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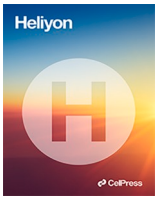

The sexually divergent cFos activation map of fear extinction

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ABSTRACT

Objective: Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder that can develop after experiencing or witnessing a traumatic event. Exposure therapy is a common treatment for PTSD, but it has varying levels of efficacy depending on sex. In this study, we aimed to compare the sexual dimorphism in brain activation during the extinction of fear conditioning in male and female rats by detecting the c-fos levels in the whole brain.

Methods: Thirty-two rats (Male: n = 16; Female: n = 16) were randomly separated into the extinction group as well as the non-extinction group, and fear conditioning was followed by extinction and non-extinction, respectively. Subsequently, brain sections from the sacrificed animal were performed immunofluorescence and the collected data were analyzed by repeated two-way ANOVAs as well as Pearson Correlation Coefficient.

Results: Our findings showed that most brain areas activated during extinction were similar in both male and female rats, except for the reuniens thalamic nucleus and ventral hippocampi. Furthermore, we found differences in the correlation between c-fos activation levels and freezing behavior during extinction between male and female rats. Specifically, in male rats, c-fos activation in the anterior cingulate cortex was negatively correlated with the freezing level, while c-fos activation in the retrosplenial granular cortex was positively correlated with the freezing level; but in female rats did not exhibit any correlation between c-fos activation and freezing level. Finally, the functional connectivity analysis revealed differences in the neural networks involved in extinction learning between male and female rats. In male rats, the infralimbic cortex and insular cortex, anterior cingulate cortex and retrosplenial granular cortex, and dorsal dentate gyrus and dCA3 were strongly correlated after extinction. In female rats, prelimbic cortex and basolateral amygdala, insular cortex and dCA3, and anterior cingulate cortex and dCA1 were significantly correlated.

Conclusion: These results suggest divergent neural networks involved in extinction learning in male and female rats and provide a clue for improving the clinical treatment of exposure therapy based on the sexual difference.

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1. Introduction

Post-traumatic stress disorder (PTSD) is a mental disorder developed following the individual experienced or witnessed an extremely stressful event, including an egregious threat or even a devastating disaster [1–3]. The overall lifetime prevalence of PTSD in the general population ranges from 1 % to 14 %, with a higher prevalence in women than men [1–4]. PTSD is characterized by hyperarousal, flashback, and the avoidance of related events and memories [5,6]. Exposure therapy, a behavior treatment that aims to weaken the associations between stressful events and related cues by repeated exposure to the environment, is a widely used method for treating PTSD. However, the biological mechanism underlying exposure therapy is still not fully understood [7–14]. Therefore, research progress on fear memory and its extinction is essential for improving the understanding and treatment of PTSD [2,3,7–12].

Pavlovian fear conditioning is a commonly used paradigm in fear memory research [9–12,15–23]. This paradigm involves pairing a neutral stimulus with a threatening stimulus to create a conditioned response (CR), mimicking the process of fear memory acquisition [13,17,20,22,24]. Subsequent extinction training leads to significant reductions in learned fear behavior [13,22,24,25]. This model reflects the behavioral and physiological changes observed in animals after acquiring fear memory, and its validity has been verified in previous studies [15,18–21,26–29]. Recently, a few studies have reported different findings in fear-related studies that may contribute to gender differences and highlight the influence of gender on fear-related behaviors [19,21,30].

Previous studies have reported sex differences in rodents in the fear memory task [30–41]. For instance, in context fear conditioning, the freezing level of male rats was significantly higher than that of females, whether the stage was acquisition or extinction [42–45]. However, other studies found that the freezing level of females was significantly higher than that of males [46,47]. Considering that the neural circuits involved in fear memory may differ between the sexes [14,30,36,41,47–49], we aimed to investigate the effect of sex on behavior by comparing the differences in the activation levels of various brain regions in male and female rats after extinction training. We used c-fos mapping and functional connectivity analysis to compare the sexual dimorphism of the extinction paradigm at the brain level by detecting the expression levels of c-fos in male and female rats after the extinction of fear conditioning training.

2. Methods

2.1. Subjects

Both male and female Sprague-Dawley rats, at 8–10 weeks old, were obtained from the Laboratory Animal Center, Peking University Health Science Center. Each rat was fed in a single cage at a temperature of 23 ± 2 °C and humidity of 50 ± 5 % with a 12 h:12 h light: dark cycle (lights off at 8:00 and lights on at 20:00). Food and water were administered ad libitum. Daily vaginal swabbing and smears were performed in all female rats to ensure that they were in non-estrus during the behavioral experiments [50–53]. All behavioral experiments were performed under the dark phase (9:00 a.m. to 11:00 a.m.). Animal care and experimental procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All experiments were permitted by the Biomedical Ethics Committee of Peking University.

2.2. Auditory fear conditioning (AFC)

2.2.1. Apparatus

The equipment used for fear conditioning is the same as used in past experiments [54,55]. Specifically, the fear conditioning was conducted using The Startle and Fear-conditioning system (Panlab, Barcelona, Spain). The device includes four Plexiglas chambers (30 cm height × 30 cm width × 30 cm length) with floors consisting of metal stainless-steel rods connected to a shock generator. Each chamber is enclosed in a ventilated soundproof box. Across all procedures, there were two different contexts (A and B). Context A (conditioning context), a chamber with a grid floor, consisted of the original conditioning chamber with a 20 % ethanol odor. And context B, several modifications were introduced based on the original training chambers to create a novel extinction training context. Specifically, the stainless-steel rod floor was covered with black Plexiglas, and the odor was replaced by a 2 % acetic acid.

