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Original Article

Sex differences in acetylcholinesterase modulation during spatial and fear memory extinction in the amygdala; an animal study in the single prolonged stress model of PTSD

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Abstract

Background and purpose: Men and women show different reactions to trauma and that is believed to be the reason behind the higher prevalence of post-traumatic stress disorder (PTSD) in women. Cholinergic signaling has long been known to be involved in the processing of fear-related information and the amygdala is a critical center for fear modulation. The main goal of the current research was to find (a) whether trauma results in different learning/extinction of fear or spatial-related information among male and female rats and (b) if trauma is associated with different acetylcholinesterase (AchE) activity in the amygdala.
Experimental approach: We used single prolonged stress (SPS) as a PTSD model in this study. Normal and SPS animals of both sexes were tested in contextual and spatial tasks (learning and extinction). AchE activity in the amygdala was also measured during each process.
Findings / Results: Results indicated that fear and spatial learning were impaired in SPS animals. SPS animals also had deficits in fear and spatial memory extinction and the effect was significantly higher in female-SPS than in the male-SPS group. In the enzymatic tests, AchE activity was increased during the fear extinction test and incremental changes were more significant in the female-SPS group.
Conclusion and implications: Collectively, these findings provided evidence that sex differences in response to trauma were at least partly related to less fear extinction potential in female subjects. It also indicated that the extinction deficit was associated with reduced cholinergic activity in the amygdala of female animals.

Keywords: Acetylcholinesterase; Amygdala; Extinction; Post-traumatic stress disorder; Sex differences.

INTRODUCTION

Dysregulation of fear learning or its extinction is considered an important underlying cause for the development of stress-related conditions like post-traumatic stress disorder (PTSD) (1). Extinction was primarily defined for conditioned responses. In the classical conditioning theory, a conditioned response is the response of the subject to a benign conditioned stimulus, like a tone, after it is presented several times with an aversive unconditioned stimulus, such as a foot shock. It is observed that if the subjects receive non-reinforced presentations of conditioned stimulus, the conditioned response will become extinct (2).

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Extinction can also occur in other learning paradigms. For example, in the Morris water maze (MWM), used for the assessment of spatial memory, the escape platform acts as a reinforcing stimulus. If, after learning sessions, the animals are exposed to swim trials with the platform being removed, they start to spend less time in the target quadrant, indicating the extinction of the spatial memory. It was first believed that similar brain structures and networks are involved in spatial and non-spatial learning acquisition and extinction, but later findings showed that there are numerous differences (3).

There is a huge amount of evidence that supports the role of the cholinergic system in the cognitive functions of the brain (4). Learning is an important cognitive process that involves four main brain structures; the prefrontal cortex, hippocampus, amygdala, and cerebellum. Among these regions, the first three receive cholinergic projections from basal forebrain cholinergic nuclei and are especially involved in cholinergic regulation of fear learning or extinction (5). Cholinergic neurons of the forebrain are grouped into 4 clusters (Ch1-Ch4) and the fourth subgroup (Ch4) sends projections into the amygdala, mostly to its basolateral region (6). Amygdala is a limbic structure that, among other roles, is involved in both spatial memory (7) and conditioned fear extinction (8). Several pieces of evidence support the role of nicotinic and muscarinic acetylcholine (Ach) receptors (especially in the basolateral area) in controlling the formation and extinction of fear memories (9-14). Measuring Ach concentrations by microdialysis also reveals the importance of the cholinergic system in these processes (11). There is no substantial evidence for the role of the amygdalar cholinergic system in the modulation of spatial navigation (especially spatial memory extinction), on the contrary, there is ample evidence for the role of the hippocampal cholinergic system in spatial processes (15).

