating extinction of the traumatic CS-US association (VanElzakker et al., 2014). Freezing behavior has traditionally been utilized as an indicator of fear memory in rodents, irrespective of their sex (Trott et al., 2022). However, it has been previously demonstrated that a sex-dependent fear response occurs (Bauer, 2023), with females showing preferentially active fear reactions and males exhibiting more passive reactions such as freezing (Bangasser, 2015). Of note, previous studies suggest that freezing behavior may not be the unique and complete quantifiable index of trauma-related fear (Brudzynski, 2001; Fitch et al., 2002; Seemiller et al., 2021).

Rats emit ultrasonic vocalizations (USVs) as a major means of communication and adult rats can produce different types of USVs according to their affective states (Brudzynski, 2013; Burgdorf et al., 2008; Portfors, 2007). For instance, alarm 22-kHz USVs, produced in potentially threatening situations, are produced in a range between 18 and 32 kHz and are generally long (>300 ms) (Alexandrov et al., 2023; Fendt et al., 2018; Litvin et al., 2007; Schwarting, 2018b; Schwarting et al., 2007; TakahashixKashino and Hironaka, 2010), whereas 50-kHz USVs (ranging from 32 to 70 kHz) are emitted in a variety of positive rewarding conditions and they are shorter than the 22-kHz ones (20–80 ms) (Simola and Costa, 2018; Wöhr, 2018). Notably, sex differences also occur in this regard. We and others have previously reported that females emit a reduced number of 22-kHz USVs compared to males in fear conditioning paradigms (Borta et al., 2006; Koo et al., 2004; Laine et al., 2022; Litvin et al., 2007; Riccardi et al., 2021; Schwarting, 2018b, 2018a; Tryon et al, 2021, Tryon et al., 2022; Willadsen et al., 2021). However, the reasons behind these differences are still quite unexplored (Lenell et al., 2021).

One of the primary difficulties in PTSD research lies in accurately replicating the intricate nature of the human pathology within animal models (Deslauriers et al., 2018; Dunsmoor et al., 2022; Verbitsky et al., 2020). Clinically, PTSD encompasses a broad spectrum of factors, such as the subjective experience of trauma, emotional memory, physiological responses, and psychosocial influences (Richter-Levin et al., 2019). Replicating the complexities of PTSD at the preclinical level presents insurmountable challenges. However, animal models are essential for discovering effective treatments and achieving a comprehensive understanding of the disorder's underlying biology. We previously developed a model that incorporates unpredictable high intensity footshock exposure paired with social isolation, which captures aspects of the chronicity and some cognitive and emotional alterations associated with the human condition (Berardi et al., 2014; Morena et al., 2018). One limitation of this model is that it examines the entire population rather than focusing exclusively on individuals who are susceptible to developing PTSD. This broad approach may dilute the specificity of findings related to those most at risk. However, employing this model allows for a more comprehensive understanding of the range of responses to trauma, which can be crucial in identifying potential biological and behavioral factors that contribute to PTSD susceptibility. By studying a wider population, we can uncover variations in trauma responses that may inform future research and therapeutic strategies tailored to different risk profiles. It is important to note that another limit of this protocol is that it was initially tested exclusively in male rats, resulting in a lack of representation from female animals.

With this background, our study had a dual objective. Firstly, we aimed to investigate potential sex differences in our animal model of traumatic stress concerning fear memory processes and their expression. Secondly, leveraging our prior findings associating alarm USVs with fear extinction (Riccardi et al., 2021), we sought to examine the relationship between fear memory-related parameters and the emission of 22-kHz USVs, while also exploring any other sex difference in the expression of fear in our traumatic stress model.

Experimental procedures

Animals

Male and female adult Sprague–Dawley rats (350–400 g and 250–300 g at the time of the behavioral experiments, respectively; n = 8–10 per group) from Charles River Laboratories (Calco, Italy) were pair housed in separate temperature-controlled (21±1 °C) rooms and maintained under a 12 h/12 h light/dark cycle (07:00 AM to 7:00 PM lights on). Food and water were available ad libitum. All tests were carried out during the light phase of the cycle between 10:00 a.m. and 2:00 p.m. Animals were randomly assigned to the experimental groups. Male and female rats were tested separately, in different cohorts and different days. Freely cycling females were used in this study.

Behavioral procedures

All rats were subjected to a slightly modified traumatic stress model previously developed in our laboratory (Berardi et al., 2014; Colucci et al., 2020; Morena et al., 2018), adapted to make it suitable to test whether the exposure to a 'shock reminder' could affect fear memory phases in both trauma-exposed (FS) and no-trauma exposed (No-FS) rats (see the paragraph "Trauma Exposure" for the details) (Fig. 1). The experimental apparatus consisted in a metal trough shaped alley (60 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) connected to an animal shocker. All the experimental sessions were video-recorded and subsequently scored by two well-trained researchers blind to the experimental conditions. After each session, fecal boli were removed and the apparatus was cleaned with a 70 % ethanol solution.

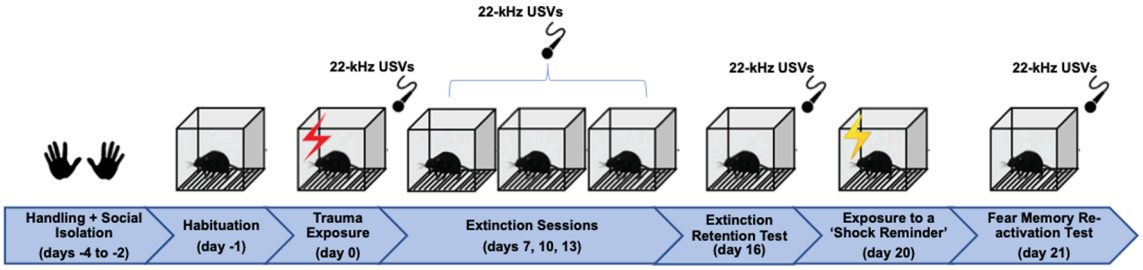

Fig. 1. Schematic representation of the experimental timeline.