2.2.2. Protocols

Fear conditioning. The animals were placed in training chamber A and allowed to explore it for 10 min. Fear conditioning was then conducted with three 20 s, 2 kHz, 80 dB tones (CS), each co-terminated with a 1 s, 0.8 mA foot-shock (US). The interval between each CS was 120 s. After conditioning, the rats were allowed to explore the conditioning chamber for an additional 2 min before returning to their home cages.

Extinction training. Extinction of conditioned fear was performed on day 2 (24 h intervals). During extinction, rats were placed inside the extinction chamber (Context B). The extinction session consisted of 18 presentations of CS without US, and each CS was separated from other CS via 120s intervals.

2.3. Immunofluorescence

All rats were anesthetized and transcardially perfused with phosphate-buffered saline (PBS, PH 7.4) within 60–90 min at the end of behavioral experiments, followed by 4 % paraformaldehyde (PFA) in 0.1 M PBS, and then brains were post-fixed for 24 h in 4 % PFA

and immersed in 30 % sucrose solution to dehydrate. Twelve pieces of Coronal sections of each region of interest (30 μm, three animals per group) were obtained utilizing freezing microtome and washed with PBS three times for 5min each. Sequentially, sections were blocked in 10 % goat serum with 0.2 % Triton-X for 3 h and then incubated at 4 °C with primary antibody against c-Fos (1:1000, Abcam ab190289) overnight. After being washed in PBS, tissues were incubated in secondary antibody Alexa Fluor 488 (1:500, thermo A32731) for 3 h at room temperature. Finally, sections were washed in PBS for 4 times prior to being mounted with Mounting Medium (antifading (with DAPI), Solarbio, S2110-5 ml), and images were captured using an Olympus BX53 fluorescent microscope (Olympus Corporation, Tokyo, Japan) [56,57].

We used lmageJ to determine c-fos activation. Specifically, the images of regions of interest were captured from sections in accordance with The Rat Brain in Stereotaxic Coordinates (fifth edition). We captured images for c-fos, and DAPI and then merged. Each captured image of c-fos was saved and the background was filtered using ImageJ software. The highest level of immunofluorescence expression was defined as the assumed c-fos positive cell, and the calculation of positive neurons was performed artificially. The DAPI counting was performed using captured images of DAPI, similar to counting assumed c-fos neurons. The c-fos and DAPI cells were counted using the counting program within the ImageJ software. Sequentially, the assumed c-fos positive cell was compared with the merged image to ensure the actual c-fos positive neuron. The final counting of actual c-fos positive neuron was presented as the average from four section in each animal, as did the DAPI. The result was shown as a percentage of the c-fos counting divided by the DAPI counting from twelve slices.