It is hypothesized that impaired fear extinction is an underlying cause of the persistence of fearful memories in PTSD patients (16). Knowing that PTSD is about three times more prevalent in women than men (17) and considering the role of the amygdalar cholinergic system in controlling the extinction process, we hypothesize that gender variation in amygdalar cholinergic regulation plays a role in the different sexual prevalence of the disease. Cholinergic transmission can be regulated in different levels from production to receptor activation and biotransformation of Ach. We measured cholinesterase activity of the amygdala as an index for cholinergic activity in the region, more cholinesterase activity means less cholinergic transmission and vice versa. Fear-related classical conditioning model and spatial MWM models of learning were chosen to evaluate possible distinct roles of the cholinergic system in fear and spatial memories. Single prolonged stress (SPS) was used as the PTSD model in our experiments. SPS is a widely used PTSD model that comprises several neuroendocrine and behavioral features of human PTSD, including those related to learning and memory (18).

MATERIAL AND METHODS

Animals, grouping, and timeline of the procedures

Male and female Wistar rats (230-280 g) were used in this study. Rats were kept in plexiglass cages (4 per cage) in animals' houses with controlled temperatures (22-25 °C) and 12/12-h. light/dark cycle. Animals were kept and treated in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee at the Medical University of Kermanshah I.R. Iran (Ethical No. 1399.552). Sixteen groups of rats were used in the experiments; four groups (n = 8) were used in the contextual conditioning procedures; four groups (n = 8) were used in the spatial memory tests, and two groups (n = 6) were included for acetylcholinesterase (AchE) analysis in the middle of the learning procedures and two groups (n = 6) were used for AchE analysis at the end of behavioral tests. There were two male (control and SPS) and two female (control and SPS) groups in each paradigm (Fig. 1). Animals that were used in biochemical assays underwent all relevant behavioral tests before being sacrificed.

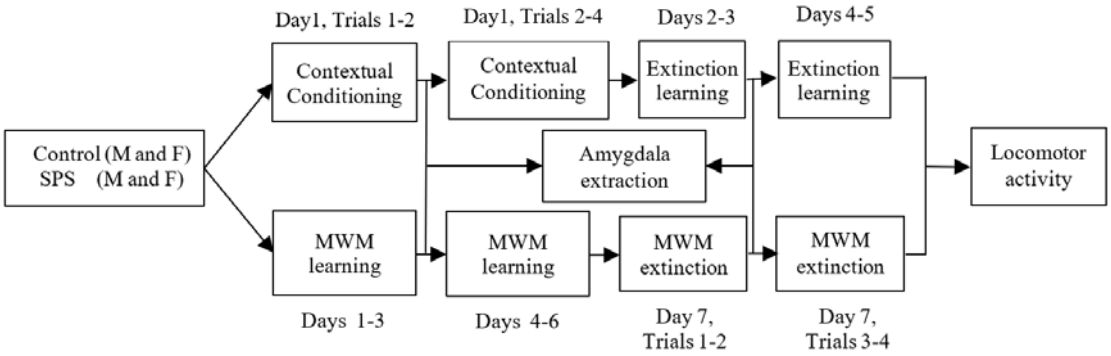

Fig. 1. Timeline depicting the course of behavioral and biochemical assays. Behavioral tests started for SPS animals seven days after the SPS exposure. MWM, Morris water maze; SPS, single-prolonged stress.

Single prolonged stress

The SPS procedure was used to induce PTSD phenotype in the animals. First rats were confined in a restrainer for 2 h. Next, rats were put in a water bath (depth 35 cm, temperature 25 °C) and forced to swim for 20 min. After the swimming step, the animals were towel-dried and remained in their cages to rest for 15 min. The last step included exposing the animals to diethyl ether until they lost consciousness. Behavioral tests were performed seven days after the SPS procedure (19).

Conditioning chamber

We used an Ugo Basile contextual conditioning (model 46002) chamber to induce contextual fear conditioning in rats. It consists of a Plexiglas container (26 × 26 × 30 cm) with a stainless-steel electrified grid floor at the bottom. Electric shocks were used as the unconditioned stimulus. An LED light was used for illuminating the box and a noiseless fan provided the chamber with fresh air.

Conditioning and extinction procedures

Conditioning and extinction procedures were adopted from the previous report (20) with slight modifications. First, rats were acclimatized to the conditioning chamber for 1 min, next they went through the training phase by receiving four trials of conditioning. In each trial, they were put in the chamber for a period of 120 s at the end of which they received a foot shock (0.5 mA, 2 s). The percentage of freezing time (time in which the animals stay immobile) is recorded in relation to the total time in each trial. The inter-trial interval was 4 h. The extinction test was performed 24 h after the last training trial. Rats were returned to the conditioning chamber and freezing was recorded in a 5-min period. This procedure was repeated for 4 consecutive days (Fig. 1).