Housing

All rats were individually housed for 2 days prior to the habituation session and remained singly housed until the end of the behavioral testing. As we have previously shown (Berardi et al., 2014), social isolation is necessary to develop enduring signs of emotional distress upon exposure to a traumatic event.

Habituation

On day −1, rats were individually habituated for 5 min to the test apparatus. Then, they were returned to their home-cages.

Trauma exposure

On day 0, rats were randomly divided into two groups. The first one (No-FS) was placed in the apparatus for a total duration of 6 min without receiving any footshock. The second one (FS), instead, once in the apparatus, was left undisturbed for the first 2 min, and then, 5 footshocks (2 s, 0.8 mA) were randomly delivered. After the last footshock (always administered at the fifth min), rats were kept in the apparatus for 60 s to facilitate context association to the aversive stimuli. Both passive (number and percentage of time spent in freezing behavior, considered as the absence of any movement except for those necessary for respiration (Fanselow, 1980) and active (number as well as percentage of time spent in jumps, rapid movements and attempts to escape from the experimental apparatus) behaviors were measured throughout the entire trauma exposure session. For FS groups, two indices have been assessed: index of passive and active trauma responses, calculated respectively as the ratio between the time spent in freezing or in active responses and the sum of the time spent in both passive and active behaviors.

Extinction sessions and extinction retention test

Each extinction session consisted of a 10 min re-exposure to the experimental apparatus, with the first carried out 7 days after the trauma exposure session (day 7) and each subsequent session was separated from the preceding one by a 72-h interval (days 10 and 13). To evaluate memory retention, rats were subjected to the experimental apparatus 16 days after trauma exposure for 10 min. During each extinction session and extinction retention test, percentage of time spent in freezing behavior has been evaluated. In order to measure the magnitude of extinction in FS rats, an Extinction Index was calculated as: 100– 100*(Y/X), where X and Y were the percent time spent freezing on extinction days 7 and 10, 13 or 16, respectively (Wilson et al., 2013). Active responses were not calculated since none of the experimental subjects exhibited this type of behavior during these sessions of the experimental protocol.

Exposure to a 'shock reminder' and fear memory re-activation test

Three weeks after trauma, all animals (both No-FS and FS rats) were exposed to a 'shock reminder', with a slightly modified procedure as previously described (MacCallum et al., 2024). Briefly, animals were individually placed in the apparatus and, after 1 min, they received a single mild footshock (2 s, 0.4 mA) as 'shock reminder' 20 days after trauma exposure. Then, all rats were kept in the apparatus for additional 60 s. The subsequent day (fear memory re-activation test), animals were re-exposed to the same context for a 10-min session and freezing behavior was scored. Active behaviors were not included in the analysis because neither males nor females exhibited these behaviors during these sessions.

Ultrasonic vocalizations (USVs)

USVs were monitored during trauma exposure session as well as 7, 10, 13, 16 and 21 days after trauma exposure (see Fig. 1) by an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) placed beside the apparatus and recorded with Avisoft-Recorder USGH 4.3 (sampling rate: 214,285 Hz; format: 16 bit). By using Avisoft SASLabPro 5.2, a fast Fourier transformation was conducted (512 FFT-length, 100 % frame, Hamming window, 75 % time window overlap), resulting in high-resolution spectrograms (frequency resolution: 0.488 kHz, time resolution: 0.512 ms). A lower cutoff frequency of 15 kHz was used to reduce background noise. Acoustic parameter of USVs number was automatically measured by the software Avisoft SASLabPro 5.2.

Statistical analysis

Statistical analysis was performed using StatView and GraphPad Prism 9 statistical softwares. Data are expressed as mean ± standard error of the mean (SEM). Comparisons between FS males and females for index of passive (or active) responses were performed by unpaired *t* test. ANOVA for Repeated Measures (RM ANOVA) or two-way ANOVA were used when appropriate. The source of the detected significances was determined by Tukey-Kramer *post hoc* tests for between and within-group differences. P-values of less than 0.05 were considered statistically significant. When appropriate, freezing behavior and USVs were also analyzed with a chi squared (χ^2) test. For the extinction sessions, χ^2 test was performed only for FS groups, as No-FS animals showed very low or even absent levels of freezing behavior and USVs. The number of rats per group is indicated in the figure legends.