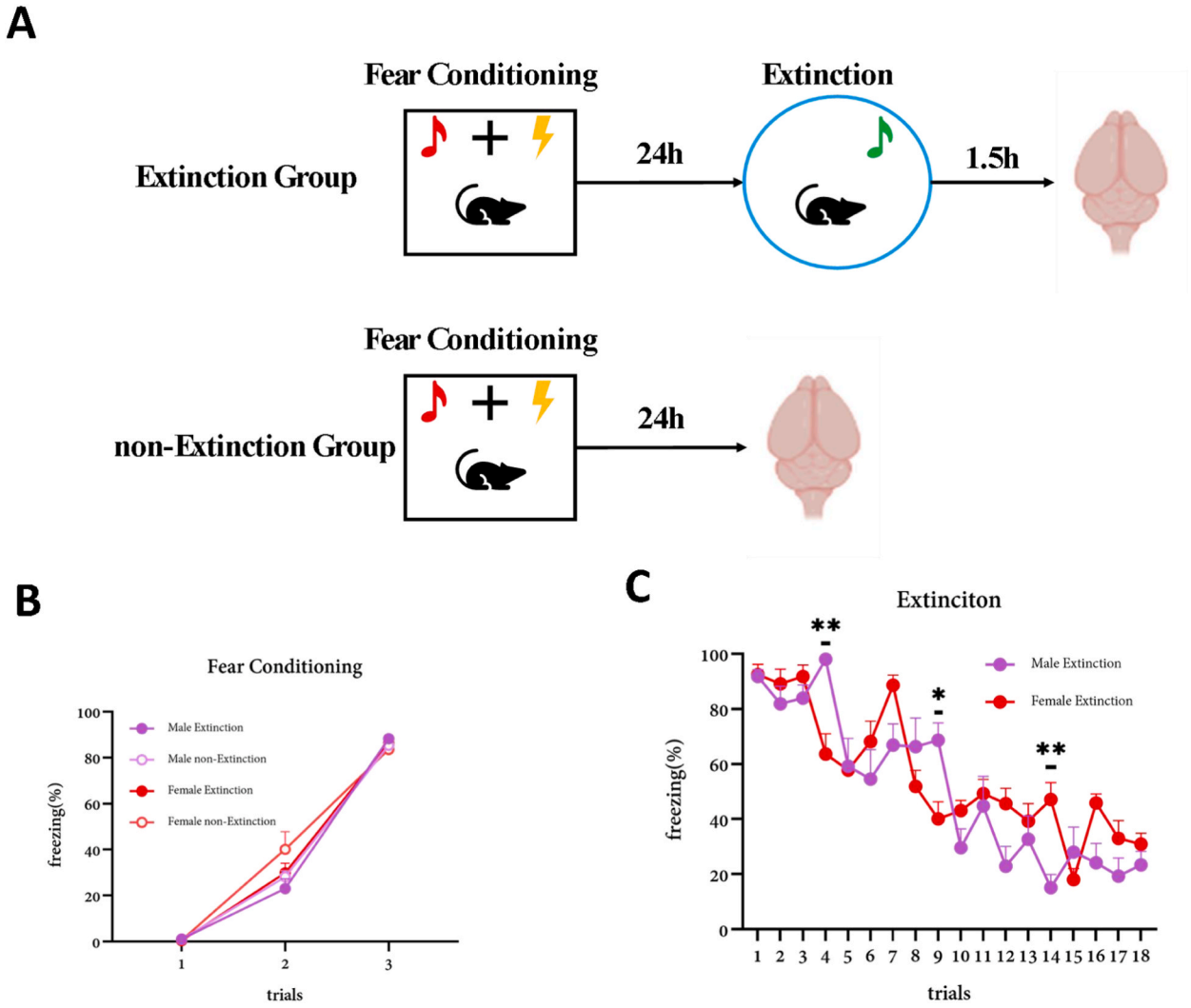

Fig. 1. The freezing levels of males and females in fear conditioning and extinction training. (**A**) Schematic representation of fear conditioning and extinction protocols in B and C. (**B**) The freezing levels across fear conditioning in both male and female groups. There was no significant difference between these groups in the last trial of fear conditioning. (n = 8 per group) (**C**) The freezing levels across extinction training in male and female rats. As conditioned fear, all rats successfully showed extinction. (n = 8 per group). Two-way ANOVA and Bonferroni multiple comparison tests were used. Data are represented as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001.