MWM apparatus

As described elsewhere (21), the maze consists of a circular water tank (diameter 120 cm, height 80 cm) that is filled with tap water (depth 45 cm, temperature 23 ± 2 °C). The pool is virtually divided into four quadrants of northeast, northwest, southeast, and southwest. A circular platform (diameter 17 cm) is positioned in the center of the northeast quadrant in a way that it is 2 cm below the water surface level and invisible to the animals. The pool is located in a black wall room with some markers (white geometrical signs) being pinned to the walls as spatial clues. A video camera is positioned on the top center of the pool and records animal behavior. The recorded video is analyzed by the video-tracking system EthoVision XT6 (Noldus Information Technology, Netherlands).

Spatial learning and extinction procedures

Spatial training (learning) was carried out on 6 consecutive days (Fig. 1). Each day rats had a session of four training trials. In each trial, rats were floated on the water from a randomly determined point in one of the quadrants (with its face towards the tank wall) and allowed to swim for a 60 s period. If during this period, they could find the platform they were left there for 15 s, and then they were towel-dried and returned to their cages, otherwise, they were

guided towards the platform and left for 15 s. Trials were 3 min apart for each rat. To test the visual and locomotor competency of the animals, a separate trial is performed while the platform was tagged with a ping-pong ball affixed on the top of a white rod. Distance to the platform, distance from the centers of the rat and the platform in each session, was analyzed as the learning capability of the animals. One day later, the extinction trials were performed with the platform being removed from the pool. Rats had four sessions and each session contained four trials of 60 s duration. The intertrial interval was 2 h (22).

AchE activity measurement

Total AchE activity in the amygdala was measured using the spectrophotometric Ellman's test (23). Briefly, rats (n = 6) were decapitated in the mid-learning phases or 1 h after the extinction trials (Fig. 1). The brain was removed from the skull and the cerebellum was dissected with a mini spatula. Next, a razor blade was used to make a coronal transection at the middle cerebral artery. Caudal sections (2.5 mm thick) of the remaining brain were made by the razor blade and the amygdala was excised from the peeled slices (24). Tissues were homogenized in an ultrasonic homogenizer (Bandelin sonoplus 2000.2, Germany), thereafter they were immersed in 1 mL of 0.05 M phosphate buffer (pH = 7). Tissue homogenates were centrifuged at 12000 rpm (-4 °C) for 45 min. The supernatant was used as the source of the enzyme AchE. Each sample was diluted at a 1:10 ratio in phosphate buffer (0.05 M) before being used in the colorimetric procedure. Acetylthiocholine (1 mM) was used as the enzyme substrate. It is hydrolyzed to thiocholine and acetic acid in the presence of AchE. Next, 5,5′-dithiobis-(2-nitrobenzoic acid) (1 mM) was added to the mixture. It reacts with thiocholine and is transformed into the yellow 5-thio2-nitrobenzoinc acid. The mixture was incubated for 10 min at 37 °C and finally, the absorbance was read at 412 nm wavelength. Protein content in each sample was assayed by the Bradford method (25). The total activity of the enzyme was divided by the protein concentration to find the AchE-specific activity of the enzyme.

Locomotor activity test

The locomotor activity test was used to assess the baseline level of motor activity in the animals. The apparatus consisted of an open box (50 × 50 × 35 cm) with its floor divided (by photoelectric beams of light) into 25 equal squares (Ugo Basile activity cage 47420, Italy). Rats are put in the box for 5 min and the total number of crossings of animals to different squares was recorded as the animals' locomotor activity.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0.2 (GraphPad Software, California, USA). The contextual conditioning test data for the training and extinction sessions (or days) were compared by the three-way repeated-measures analysis of variance (ANOVA) with the trial, sex, and disease state as the main factors. The spatial memory test data for the learning section were compared by the three-way repeated-measures ANOVA with day, sex, and the disease state as the main factors. Data for the extinction section was analyzed by the two-way analysis of variance with the disease state and sex being the main factors of comparison. Data related to the AchE and locomotor activity were also compared by the two-way ANOVA with the disease state and sex as the main factors. Tukey's post hoc test was used to assess the significance of differences between group means All data were shown as the mean ± SD, and the *P*-values < 0.05 were considered significant.