Results

Sexually divergent expression of fear responses during trauma exposure

We first assessed whether there was a sex-specific fear response to the traumatic event during the trauma exposure session in our traumatic stress model, and if there were any differences in USVs emission among groups. As shown in Fig. 2a, two-way ANOVA for time spent in passive response (Fig. 2a) indicated an effect of sex ($F_{(1,32)} = 14.420$, $P < 0.001$), trauma ($F_{(1,32)} = 33.530$, $P < 0.0001$) and interaction between these two factors ($F_{(1,32)} = 13.370$, $P < 0.001$). *Post hoc* analysis revealed that FS

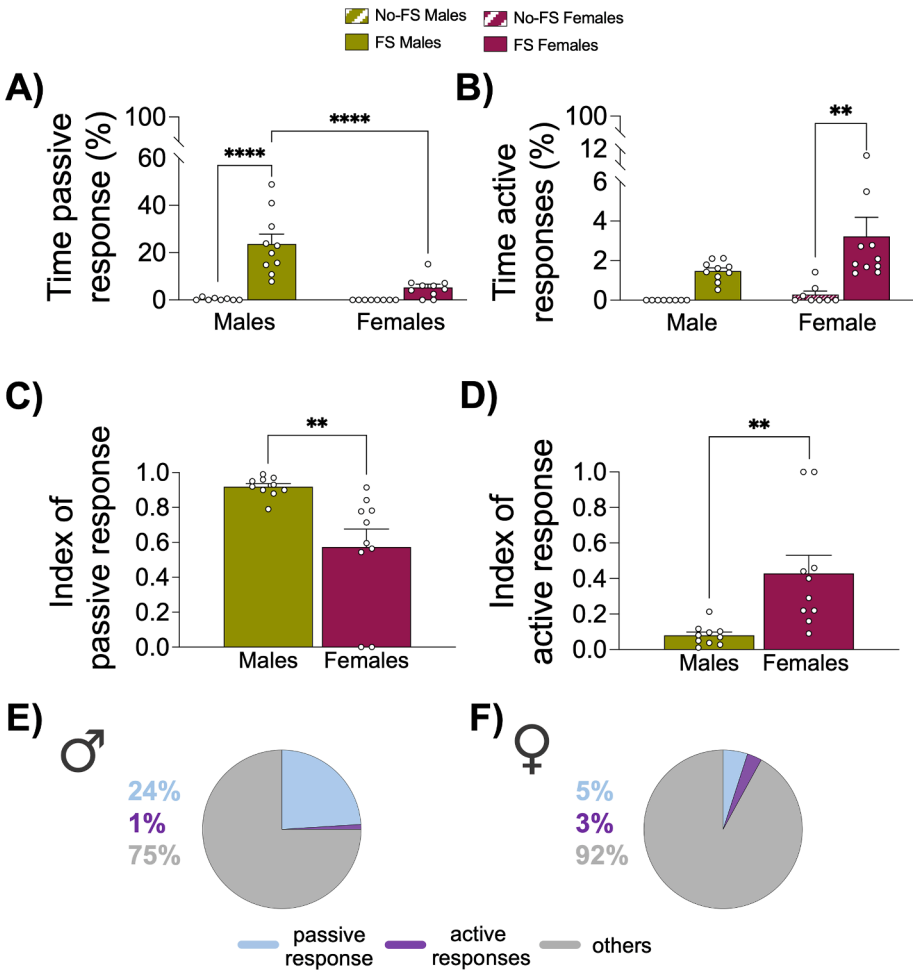

Fig. 2. Sex differences during trauma exposure in our PTSD model. **a)** Percentage of time spent in passive response during trauma exposure; **b)** percentage of time spent in active responses during trauma exposure; **c)** index of passive trauma response calculated as: passive response (s)/passive response (s) + active responses (s); **d)** index of active trauma response calculated as: active responses (s)/passive response (s) + active responses (s); graphic representation of the time spent in passive or active responses during trauma exposure session in **e)** FS males and **f)** FS females, calculated as passive (or active) response (s) x 100/total trauma exposure duration (s). ** $P < 0.01$; **** $P < 0.0001$. Data represent mean ± SEM (n = 8–10 rats per group).

males exhibited more time spent in passive response than both No-FS males ($P < 0.0001$) and FS females ($P < 0.0001$). Two-way ANOVA for time spent in active responses (Fig. 2b) indicated an effect of trauma ($F_{(1,32)} = 15.850$, $P < 0.001$), but not significant effects of sex ($F_{(1,32)} = 3.346$, $P = 0.077$) and interaction between trauma and sex ($F_{(1,32)} = 1.761$, $P = 0.194$). *Post hoc* analysis revealed that FS females spent more time in active responses than the corresponding No-FS group ($P < 0.01$). Unpaired *t* test for index of passive (Fig. 2c) and active (Fig. 2d) trauma responses revealed a significant difference between male and female FS groups ($t_{(18)} = 3.309$, $P < 0.01$ and $t_{(18)} = 3.335$, $P < 0.01$, respectively). Total percentage of time spent in passive and active responses in FS males and FS females is represented in Fig. 2e and f, respectively. Two-way ANOVA for number of active responses (Table 1a) indicated an effect of sex ($F_{(1,32)} = 12.280$, $P = 0.001$), trauma ($F_{(1,32)} = 81.690$, $P < 0.0001$) and interaction sex x trauma ($F_{(1,32)} = 5.175$, $P = 0.030$). *Post hoc* analysis revealed that FS male rats showed more active responses than the corresponding No-FS group ($P < 0.001$), and that FS females responded more actively to trauma than No-FS female rats ($P < 0.0001$) and FS male rats ($P < 0.001$). Two-way ANOVA for number of USVs emitted during trauma exposure (Table 1b) indicated an effect of trauma ($F_{(1,32)} = 149.000$, $P < 0.0001$), but not of sex ($F_{(1,32)} = 1.192$, $P = 0.283$) or interaction trauma x sex ($F_{(1,32)} = 1.192$, $P = 0.283$). *Post hoc* analysis revealed that, regardless of sex, FS animals emitted more USVs than the corresponding No-FS groups ($P < 0.0001$).

Table 1
a) Number of active responses during trauma exposure session. Males show a lower number of active responses than females; **b) Number of 22-kHz USVs emitted during trauma exposure.** Both FS male and female rats emitted a higher number of USVs than the No-FS counterparts.