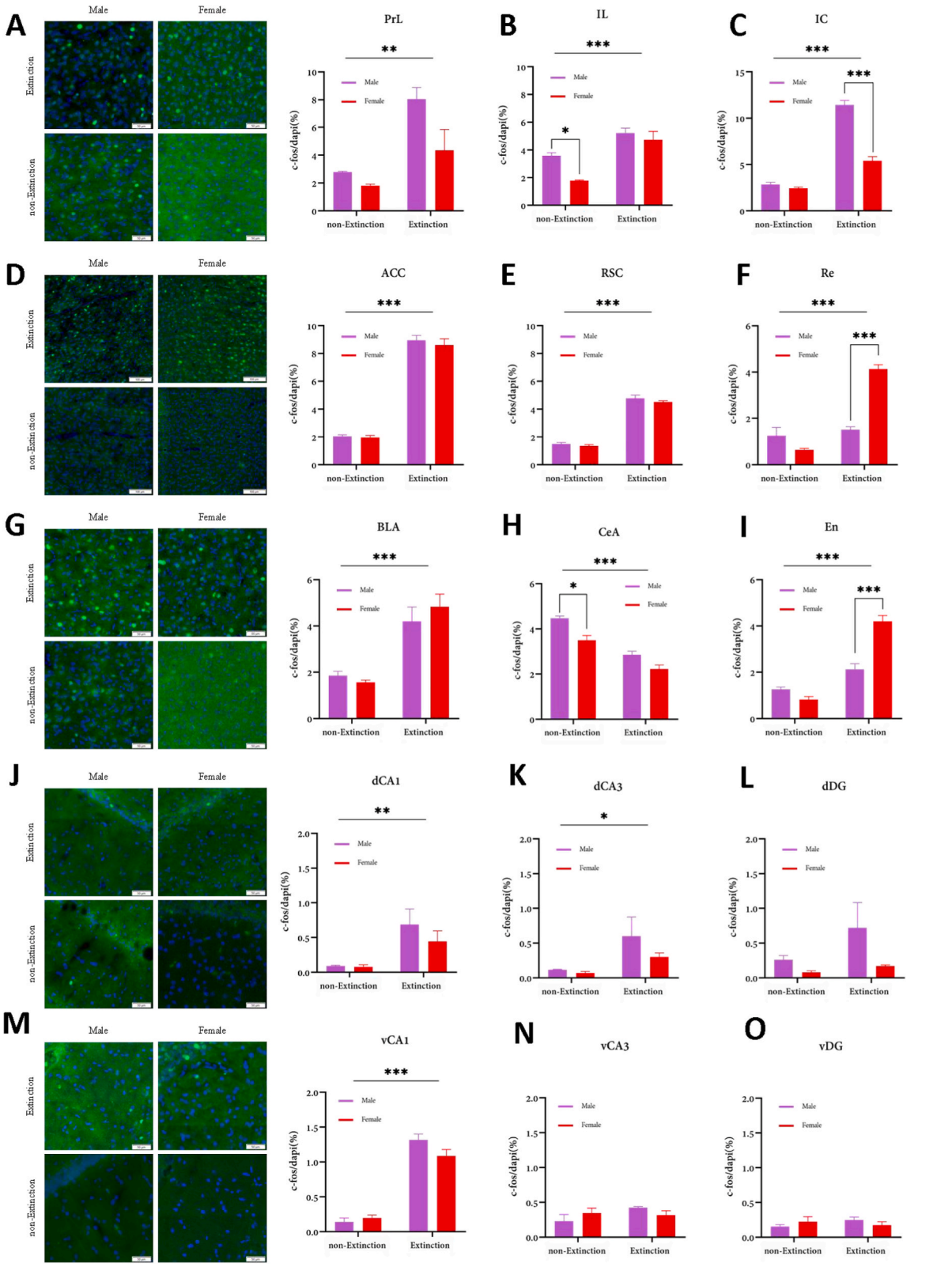

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Fig. 2. Compared the c-fos activation of 15 regions in male and female rats before and after extinction training.The immunofluorescence images of PrL(**A**), ACC(**D**), BLA(**G**), dCA1(**J**), and vCA1(**M**) left (male extinction group, left above; male non-extinction group, left bottom; female extinction group, middle above; female extinction group, middle bottom) in male and female rats are shown in figures; the statistical result of c-fos in male rats are shown as bar graphs, (**A**, right) PrL, (**B**) IL, (**C**) IC, (**D,** right) ACC, (**E**) RSC, (**F**) Re, (**G,** right) BLA, (**H**) CeA, (**I**) En, (**J,** right) dCA1, (**K**) dCA3, (**L**) dDG, (**M,** right) vCA1, (**N**) vCA3, (**O**) vDG. n = 3 per group. Two-way ANOVA and Bonferroni multiple comparison test were used. Data are represented as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001.

2.4. Statistical analysis

Data analysis was performed using GraphPad PRISM 8 software, ImageJ, and Jupyter Notebook. All the data showed a normal distribution and was expressed as mean ± SEM. The behavioral data and immunofluorescence data were analyzed using two-way repeated measures ANOVA as well as Bonferroni multiple comparisons and showed as line charts or bar charts by GraphPad PRISM 8 software, and the significant difference was defined as $p < 0.05$. For inter-regional correlation analysis and the freezing-region correlation analysis, we performed Pearson correlation analysis between all 15 regions or freezing in each group using Jupyter Notebook by Pandas corr(.)-function as well as using scipy.stats pearsonr(.)-function and then displayed as the heatmap using seaborn sns.heatmap(.)-function. The significant difference was also defined as $p < 0.05$ and the high correlation was defined as $r \geq 0.6$.

3. Results

3.1. No differences in fear acquisition and extinction between male and female rats

To investigate potential sexual differences in the activation of brain regions involved in conditioned fear extinction show, we conducted the experiment as depicted in Fig. 1A. Specifically, both male and female rats were divided randomly into two sub-groups: the extinction and the non-extinction group. On day 1, all animals underwent fear conditioning. After 24 h (day 2), all animals in the extinction group received extinction protocol before sacrificing, while the animals in the non-extinction group were directly perfused transcardially and collected the brains during the same time frame. As expected, both male and female rats could successfully learn conditioned fear memory (main effect of group: $F(3,84) = 0.8356$; main effect of trial: $F(2,84) = 679.5$, $p < 0.0001$; group × trial interaction: $F(6,84) = 2.107$, $p = 0.0608$; Fig. 1B). During the extinction conditioning process, both male and female rats exhibited a decrease in freezing levels across different trials (main effect of sex: $F(1, 252) = 4.819$, $p = 0.0291$; main effect of trial: $F(17,252) = 26.61$, $p < 0.0001$; sex × trial interaction: $F(17,252) = 3.678$, $p < 0.0001$; Fig. 1C). Notably, the animals in the extinction group of male rats displayed higher freezing levels at trial 4 (Bonferroni, $t(252) = 3.78$, $p = 0.0035$), trial 9 (Bonferroni, $t(252) = 3.125$, $p = 0.0358$), while showing a significantly lower level at trial 14 compared to the female rats (Bonferroni, $t(252) = 3.513$, $p = 0.0094$) (Fig. 1C). This disparity in fear extinction between male and female may be attributed to individual variation or the involvement of gender-specific brain networks recruited during extinction training.