RESULTS

Effects of SPS on contextual fear learning and contextual fear extinction in male and female rats

Contextual conditioning was performed 7 days after the SPS procedure (Fig. 1). The percentage of time spent in the freezing state was calculated. A three-way repeated-measures ANOVA found a significant main effect of the trial [$F(3, 84) = 291$, $P < 0.001$] such that animals spent more time freezing as they experienced more trials. There was a significant difference in the main effect of disease [$F(1, 28) = 229$, $P < 0.001$] with SPS

animals having more freezing than the control group. There was also a significant difference in the main effect of sex [F (1, 28) = 8.24, $P < 0.01$], and the male animals had a higher freezing time compared to the females. Additionally, there was a significant trial × disease interaction [F (3, 84) = 17.76, $P < 0.001$], meaning that freezing was more readily increased in SPS animals as they proceeded to further trials. Interactions for trial × sex [F (3, 84) = 0.36, $P = 0.78$] and disease × sex [F (1, 28) = 0.71, $P = 0.40$] were not significant (Fig. 2A).

The contextual fear extinction test was started one day after the conditioning procedure and continued for 4 days (Fig. 1). Statistical analysis revealed that there is a significant difference in the main effect of the day [F (3, 84) = 60.56, $P < 0.001$]. There was also a significant difference in the main effect of disease [F (1, 28) = 127.8, $P < 0.001$] which represents higher freezing in the SPS group. There is also an interaction between disease × sex [F (1, 28) = 5.03, $P = 0.03$] with female-SPS rats having higher freezing compared to the male-SPS group (Fig. 2B).

Effect of SPS on AchE activity during contextual fear learning and extinction in male and female animals

AchE activity was measured in separate group of animals (n = 6) in the middle of learning and extinction trials (Fig. 1). In the learning stage results of the two-way ANOVA indicated that neither sex [F (1, 20) = 0.18, $P = 0.67$] nor disease state [F (1, 20) = 0.096, $P = 0.75$] have significant effects on AchE activity in the amygdala (Fig. 3A).

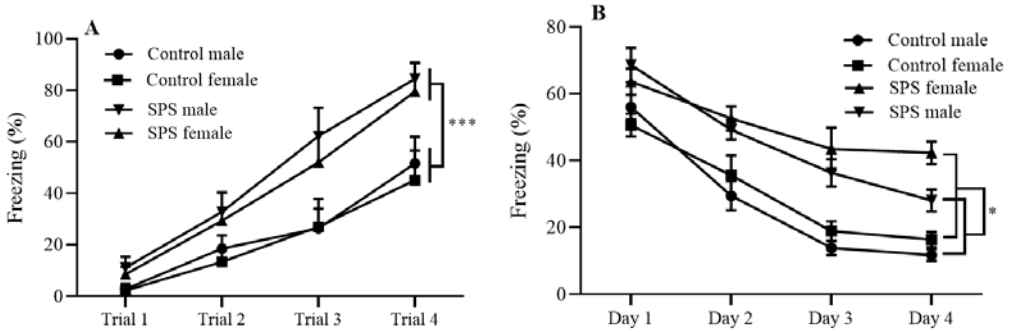

Fig. 2. Contextual fear (A) learning and (B) extinction in male and female rats. Normal and SPS rats (n = 8) from both sexes were used in the experiments. Values were calculated as the percentage of freezing behavior in the training or extinction sessions. A three-way ANOVA showed that (A) there is a significant (***$P < 0.001$) trial × disease interaction in the learning phase with SPS rats having higher freezing compared to control and (B) there is a significant (*$P < 0.05$) sex × disease interaction in the extinction phase with female-SPS having higher freezing than male-SPS. Data are presented as mean ± SD. SPS, Single-prolonged stress.