	Active responses (number)	USVs (number)
No-FS		
Males	0.00±0.00	0.00±0.00
Females	1.00±0.57	0.00±0.00
FS		
Males	5.50±0.43 ###, °°°	169.30±11.01 ####
Females	10.20±1.31 ####	141.50±19.77 ####

###, $P < 0.001$; ####, $P < 0.0001$ vs the corresponding No-FS group; °°°, $P < 0.001$ vs FS female group. Data are represented as mean ± SEM (n = 8–10 rats per group).

All together, these data suggest that males predominately respond passively to trauma, while females exhibit higher levels of active behaviors. Moreover, females also emit a lower number of USVs compared to their male counterpart.

Female rats extinguish trauma faster and more efficiently than males, in terms of reduction of both freezing and USVs emission

Here, we sought to analyze the extinction profile in male and female rats subjected to our animal model of traumatic stress, by examining both freezing and USVs emission to better investigate the emotional state of the animals. RM ANOVA for time spent in freezing (Fig. 3a) showed an effect of sex ($F_{(1,32)} = 17.930$, $P < 0.001$), trauma ($F_{(1,32)} = 114.900$, $P < 0.0001$), days after trauma ($F_{(3,96)} = 15.680$, $P < 0.0001$), interaction sex x trauma ($F_{(1,32)} = 16.220$, $P < 0.001$), days after trauma x trauma ($F_{(3,96)} = 17.580$, $P < 0.0001$), but not an effect of days after trauma x sex ($F_{(3,96)} = 1.512$, $P = 0.216$) and days after trauma x sex x trauma ($F_{(3,96)} = 1.120$, $P = 0.345$). Particularly, *post hoc* analysis revealed that FS male rats spent more time in freezing than the corresponding No-FS males at each time point and the FS females at 10, 13 and 16 days after trauma ($P < 0.0001$ for each comparison). On the other hand, FS females spent more time in freezing than the corresponding No-FS group at 7 and 10 days after trauma ($P < 0.0001$ and $P < 0.01$, respectively). These data suggest that trauma exposure induces a greater freezing response in both FS males and FS females than No-FS rats, and that this response is higher in males than in females. Moreover, *post hoc* analysis also showed that, although both FS male and FS female rats exhibited reduced freezing over the extinction sessions (days 7, 10 and 13) and extinction retention test (day 16), females, nevertheless, extinguished trauma sooner and more effectively compared to males (in FS males: $P < 0.0001$ day 7 vs day 16; in FS females: $P < 0.05$ day 7 vs day 10, $P < 0.0001$ day 7 vs days 13 and 16). Such data is also confirmed by RM ANOVA for the Extinction Index, that showed an effect of sex ($F_{(1,18)} = 11.880$, $P < 0.01$) and days after trauma ($F_{(2,36)} = 12.490$, $P < 0.0001$), but not an effect of the interaction sex x days after trauma ($F_{(2,36)} = 0.695$, $P = 0.506$). Particularly, *post hoc* analysis revealed that FS male rats had a lower extinction index than females at each time point ($P < 0.05$ at 10 and 16 days after trauma, and $P < 0.01$ at 13 days after trauma). Moreover, *post hoc* highlighted an increase of the extinction index along the extinction sessions at both 13 and 16 days after trauma in female rats ($P < 0.01$ in either case), and only at 13 for males ($P < 0.01$).

RM ANOVA for number of USVs emitted during the extinction sessions (Fig. 3c) showed an effect of sex ($F_{(1,32)} = 6.919$, $P = 0.013$), trauma ($F_{(1,32)} = 61.390$, $P < 0.0001$), days after trauma ($F_{(3,96)} = 14.180$, $P < 0.0001$), interaction sex x trauma ($F_{(1,32)} = 9.391$, $P = 0.004$), days after trauma x trauma ($F_{(3,96)} = 15.410$, $P < 0.0001$), but not an effect of days after trauma x sex ($F_{(3,96)} = 0.854$, $P = 0.468$) and days after trauma x sex x trauma ($F_{(3,96)} = 0.229$, $P = 0.876$). *Post hoc* analysis revealed that 7 days after trauma, both FS males and FS females emitted more 22-kHz USVs than the corresponding No-FS groups ($P < 0.0001$ for each comparison). However, in the following days (10, 13 and 16 days after trauma), the number of USVs remained higher in FS