3.2. Sexual difference in the activations of brain regions involved in fear extinction

To investigate the specific brain mechanisms underlying the observed differences in fear extinction between male and female rats, we analyzed the changes in c-fos expression in 15 brain regions associated with fear memory. First, we examined the involvement of prefrontal cortex, a key region in fear conditioning and extinction, in both males and females. Compared with the non-extinction group, the expression of c-fos in the prelimbic cortex (PrL, main effect of extinction: $F(1,8) = 20.65$, $p = 0.0019$) and infralimbic cortex (IL, main effect of extinction: $F(1, 8) = 39.53$, $p = 0.0002$) of male and female rats in the extinction group was significantly increased, and there were effect of sex in both brain regions (PrL: main effect of sex: $F(1,8) = 7.378$, $p = 0.0264$; sex × extinction interaction: $F(1,8) = 2.519$, $p = 0.1511$; Fig. 2A; IL: main effect of sex: $F(1,8) = 10.04$, $p = 0.0132$; sex × extinction interaction: $F(1,8) = 3.251$, $p = 0.1090$; Fig. 2B). We then compared the c-fos activity in the insular cortex (IC), a region implicated in aversion emotions. The result showed that extinction led to increased c-fos densities in IC (main effect of extinction: $F(1,8) = 253.2$, $p < 0.0001$), with males exhibiting a more increase in extinction-induced c-fos expression relative to females (main effect of sex: $F(1,8) = 78.48$, $p < 0.0001$; sex × extinction interaction: $F(1,8) = 60.34$, $p < 0.0001$; Bonferroni, extinction group $t(8) = 11.76$, $p < 0.0001$; Fig. 2C). Next, we measured the c-fos expression in the cingulate cortex, which is known to be related to emotion and memory, in extinction and non-extinction groups. In both male and female rats, the c-fos expression in the extinction group of was increased significantly in the anterior cingulate cortex (ACC, main effect of extinction: $F(1, 8) = 526.5$, $p < 0.0001$; Fig. 2D) and the retrosplenial cortex (RSC, main effect of extinction: $F(1,8) = 509.4$, $p < 0.0001$; Fig. 2E) without the main effect of sex (ACC: main effect of sex: $F(1,8) = 0.5373$, $p = 0.4845$, sex × extinction interaction: $F(1,8) = 0.1650$, $p = 0.6953$; and RSC: main effect of sex: $F(1,8) = 1.892$, $p = 0.2063$, sex × extinction interaction: $F(1,8) = 0.1766$, $p = 0.6853$). The reuniens thalamic nucleus (Re), a key brain region involved in long-term learning, exhibited an interesting pattern of c-fos expression. The c-fos expression of Re in the non-extinction group did not show a significant difference, but an increase was observed in the extinction group compared to the non-extinction group in the female rats (main effect of sex: $F(1,8) = 21.97$, $p = 0.0016$; main effect of extinction: $F(1,8) = 76.55$, $p < 0.0001$; sex × extinction interaction: $F(1,8) = 56.54$, $p < 0.0001$; Bonferroni, extinction group $t(8) = 8.632$, $p = 0.0002$; female group $t(8) = 11.50$, $p < 0.0001$; Fig. 2F). Besides the prefrontal cortex, the amygdala is a critical region of fear conditioning and extinction. The basolateral amygdala (BLA) displayed significantly increased c-fos expression in animals underwent extinction training (main effect of extinction: $F(1,8) = 43.82$,

p = 0.0002; Fig. 2G) without sex differences (main effect of sex: F(1,8) = 0.1646, p = 0.6956; sex × extinction interaction: F(1,8) = 1.186, p = 0.3079). However, the c-fos expression in the central amygdala (CeA) exhibited a decline in the extinction group of male and female group (main effect of extinction: F(1,8) = 75.89, p < 0.0001; Fig. 2H), and significant difference was observed between male and female rats in the non-extinction group (main effect of sex: F(1,8) = 23.62, p = 0.0013; sex × extinction interaction: F(1,8) = 1.067, p = 0.3318, Bonferroni, non-extinction group t(8) = 11.76, p = 0.0188). The entorhinal cortex (En), a region associated with learning, showed increased expression of c-fos in both male and female rats undergoing extinction, with females showing a greater increase of c-fos expression relative to males (main effect of sex: F(1,8) = 18.13, p = 0.0028; main effect of extinction: F(1,8) = 123.2, p < 0.0001; sex × extinction interaction: F(1,8) = 43.51, p = 0.0002; Bonferroni, extinction group t(8) = 7.675, p = 0.0004; Fig. 2I). Finally, we examined the c-fos expression pattern in the sub-regions of the hippocampi. In the dorsal hippocampi, extinction training led to an increased level of the c-fos expression in both dCA1 (main effect of extinction: F(1,8) = 12.49, p = 0.0077; Fig. 2J) and dCA3 (main effect of extinction: F(1,8) = 6.287, p = 0.0365; Fig. 2K) without sex differences (dCA1: main effect of sex: F(1,8) = 0.8885, p = 0.3735; sex × extinction interaction: F(1,8) = 0.7428, p = 0.4138; and dCA3: main effect of sex: F(1,8) = 1.467, p = 0.2604; sex × extinction interaction: F(1,8) = 0.8116, p = 0.3939). However, no difference on c-fos activity changes was observed between the extinction group and the non-extinction group in dDG (main effect of sex: F(1,8) = 3.919, p = 0.0831; main effect of extinction: F(1,8) = 2.187, p = 0.1775; sex × extinction interaction: F(1,8) = 1.010, p = 0.3443; Fig. 2L). For the ventral hippocampi, we found that the c-fos activity in vCA1 increased significantly in the extinction group compared to the non-extinction group (main effect of extinction: F(1,8) = 210.9, p < 0.0001), without the effect of sex (main effect of sex: F(1,8) = 1.503, p = 0.2551; sex × extinction interaction: F(1,8) = 4.065, p = 0.0785; Fig. 2M). There was no significant difference in the c-fos activity of vCA3 (main effect of sex: F(1,8) = 0.002548, p = 0.9610; main effect of extinction: F(1,8) = 1.526, p = 0.2517; sex × extinction interaction: F(1,8) = 2.823, p = 0.1315; Fig. 2N) and vDG (main effect of sex: F(1,8) = 0.002664, p = 0.9601; main effect of extinction: F(1,8) = 0.1711, p = 0.6900; sex × extinction interaction: F(1,8) = 2.180, p = 0.1781; Fig. 2O) between groups.