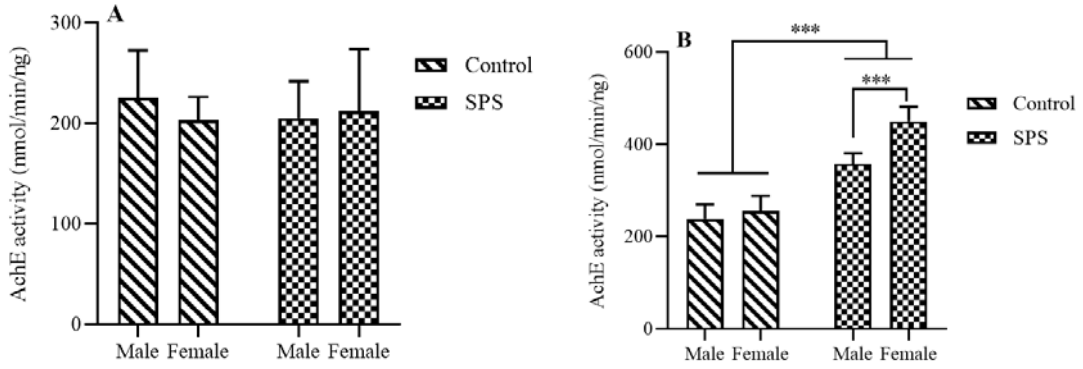

Fig. 3. AchE activity was measured halfway through contextual (A) learning and (B) extinction procedures (n = 6). A two-way ANOVA showed that (A) there is no significant effect for the main factors of disease and sex in the learning steps but (B) there is a significant (***$P < 0.001$) effect for the main factors of sex and disease in the extinction phase with SPS rats having higher AchE activity and female-SPS being significantly higher than male-SPS. Data are presented as mean ± SD. AchE, Acetylcholinesterase; SPS, single-prolonged stress.

In the extinction phase, there is a significant difference for the main effects of disease [F (1, 20) = 165, $P < 0.001$] and sex [F (1, 20) = 20.08, $P < 0.001$], and there is an interaction for disease × sex [F (1, 20) = 9.29, $P < 0.01$]. Pairwise comparisons showed that there is a significant difference ($P < 0.001$) between AchE activity in male-SPS and female-SPS groups (Fig. 3B).

Effects of SPS on spatial learning and extinction in male and female rats

Seven days after the SPS exposure animals went through the spatial learning experiment that took place in 6 days (Fig. 1). According to the results from a three-way repeated measures ANOVA, there are significant main effects of day [F (5, 140) = 183.8, $P < 0.001$], disease [F (1, 28) = 81.78, $P < 0.001$], and sex [F (1, 28) = 14.53, $P < 0.001$] on the distance to platform in all animal groups. There is also a significant interaction for day × disease [F (5, 140) = 2.54, $P < 0.05$] (Fig. 4A). One day after the last day of spatial learning, spatial memory extinction was conducted as described before (Fig. 1). Two-way ANOVA found that disease [F (1, 28) = 90.24, $P < 0.001$] and sex [F (1, 28) = 19.80, $P < 0.001$] have significant effects on the time spent in the target quadrant and there was a disease × sex interaction [F (1, 28) = 4.98, $P < 0.05$]. Post hoc comparisons revealed that male-SPS rats had less distance to the platform ($P < 0.001$) compared to the female-SPS (Fig. 4B).

Effect of SPS on AchE activity during spatial memory learning and extinction in male and female animals

Two distinct groups of animals (n = 6) were used to measure the AchE activity in the spatial tasks. As depicted in Fig. 5, there was no significant difference for the main effects of disease or sex in learning [sex; F (1, 20) = 0.05, $P = 0.81$, disease; F (1, 20) = 0.02, $P = 0.88$, Fig. 5A] or extinction [sex; F (1, 20) = 0.28, $P = 0.60$, disease; F (1, 20) = 4.9, $P < 0.05$, Fig. 5B] tests on AchE activity in the amygdala.