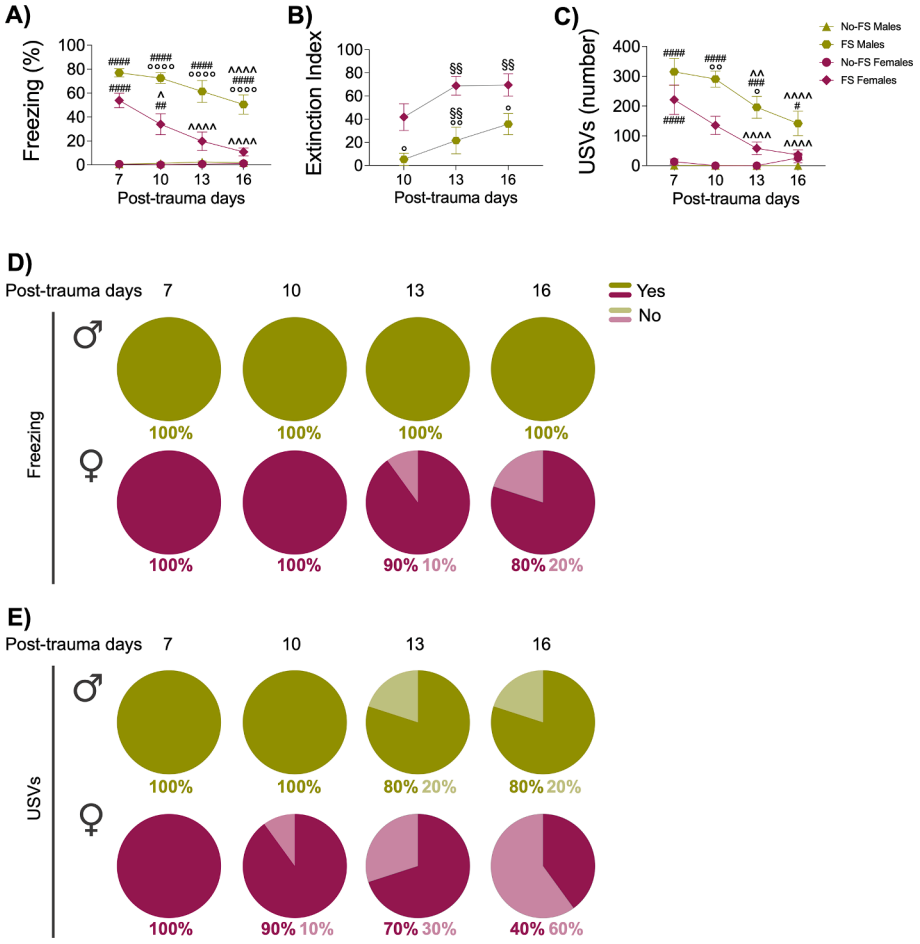

Fig. 3. Extinction of fear memory in male and female rats. **a)** Percentage of time spent in freezing during extinction sessions and extinction retention test; **b)** extinction index of FS male and female rats; **c)** number of 22-kHz USVs emitted during the extinction sessions and extinction retention test; **d)** percentage of FS males and FS females who froze or not at extinction sessions (days 7, 10, 13) and extinction retention test (day 16); **e)** percentage of FS males and FS females who emitted alarm calls or not at extinction sessions (days 7, 10, 13) and extinction retention test (day 16); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ vs the corresponding No-FS group;^$P < 0.05$,^^$P < 0.01$,^^^^$P < 0.0001$ vs the same group at day 7; §§ $P < 0.01$ vs the same group at day 10; ° $P < 0.05$, °° $P < 0.01$, °°°° $P < 0.0001$ vs the corresponding female group. Data represent mean ± SEM (n = 8–10 rats per group).

males, and not FS females, compared to the corresponding group ($P < 0.0001$, $P < 0.001$ and $P < 0.05$ at 10, 13 and 16 days after trauma, respectively). Moreover, both FS males and FS females presented an overall reduction of the number of USVs emitted across the extinction sessions (days 7, 10 and 13) and extinction retention test (day 16) (in males: $P < 0.01$ for day 7 vs day 13, $P < 0.0001$ day 7 vs day 16; in females: $P < 0.0001$ for day 7 vs day 13, and $P < 0.0001$ for day 7 vs day 16). Furthermore, FS male group exhibited a higher number of USVs than FS females at both 10 ($P < 0.01$) and 13 days ($P < 0.05$) after trauma.

χ^2 test for freezing (Fig. 3d), during all the extinction sessions and extinction retention test, revealed that all FS male rats showed freezing behavior (100 %), while, for FS females, all of them exhibited freezing behavior at 7 and 10 days after trauma (100 %), 90 % of them exhibited freezing behavior at 13 days after trauma, and 80 % of them exhibited freezing behavior at 16 days after trauma. Nevertheless, at each time point, both sexes had the same probability to freeze ($\chi^2 = 1.053$, $P = 0.305$ and $\chi^2 = 2.222$, $P = 0.136$, respectively at 13 and 16 days after trauma).

Regarding USVs emission (Fig. 3e), all the FS males (100 %) vocalized at 7 and 10 days after trauma, while at 13 and 16 days after trauma, 80 % of them emitted USVs. Considering FS females, instead, the percentage of rats who vocalized was 100 %, 90 %, 70 % and 40 % across the extinction sessions and extinction retention test. However, also in this case, the probability to vocalize in the two sexes was the same ($\chi^2 = 1.053$, $P = 0.305$; $\chi^2 = 0.267$, $P = 0.606$; $\chi^2 = 3.333$, $P = 0.068$, respectively for 10, 13 and 16 days after trauma).

Taken together, the present findings demonstrate that USVs emission mirrors the extinction profile, and that females are able to extinguish trauma better and faster than males, not only in terms of reduction of freezing, but also of 22-kHz USVs emission.

Exposure to a 'shock reminder' induces fear memory re-activation only in FS males, in terms of reduction of both freezing and USVs emission, and it elicits a conditioned fear response in No-FS male rats