3.3. Sexual difference in Pearson Correlation Coefficient between brain regions and freezing levels

To explore the relationship between behavior and brain region activation, we conducted the Pearson correlation analysis between these 15 brain regions and the freezing levels (Fig. 3). For the male rats, ACC activation was negatively correlated with freezing levels, but RSC showed a positive correlation coefficient with the freezing levels (ACC, r = −1, p = 0.02106; RSC, r = 1, p = 0.00798; Fig. 3 above). While, regions from female rats did not exhibit any significant correlation coefficient with freezing levels (Fig. 3 bottom).

3.4. The sexual differences in inter-regional functional connections during fear extinction and non-extinction processes

Finally, to gain deeper insight into the functional connections within these 15 regions, we performed the Pearson correlation

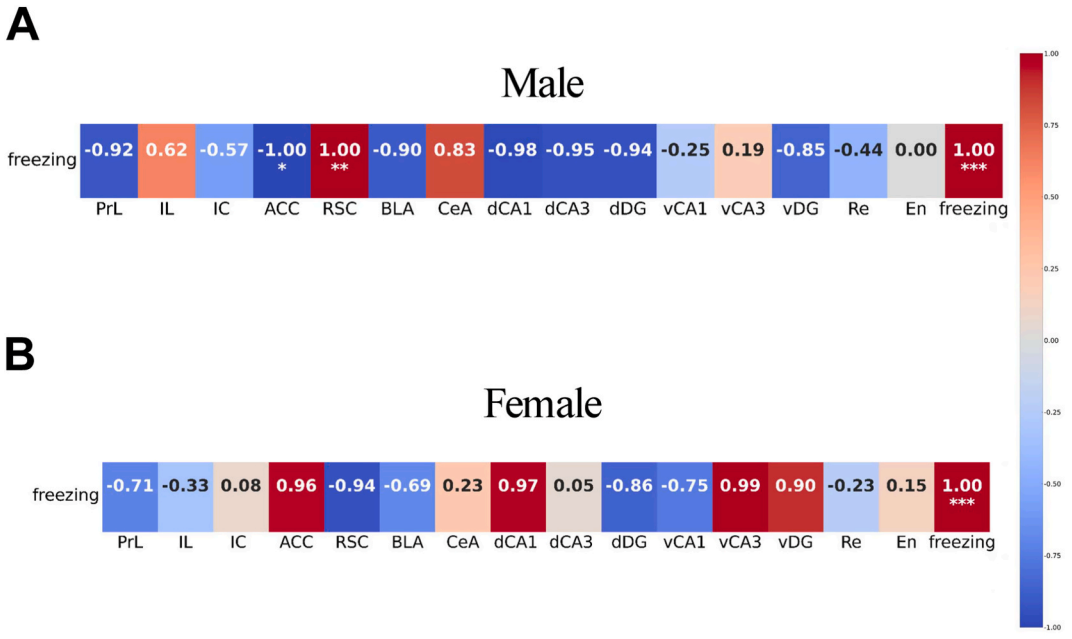

Fig. 3. The correlation analysis of freezing and brain regions involved.As the freezing times of the last 3 trials have dropped below 60 % in both males and females in the extinction session, we performed Pearson correlations analysis between the mean values of the last 3 trials and c-fos intensities of all regions of male (above) rats as well as female (bottom) rats. The results are shown as a heatmap using Python code. The red color represented positive correlations, whereas the blue color represented negative ones. $-1 \leq r \leq 1$, *p < 0.05, **p < 0.01, ***p < 0.001.