Effect of SPS on locomotor activity in male and female rats

Locomotor activity was measured 1 h after the last extinction trial. As shown in Fig. 6A and B, the mean for total number of crossings was not different among different sexes or disease states in cohorts that underwent contextual [disease state; F (1, 14) = 0.22, $P = 0.64$, sex; F (1, 14) = 1.43, $P = 0.25$] or spatial [disease; F (1, 14) = 1.19, $P = 0.29$, sex; F (1, 14) = 1.46, $P = 0.24$] paradigms.

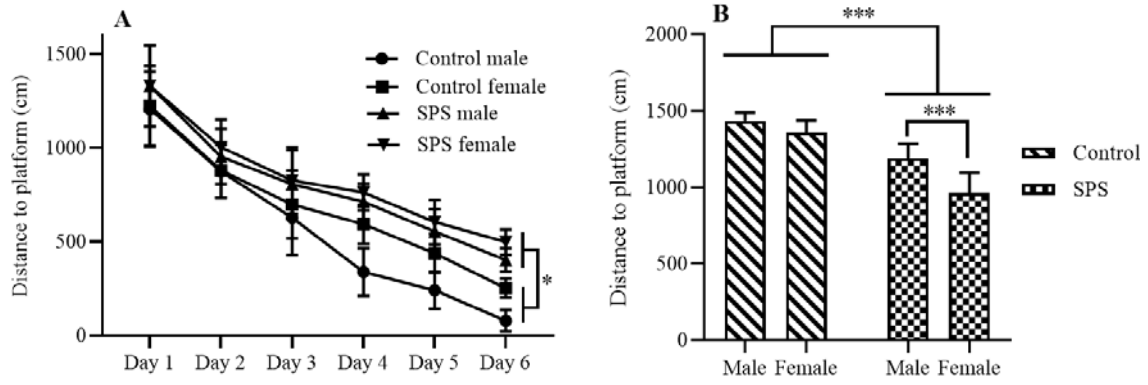

Fig. 4. Spatial (A) learning and (B) extinction in male and female rats. Normal and SPS rats (n = 8) from both sexes were used in the experiments. (A) A three-way ANOVA revealed that there is a significant (*$P < 0.05$) day × disease interaction in the learning phase with SPS rats having more average distance to the platform. (B) A two-way ANOVA showed that there is a significant (***$P < 0.001$) effect for the main factors of disease and sex in the extinction steps with female-SPS having an average of less distance to the platform compared to male-SPS. Data are presented as mean ± SD. SPS, Single-prolonged stress.

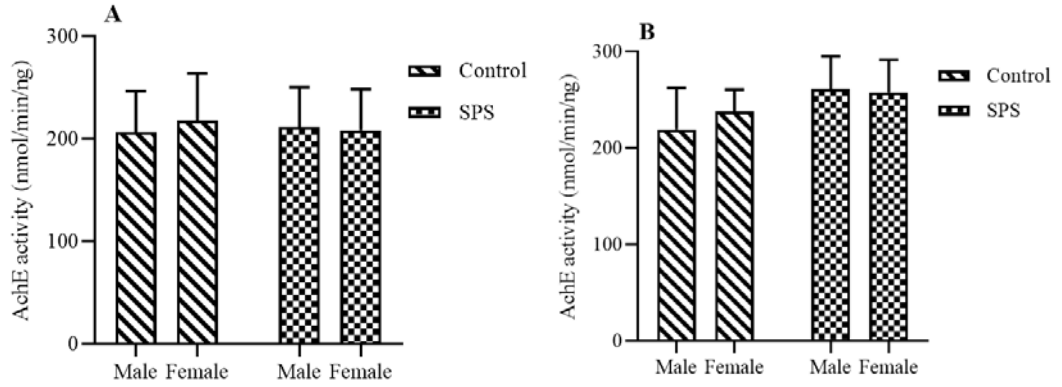

Fig. 5. AchE activity was measured halfway through (A) spatial learning and (B) extinction procedures (n = 6). A two-way ANOVA showed that AchE activity does not change during learning or extinction tests in (A) spatial learning or (B) spatial memory extinction tests. AchE, Acetylcholinesterase; SPS, single-prolonged stress.