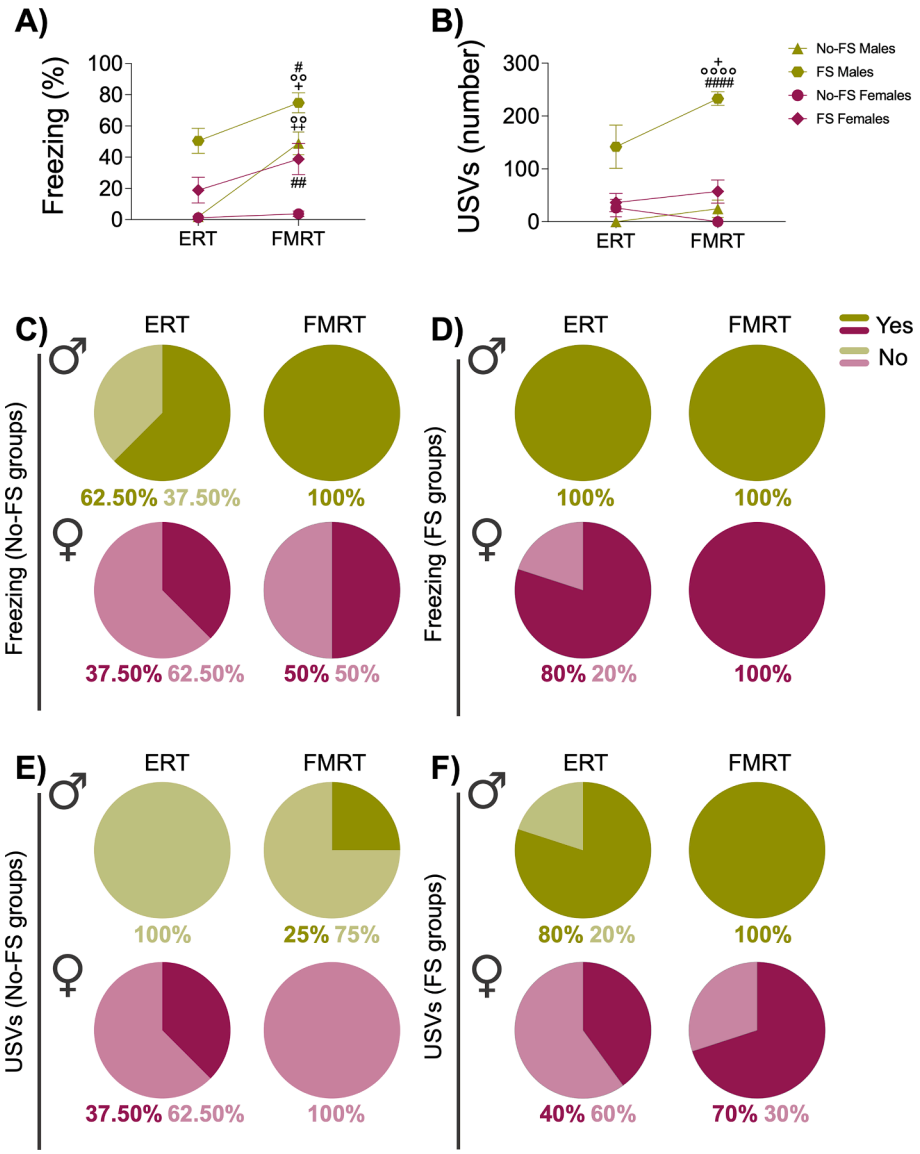

Fig. 4. Re-activation of fear in male and female rats. **a)** Percentage of time spent in freezing at extinction retention test (ERT) and fear memory re-activation test (FMRT); **b)** number of 22-kHz USVs emitted at ERT and FMRT; **c)** percentage of No-FS males and No-FS females who froze or not at ERT and at FMRT; **d)** percentage of FS males and FS females who froze or not at ERT and at FMRT; **e)** percentage of No-FS males and females who emitted alarm calls or not at ERT and at FMRT; **f)** percentage of FS males and FS females who emitted alarm calls or not at ERT and at FMRT. # $P < 0.05$, ## $P < 0.01$, #### $P < 0.0001$ vs the corresponding No-FS group; + $P < 0.05$, ++ $P < 0.01$ vs the same group at ERT; °° $P < 0.01$, °°°° $P < 0.0001$ vs the corresponding female group. Data represent mean ± SEM (n = 8–10 rats per group).

only, in terms of enhancement of freezing but not USVs emission.

We tested whether the exposure to a mild stressor ('shock reminder'; 2 s, 0.4 mA) could re-activate the traumatic memory in the FS rats. As shown in Fig. 4a, RM ANOVA for time spent in freezing revealed an effect of sex ($F_{(1,32)} = 22.450$, $P < 0.0001$), trauma ($F_{(1,32)} = 28.350$, $P < 0.0001$), session days ($F_{(1,32)} = 42.290$, $P < 0.0001$), interaction session days x sex ($F_{(1,32)} = 11.500$, $P = 0.009$) and session days x sex x trauma ($F_{(1,32)} = 7.747$, $P = 0.009$), but not a significant effect of session days x trauma ($F_{(1,32)} = 0.137$, $P = 0.713$) and sex x trauma ($F_{(1,32)} = 0.829$, $P = 0.369$). *Post hoc* analysis showed that FS male rats spent more time in freezing when they were re-exposed to the aversive context (fear memory re-activation test, 1 day after the exposure to the 'shock reminder') vs the extinction retention test (16 post-trauma days) ($P < 0.05$), indicating fear memory re-activation. No-FS males showed higher freezing in the context during fear memory re-activation test vs the extinction retention test ($P < 0.01$), indicating a conditioned fear response. Furthermore, both male and female FS groups exhibited greater freezing levels than the corresponding No-FS groups during fear memory re-activation test ($P < 0.05$ and $P < 0.01$, respectively for males and females). Sex differences have been observed at fear memory re-activation test, in both No-FS and FS groups, with males showing higher freezing levels than the corresponding No-FS and FS females ($P < 0.01$ for each comparison).