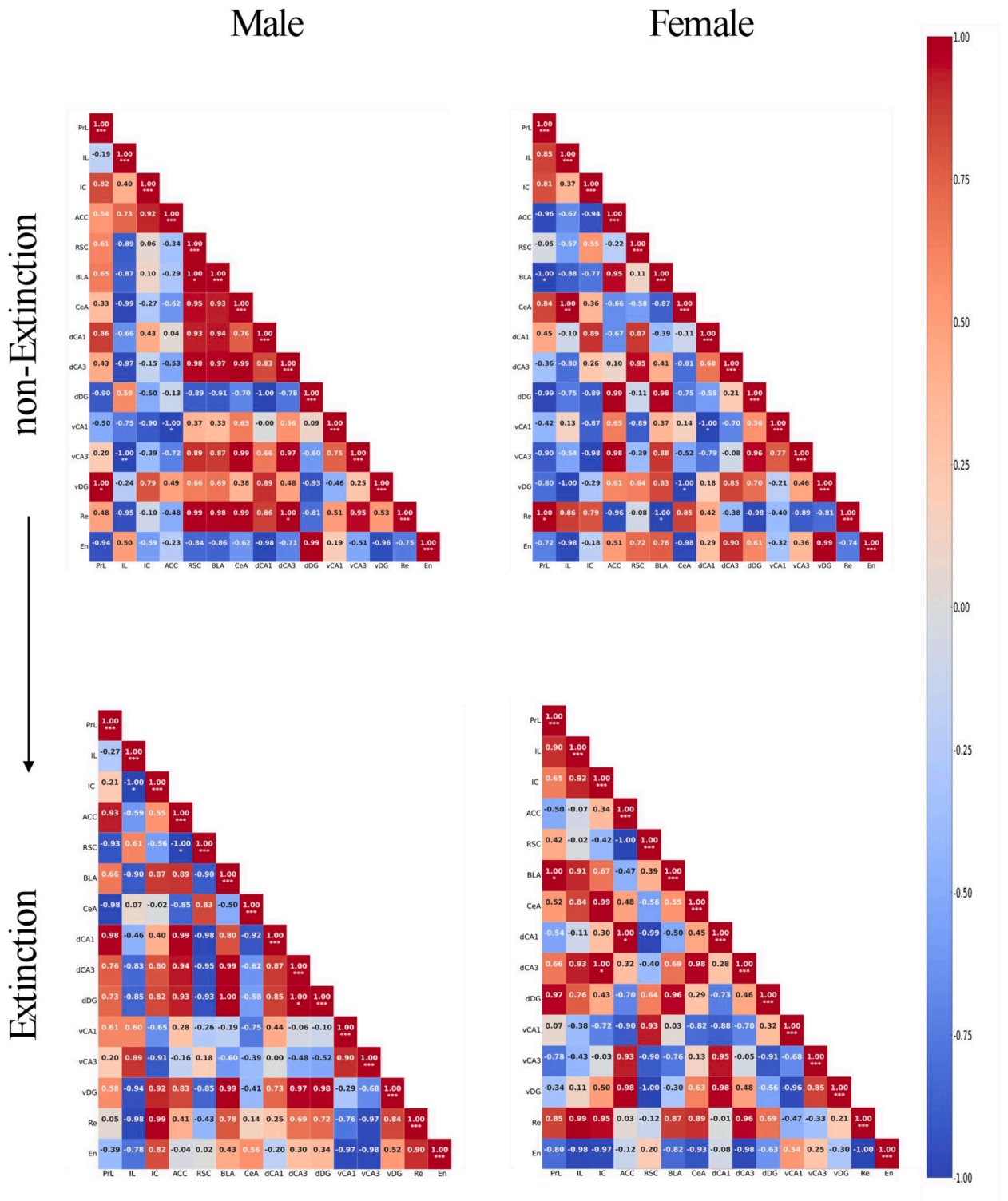

Fig. 4. The Pearson correlations of inter-regional networks of male and female rats before and after extinction training. The heatmap of Pearson correlations showing inter-regional correlations of c-Fos activation density in non-extinction group (above) and extinction group (bottom) in male (left) and female (right) rats. Axes represent brain regions. Colors reflect Pearson correlation coefficients (scale, right), and data, as well as asterisk within squares, correspond to R values of correlations and significance, respectively. $-1 \leq r \leq 1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

analysis and compared the changes in inter-regional correlations in extinction and non-extinction groups of male and female rats (Fig. 4). In the non-extinction group of male rats, we observed strong positive correlations between PrL and vDG ($r = 1$, $p = 0.0351$), BLA and RSC ($r = 1$, $p = 0.0288$), Re and dCA3 ($r = 1$, $p = 0.358$) as well as a strong negative correlation between IL and vCA3 ($r = -1$, $p = 0.0058$), ACC and vCA1 ($r = -1$, $p = 0.0246$; Fig. 4 lift above). In the extinction group (Fig. 4 left bottom), we observed three pairs of correlation coefficient. Specifically, IL was negatively correlated with IC ($r = -1$, $p = 0.0366$) in the cortex. ACC was negatively correlated with RSC ($r = -1$, $p = 0.013$) in the cingulate cortex. And dCA3 was positively correlated with dDG ($r = 1$, $p = 0.0289$) in the hippocampi.

In female rats, we observed distinct patterns of functional connections in both the non-extinction and extinction groups. In the non-extinction group (Fig. 4 right above), PrL exhibited strong positive correlation with Re ($r = 1$, $p = 0.019$) as well as negative correlation with BLA ($r = -1$, $p = 0.039$); meanwhile BLA and Re also carried negative correlation ($r = -1$, $p = 0.02$), indicating that PrL, BLA and Re might work synchronous as a small network to store the fear-related memory. CeA was positively correlated with IL ($r = 1$, $p = 0.0081$) and negatively correlated with vDG ($r = -1$, $p = 0.0456$), respectively, indicating that CeA might serve as the transit point between IL and vDG. In addition, the dorsal and ventral regions were negative correlation in CA1 ($r = -1$, $p = 0.0187$). Then in the extinction group (Fig. 4 right bottom), the functional connections between PrL and BLA shifted from negative to positive correlation ($r = 1$, $p = 0.0225$). Two of hippocampi subregions formed positive correlations with IC and ACC respectively. They are dCA3 and IC ($r = 1$, $p = 0.0153$) as well as dCA1 and ACC ($r = 1$, $p = 0.0269$).