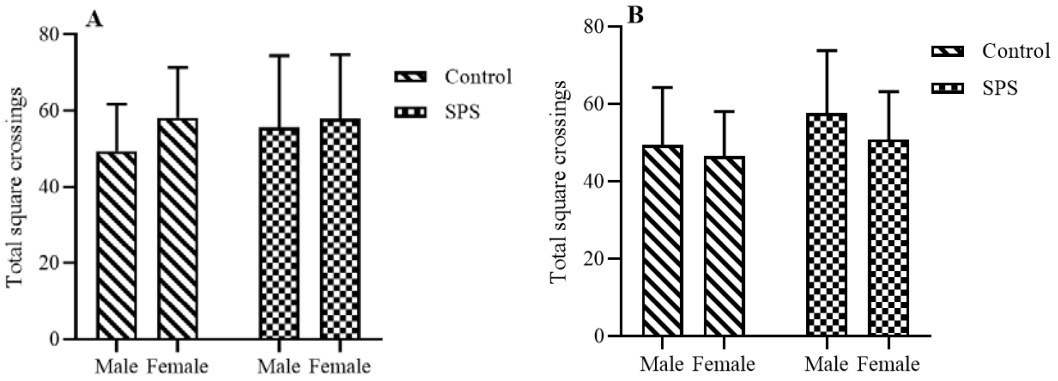

Fig. 6. Locomotor activity between different sexes in SPS and control animals. A two-way ANOVA indicated that there is not a significant difference between groups in locomotor activity in (A) contextual or (B) spatial experiments. Data are shown as mean ± SD. SPS, Single-prolonged stress.

DISCUSSION

This study aimed to find the possible gender differences in the modulation of contextual and spatial learning and extinction in the SPS model of PTSD. We also explored the association of cholinesterase activity in the amygdala with gender differences in SPS animals. The main findings from this study were that (1) SPS enhances contextual fear learning and impairs contextual fear extinction in both male and female subjects. The impairment of fear extinction is more pronounced in the female-SPS group, 2) impaired extinction of fear in SPS animals co-occurs with enhanced AhE activity in the amygdala which is significantly higher in the female-SPS group, 3) learning and extinction of spatial memory are also different in SPS and non-SPS animals with female-SPS having the mostly impaired extinction memory and 4) AchE activity is not different among SPS or sex groups during spatial learning and extinction.

It is strongly believed that the development of PTSD is coupled with some maladaptive fear phenotypes manifested in individuals exposed to a traumatic experience. The maladaptive response stems from dysregulation of fear memory processes like the acquisition or extinction of fear memory. Since PTSD is more common in women than men, it is reasonable to think that there are gender differences in the processing of information in the brain parts devoted to processing the memory of fear. Amygdala is a region that is extensively explored for its role in fear memory processing (26). There is evidence that in the basolateral region of the amygdala activation of both

nicotinic and muscarinic receptors facilitates the formation of memory by enhancing the signal-to-noise ratio in its principal neurons (27). Other studies show that increased ACh release in the basolateral region results in enhanced contextual fear conditioning (11,13) and extinction (9). These studies together with several other pieces of evidence that found sex differences in the functional and structural role of the amygdala in fear modulation (28) imply that cholinergic transmission in the amygdala might be a proper investigating target for physiologic differences in the regulation of fear memory in PTSD subjects.