To analyze in more detail the emotional state of the animals during all the fear re-activation memory phases of our experimental protocol as well as to assess whether there could be a link with the fear memory expression and emission of alarm calls, we measured 22-kHz USVs emission. As shown in Fig. 4b, RM ANOVA for USVs emission revealed an effect of sex ($F_{(1,32)} = 16.650$, $P < 0.001$), trauma ($F_{(1,32)} = 37.160$, $P < 0.0001$), session days ($F_{(1,32)} = 4.441$, $P = 0.043$), interaction session days x sex ($F_{(1,32)} = 5.298$, $P = 0.028$), session days x trauma ($F_{(1,32)} = 4.665$, $P = 0.038$), sex x trauma ($F_{(1,32)} = 16.980$, $P < 0.001$), but not for session days x sex x trauma ($F_{(1,32)} = 0.139$, $P = 0.712$). *Post hoc* analysis showed that only FS male rats showed fear memory re-activation in terms of USVs emission ($P < 0.05$), and that they vocalized more than the No-FS corresponding group ($P < 0.0001$) during fear memory re-activation test. Finally, FS males emitted higher number of 22-kHz USVs than FS females at the fear re-activation memory test ($P < 0.0001$).

In Fig. 4c, χ^2 test for freezing indicated that in No-FS groups at the extinction retention test, both sexes had the same probability to freeze ($\chi^2 = 1.000$, $P = 0.317$), with 62.50 % of males who froze and 37.50 % of females. For fear memory re-activation test, instead, all the male rats exhibited freezing behavior (100 %), while only 50 % of females did it. Thus, males had a greater probability to freeze compared to females ($\chi^2 = 5.333$, $P = 0.021$). Taking into account USVs emission (Fig. 4e), both at extinction retention and at fear memory re-activation test, No-FS rats had the same likelihood to vocalize ($\chi^2 = 3.692$, $P = 0.055$ and $\chi^2 = 2.286$, $P = 0.131$, respectively). At the extinction retention test, none of the males vocalized (0 %) and 37.50 % of females emitted USVs, respectively, while, at fear memory re-activation test, 25 % of male rats vocalized, and none of the females (0 %) did it. Data related to the FS groups at the extinction retention test (post-trauma day 16) have been reported in the previous paragraph. Considering FS groups at fear memory re-activation test, males and females had the same probability to freeze (Fig. 4d). Both FS male and FS female groups (100 %) did freeze (Fig. 4d). Concerning the emission of alarm USVs 100 % of FS male rats and 70 % of FS females emitted USVs ($\chi^2 = 3.529$, $P = 0.060$ for fear memory re-activation test; Fig. 4f).

All together these data demonstrate that the exposure to a 'shock reminder' re-activates fear related memory in previously traumatized rats in terms of both freezing and USVs emission and increases freezing levels (conditioned fear response) in rats that were never exposed to a trauma before. Interestingly, these effects were sex divergent as they were observed in male rats only.

Discussion

Collectively, the present findings have shown a pronounced sex difference in fear expression, extinction, and re-activation in our traumatic stress paradigm, consisting in a footshock-based model (e.g., contextual fear conditioning paradigm) paired with social isolation. While males predominately expressed freezing behavior as trauma response, females exhibited more active fear responses. Moreover, females extinguished the fear memory faster and more efficiently than the male counterpart and only male rats exhibit fear memory re-activation after exposure to a 'shock reminder'.

Compelling research has demonstrated that women have a two-fold greater risk to develop PTSD than men (Olff, 2017; Tolin and Foa, 2006). Notwithstanding, while important evidence highlighted sex differences in stress response (Bangasser and Wicks, 2017; Lu et al., 2015), females are still underconsidered in preclinical studies (Deslauriers et al., 2018). We have recently developed an animal model of PTSD able to capture some of the most important cognitive and emotional alterations of the human pathology, while at the same time mirroring the chronic nature of the disorder (Berardi et al., 2014; Morena et al., 2018). This model involves the exposure of rats to a sequence of inescapable footshocks coupled with social isolation, thereby establishing a durable memory trace of the traumatic event. However, until now, our model has been somewhat incomplete as it has solely focused on male subjects, neglecting female inclusion.

The findings of the present study revealed an intriguing sex dichotomy between passive and active responses to trauma. Specifically, male rats exhibited a predominantly passive response characterized by elevated freezing levels, whereas females displayed a more pronounced active response. While these two behaviors may appear to be opposites, they can be viewed as complementary facets of the same underlying mechanism. Freezing, typically associated with a passive response, and active behaviors such as darting, reminiscent of an escape-like reaction (Gruene et al., 2015), are indeed both conditioned responses (Greiner et al., 2019; Pellman et al., 2017). Active responses express fear acquisition and are influenced by similar situational factors that also affect freezing behavior (Mitchell et al., 2022). Our study complements recent translational findings, indicating that females adopt a distinct response strategy to fear, predominately manifesting an active behavior. This pattern translates into darting behavior in rodents and a tend-and-befriend response in humans (Morena et al., 2021; Olff, 2017; Taylor et al., 2000; Velasco et al., 2019). It has been speculated that the disparities in fear reaction between the two sexes may arise from oxytocin's capacity to activate a subset of neurons within the centro-lateral amygdala, indirectly inhibiting the centro-medial amygdala (Terburg et al., 2018; Viviani et al., 2011). Such neural modulation might explain the reduction in the passive fear responses, such as freezing and fear-potentiated startle, and the increase in the active ones.