4. Discussion

Conditioned fear and extinction are two important memory processes in the studies of fear-related memories. While previous studies have used male animals to control for the effect of estrogen on behavior, there is a need for studies on females. Thus, we compared the differences in behavior and activated brain regions between male and female rats conditioned during fear acquisition and extinction training. We observed no significant differences in fear acquisition between males and females. Although the freezing level of both sexes showed significant decrease in the extinction training, the freezing level in different trials showed significant differences during the extinction process. Possible explanations for these differences may include variations in the encoding mechanisms of extinction learning in male and female rats or their different biological bases. Further examination of c-fos activation using immunofluorescence staining revealed differences in activation between brain regions during the extinction between the sexes.

Studies have shown that male and female rodents exhibit differences in the activation of brain regions involved in fear conditioning, such as the hippocampus, amygdala, and prefrontal cortex [48]. We found that both sexes exhibited similar levels of fear acquisition, but the decrease in freezing levels during extinction training differed in some trials between the two groups. Additionally, we observed significant differences in c-fos expression in key brain regions involved in fear extinction, including the prefrontal cortex, amygdala, and hippocampus [25]. Traditionally, for two subregions of the medial prefrontal cortex, PrL plays a crucial role in the fear formation, while IL is involved in fear extinction [25,58]. Here, we found that both PrL and IL were significantly activated during the extinction process. This result might due to two reasons. The first explanation is that the process of extinction is same as the retrieval of fear, so PrL is activated in this period to respond to the retrieval of fear. The other explanation may be that the original fear memory and the new extinction memory, are two mutually inhibitory associative memories, which are activated simultaneously to encode and process memories. Previous studies have shown that BLA is involved in the extinction process and regulates the expression of fear emotion by regulating CeA region [59]. We also observed significant changes in c-fos expression in the BLA and CeA regions of the amygdala, with increased activation in the BLA and decreased activation in the CeA during extinction. This indicates that the BLA is involved in learning and processing extinction memories, while CeA, which is responsible for expressing fear memories, is inhibited. We also explored the activation of ACC and IC, which were related to emotional processing [60–63], as well as the activation of En and RSC which were involved in learning and memory [64–66]. We found that the four brain regions were significantly activated during the extinction process, which was in line with the previous study [41]. As a key region for storing spatial memory [67,68], the expression of c-fos in the hippocampus was also significantly higher than in the non-extinction group after the process of auditory fear conditioning and extinction. This might indicate that auditory fear extinction is a complex process that includes contextual cues during learning. Interestingly, we observed sex differences in Re activation, with increased c-fos expression in female rats during extinction. Previous studies have found that Re plays an important role in storing long-term memories [69], but our results suggest that Re may also be involved in learning short-term memories, at least in female rats. Therefore, because of this difference, we can further study the specific role of Re in female rats learning short-term memories.

We also conducted the Pearson correlation coefficients between the c-fos expression level in each brain area and the freezing level during the extinction process. Correlation analysis is widely used to study the correlation among multiple brain regions, and the results can help to understand the mechanism and function of complex brain networks [70,71]. Although previous studies have applied correlation network analysis to study the neural circuits of conditioned fear and extinction [70], there is limited research comparing the differences in fear between the sexes using this approach. Based on the methods of other studies [70,72], we used correlational matrix analysis in our study to compare fear differences between the sexes for the first time. The results showed that in male rats two subregions of the cingulate cortex were strongly correlated with the freezing level, whereas female rats carried no region correlated with the freezing level. Furthermore, we compared the differences in functional connectivity between the groups by conducting an inter-brain correlation analysis. The results showed distinct correlation matrices between the non-extinction group and extinction in both males and females. In the extinction learning process, three pairs of correlation coefficient exhibited in the male group were IL-IC, ACC-RSC, and dCA3-dDG, while three pairs of correlation coefficient exhibited in the female group were PrL-BLA, IC-dCA3, and ACC-dCA1. IC, ACC, and dCA3 were involved in the correlation coefficient with different paired regions in both males and females,

indicating that these three regions might be critical regions with sexual divergence in extinction learning. Therefore, subsequent studies could also focus on exploring the sexual divergence of correlations of distinct regions in extinction learning networks.

5. Conclusion

In conclusion, our study has revealed differences in freezing levels and activations of multiple brain regions involved in extinction learning between male and female rats. These differences suggest that distinct mechanisms underlie the encoding of extinction memory between the sexes. In this study, we have discussed the sexual divergent of before and learning extinction, meanwhile the retrieval of extinction may also carry sexual divergent. Thus, the sex difference in extinction retrieval would be explore in the next step. Furthermore, we have to note that animals in the extinction group experience novel environment, the extinction context, which could lead to c-fos expression in certain brain regions, but the non-extinction group do not. We could not exclude the effect of novel context, so this factor should be included in the retrieval study too. Besides the effect of new environment, it is also important to note that the female rats selected for this study were all in the non-estrus period and had low estrogen levels. Future studies could explore the impact of hormones on learning and memory as well as on fear behavior by controlling the estrus cycle of female rats, as previous research has demonstrated [73–75].

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Kai Zhang: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Project administration. **Dan Shen:** Writing – review & editing, Data curation, Methodology. **Shihao Huang:** Writing – review & editing. **Javed Iqbal:** Writing – review & editing. **Gengdi Huang:** Writing – review & editing. **Jijian Si:** Writing – review & editing. **Yanxue Xue:** Conceptualization, Funding acquisition. **Jian-Li Yang:** Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Yanxue Xue reports financial support was provided by National Natural Science Foundation of China. If there are other authors, they 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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