Our results showed that fear learning (consolidation) is increased in SPS animals (Fig. 2A) which is in line with other reports demonstrating an enhanced contextual fear response following SPS (29-31) and agrees with the notion that prior stress exposure enhances the acquisition of new fear (32). We found that enhanced fear is more pronounced in male groups. This sexual differentiation of contextual fear conditioning is also reported in other studies (32,33) and is attributed to higher contextual learning in males, probably due to sexual dimorphism in long-term potentiation (LTP) induction in the hippocampus (33). Extinction of fear is also impaired in SPS rats and the female group has more impairment than the male (Fig. 2B). As seen in Fig. 3B the pattern of differences in AchE activity is opposite to that seen in the contextual extinction (higher AchE activity in SPS and female-SPS groups). This could imply that higher AchE activity and hence lower cholinergic drive in the amygdala is associated with extinction deficit in the SPS animals, and gender differences are also due to different enzymatic activity between males and females. The significant interaction of SPS × sex suggests that different AchE modulation is at least one reason behind dimorphic behavioral changes in SPS animals. In support of the role of the amygdalar cholinergic system in extinction, other studies have found that cholinergic hyperactivity in the basolateral, either as a result of muscarinic Ach receptor activation (12,34,35) or increased Ach release (9), is crucial for the acquisition of contextual fear extinction. Similar to the extinction phase, studies are backing up the role of muscarinic Ach receptor or Ach release in the learning phase (5). Our results showed that acquisition of fear learning is also impaired in SPS animals, independent of the sex factor (Fig. 2A). Seemingly, this is not associated with AchE activity, because enzyme activity is not changed during this process (Fig. 3A). Cholinergic regulation through AchE modulation is reported in several other investigations (21, 36-38). We have recently found that reduced hippocampal cholinesterase activity occurs concurrently with extinction and working memory deficits (36) in male rats. The hippocampus receives cholinergic inputs from the medial septum and diagonal band of Broca neurons in the basal forebrain cholinergic nuclei, while the amygdala is innervated by neurons in the nucleus basalis magnocellularis. There are also cholinergic projections from the nucleus basalis magnocellularis to the prefrontal cortex (39). Cholinergic inputs to these three structures are crucial for controlling neural plasticity, a process that is also important for learning and extinction of fear and non-fear-related memories. A lot of research is done to unravel the role of the basal forebrain cholinergic system in controlling mnemonic processes (40), but there is still a long way to go. Although the results of the present study proposed that a change in AchE regulation may underlie gender differences in contextual extinction impairments, we cannot rule out the role of other regulatory mechanisms in cholinergic transmission. Processes like Ach release or Ach receptor may also be subjected to modulation. Another limitation in our study was that we used the amygdala as one unit in our experiments and we cannot comment on AchE activity in distinct subregions of this structure.

Results of the spatial learning test indicated that SPS has led to spatial memory deficits in rats, and male and female groups are similarly affected (Fig. 4A). Defects seen in spatial memory were not associated with changes in AchE activity in the amygdala (Fig. 5A). In the extinction phase, however, SPS has induced extinction impairments in the animals and female-SPS have shown less learning capabilities compared to male-SPS (Fig. 4B).

Again, no change in AchE activity was seen among different groups (Fig. 5B). Contrary to fear-related learning, there are not many studies regarding the effect of PTSD on spatial learning in animals. In agreement with the present results, we have previously reported that spatial memory and memory extinction are affected by SPS in male rats (41). Male superiority in spatial learning abilities is reported both in humans and animals (40,42,43). Studies regarding the anatomical origin of the differences are mostly focused on the hippocampus because this is the critical structure for processing spatial information (44). In our experiments, amygdalar AchE was enhanced during contextual- (Fig. 3B) and was intact during spatial-extinction (Fig. 5B) tasks which implies that AchE modulation is only involved in fear-related, but not spatial, memory processing.

Results in the conditioning and especially the MWM test may need further interpretations if the locomotor activity of the animals is different across SPS or sex groups. As shown in Fig. 6 locomotor activity is similar across disease states or sex for conditioning or spatial tests and we can say that our results are not confounded by changes in the movement tendency of the animals.

CONCLUSION

In summary, the major findings of this study are that female rats have more fear extinction impairment compared to males and concurrently show higher AchE activity in their amygdala. It seems that there is a sex difference in the contribution of the amygdala to the contextual extinction process in SPS animals, which is probably due to lower cholinergic transmission in the female amygdala. More comprehensive studies and better insights into the role of the cholinergic system and its molecular processes will offer new manipulative targets to treat excessive fear in PTSD and other fear-related disorders.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. Mohammadi-Farani concepted and designed the study. S. Shirooie supervised the study. A. Mohammadi-Farani and S. Shirooie analyzed and interpreted the data. S. Farhangian performed the behavioral experiments and collected the data. A. Mohammadi-Farani acquired the molecular data. S. Shirooie, A. Mohammadi-Farani, and S. Farhangian contributed to preparing the first draft. A. Mohammadi-Farani revised the manuscript. The final version of the article was approved by all authors.

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