While examining a different parameter compared to the established gold standard measure of fear in rodents (Fanselow, 1980) is already a promising approach for uncovering potential sex differences in fear manifestation, other behavioral indices may be taken into account to fully encapsulate the emotional state of the animal linked to its memory elaboration (LeDoux and Pine, 2016). In this regard, USVs may represent a very useful tool (Knutson et al., 2002). We have previously demonstrated that there is a correlation between freezing behavior and the emission of 22-kHz USVs during a fear conditioning paradigm, also highlighting the sex-specific nature of this association (Riccardi et al., 2021). Regarding fear memory alterations, here we found that 22-kHz USV emission parallels the passive response (freezing) in male animals, while it mirrors the active responses in female rats during trauma presentation and fear acquisition. The increased freezing behavior observed in males and the heightened active responses observed in females were accompanied by an increased number of USVs in FS groups compared to the No-FS groups.

Rats produce 22-kHz USVs in situations potentially harmful to other

individuals or to the group. Such situations may involve social factors (e.g., encounters with aggressive peers or humans) (Brudzynski and Ociepa, 1992; Panksepp et al., 2004), or non-social factors (e.g., exposure to air puffs or withdrawal from rewarding drugs) (Brudzynski and Holland, 2005; Oliveira and Barros, 2006). Additionally, anticipation of negative emotional stimuli can trigger 22-kHz USV emission in rats (Kassai and Gyertyán, 2018). The emission of 22-kHz USVs thus indicates in rats heightened arousal to negative emotional stimuli and an aversive emotional state (Premoli et al., 2023). This aligns with the notion that 22-kHz USVs emission may be an evolutionary analogue to adult human crying (Brudzynski, 2019).

The inability to extinguish the memory associated to trauma is one of the hallmarks of PTSD (Pitman et al., 2012). In order to mimic a classical setting of exposure therapy in humans, we had previously demonstrated that, in rats, repeated spaced exposures to the traumatic context in the absence of the US (footshocks) were able to reduce freezing behavior, dampening fear memory recall and promoting extinction (Morena et al., 2018). Intriguingly, here we found significant sex differences in our animal model of traumatic stress. Specifically, females exhibited better and quicker extinction of the traumatic event compared to males both in terms of reduction in freezing behavior and USVs emission. Such data are in line with experimental studies demonstrating sex differences in fear memory dynamics, with females exhibiting a greater extinction capability than males (Baran et al., 2009; Binette et al., 2022; Mancini et al., 2021; Maren et al., 1994). However, this finding seems contradictory to human evidence, which shows a greater susceptibility to PTSD in women compared to men. While one possible explanation is that our study, as well as previous ones, have examined the entire traumatized population rather than specifically focusing on vulnerable individuals, this sex difference remains of utmost importance. Further extensive follow-up studies are needed to unravel the neural mechanisms underlying these divergent sex effects. We also analyzed 22-kHz USVs emission during each re-exposure to the traumatic context (7, 10, 13 and 16 days after trauma), and our results indicate that USVs emission, like freezing behavior, decreases across the extinction sessions.

In the present study we further aimed at investigating whether a single mild stressor could rekindle a previously extinguished conditioned response. Prior to this investigation, in our traumatic stress model we had not addressed this facet, yet it holds significance, particularly in understanding symptom relapse within PTSD. The fear memory reactivation stands as a significant challenge in the relapse of PTSD after CBT and exposure therapy (Goode and Maren, 2019; Boschen et al., 2009; Craske and Rachman, 1987; Vervliet et al., 2013a; Vervliet et al., 2013b; Yonkers et al., 2003). It entails the phenomenon whereby extinguished fear reappears upon re-exposure to trauma-associated stimuli (Krisch et al., 2020) (Zuj and Norrholm, 2019). Hence, in the present study, we re-exposed (FS groups) or exposed for the first time (No-FS groups) the animals to a mild shock 3 weeks after trauma and measured the behavioral response (i.e., freezing and 22-kHz USV emission). We found that after the exposure to a 'shock reminder' rats that were previously traumatized (FS) exhibited fear memory reactivation at both freezing and USVs emission levels. Moreover, while rats that were not exposed to trauma before (No-FS) showed increased freezing behavior when they were exposed to the aversive context the day after, indicating the formation of a conditioned fear response. Interestingly, these results were sex divergent as both FS and No-FS females did not show any behavioral effects. Gonadal hormones, brain circuit involved but also the kind of stressor and experimental paradigm (Allen and Gorski, 1990; Avery et al., 2014; Baran et al., 2009; Blume et al., 2017; Dalla and Shors, 2009; Gresack et al., 2009) play an important role as potential factors involved in this difference. In addition to that, the underrepresentation of females in preclinical research and the fact that many behavioral tests have been developed using only male rodents, has led to neglecting important behavioral manifestation of fear in females which may be different from those shown by males. This, ultimately, may lead to misinterpretation of behavioral results. For instance, female rats generally exhibit higher active behavior compared to males. Consequently, behavioral assessments relying on the animals' activity levels may identify lower or higher behavioral changes in females compared to males, depending on the direction of the effect (Dalla et al., 2024; Kokras and Dalla, 2014).

In conclusion, our data provide evidence supporting robust sex differences in the modulation of abnormal fear memory. Moreover, our results underline the importance of considering 22-kHz USVs emission as another behavioral parameter that, together with the other parameters traditionally used to measure indices of fear, may help to have a more complete picture of the spectrum of fear memory responses and allow for a better interpretation of results in both sexes, thus providing evidence as to how implement sex as a biological variable into preclinical research. All of this will pave the way for increasingly customized effective therapies.

CRediT authorship contribution statement

Eleonora Riccardi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Giulia Federica Mancini:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Arianna Pisaneschi:** Methodology, Investigation, Formal analysis, Data curation. **Maria Morena:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Patrizia Campolongo:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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