Research Square

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A Terrified Sound Stress Impairs Learning And Memory Ability of Adult Female

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Abstract

Stress, as an important environmental factor of mental health, cannot be ignored. The great physiological difference between males and females implies that the effects of stress may differ by gender. However, few studies have focused on the effects of stress on females. This study investigated the effects of a terrified sound stress on adult female mice.

Methods: 32 adults female C57BL/6 mice were randomly divided into control group (n=16) and stress group (n=16). Sucrose preference test and open field test (OFT) were carried out to evaluate the anxiety and depression of mice. Spatial learning and memory ability were measured by Morris Water maze test (MWM). Endocrine hormones were determined by enzyme-linked immunosorbent assay (ELISA). Serum differential proteins were screened by mass spectrometry (MS).

Results: Compared with control group, the sucrose preference of stress group was decreased; in MWM, the escape latency of the stress group was significantly prolonged ($P<0.05$), and the total swimming distance was significantly increased ($P<0.05$).Serum T ($P<0.05$), GnRH ($P<0.05$), FSH and LH levels decreased; thirty six differential peaks were found by MS, eight of them had high multiples of difference (> 1.2 or <0.8).

Conclusion: terrified sound stress impairs spatial learning ability and mental health of adult female mice.

Introduction

Stress is a physical or psychological stimulus that produces a psychological or physiological reaction that can lead to illness. Stress is triggered by numerous unexpected environmental stimuli such as aggressive behavior, fear, forced physical activity, sudden environmental changes, social isolation or pathological situations(1). Military personnel will experience a variety of physical and psychological stressors during their service, such as continuous stressful work conditions, extreme environments, and war deaths, which can have a chronic and profound impact on the mental health of military personnel, seriously affect their health, and even affect their professional life. Psychological stress leads to various changes including clinical depression and cardiovascular disease, it is an important factor for the development of irritable bowel syndrome and it directly influences the susceptibility to viral and bacterial infections, including the common cold, Influenza, Toxoplasma, and Salmonella infections (2-4). Increasing evidence suggests that psychological stress plays an important role in the progression of oxidative stress-related diseases including cardiovascular disorders, diabetes, and cancer as well as other diseases (5-7). In addition, psychological stress can also affect reproductive ability by affecting hormone levels. In our research group's published papers, we have found that chronic psychological stress can affect reproductive ability of male rats.

Depression, one of the most serious diseases, severely affects an individual's quality of life, and is an important reason for the rise of suicide rates in the 21st century(8). The chronic and debilitating nature of depression complicates the prognosis of many chronic diseases and exacerbates disease and disability

conditions worldwide(9). According the World Health Organization (WHO), major depression is projected to become a major cause of disability worldwide by 2030 (10). Both environmental and genetic factors contribute to the development of depression. Studies have shown that about 40% ~ 50% of depression is mediated by genes(11). Environmental factors associated with depression include stressful events, cancer, and endocrine abnormalities. Although the exact cause of depression is unknown, increasing attention has focused on the impact of psychological stress on depression. Stressful life events can induce a range of psychological and physiological changes, including activation of the nervous, endocrine and immune systems. Hyperactivity on the HPA axis is one of the most common neurobiological changes in patients with depression. HPA axis dysfunction is present in approximately 70% of patients with depression(12). Studies have confirmed that the reproductive axis HPG and reproductive hormone such as testosterone (T), follicle-stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH) and Luteinizing hormone (LH) is also affected under stress (13).Transcriptomic studies have shown that different pathways and networks are activated when men and women are stressed (14). Gender differences can lead to increased stress sensitivity in women, and studies have shown that changes in certain hormone levels increase anxiety-like behavior more in women than in men. For example, mice with overexpression of CRF in the forebrain during development displayed anxiety-related behaviors in adulthood, especially in female (15).

Animal models are useful tools for studying the neurobiology of psychological stress and mental disorders such as depression and anxiety. Various stress models, including the forced swim test, the tail suspension test, immersion in cold water, electric foot shock, restraint, social isolation, food deprivation, sleep deprivation, early-life stress, and chronic paradigms such as chronic unpredictable mild stress have been commonly used to explore the relationship between psychological stress and its effects (16, 17) Most of the other existing stress models involve the simultaneous application of both physical and psychological stressors, except mother–son isolation models. Moreover, physical stress can influence the effects of psychological stress. Thus, the effects of psychological stress alone are difficult to study. We developed a pure psychological stress model by using a terrified sound as the stressor. Previous studies from our group showed that terrified sound stress induced morphological changes in the adrenal gland, increased serum corticosterone (CORT) and norepinephrine levels, suppressed body weight gain, improved spleen and thymus indices, and changed hippocampus proteomic profiles (18-20). The studies mentioned above focused mainly on the effect of terrified sound stress on male. There are gender differences in depression, generally believed to be twice as many females experiencing major depression as male(21). In this paper, we investigate the effects of terrified sound stress on female mice, from the ethological, endocrine hormone and serum protein molecular differences to explain. We preliminarily found that terrified sound stress reduced the learning and memory ability of adult female mice, accompanied by depression-like behavior, and affected endocrine hormone and serum protein levels

Materials And Methods

Animals

Female C57BL/6 mice aged 14 weeks and weighing 24-27g were obtained from the animal experimental center of The Fourth Military Medical University of China. The mice were housed four per cage in an aseptic room kept at constant temperature (23 ± 2°C) and humidity (65%), and with a 12 h light/dark cycle (light was on from 06:00 to 18:00). Sterilized food and water were available ad libitum. The animal experimental protocols were approved specifically by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University of China (Permission No. 2011-0110). Every effort was made to minimize the number of animals used and their suffering.

Terrified sound stress procedure

After 7 days of acclimatization, mice were randomly divided into the terrified sound stress group and the control group with 16 mice in each group (Figure 1). The terrified sound stress was produced by exposing the mice to a terrified sound (frequencies from 0.5 kHz to 4 kHz) for 6 h daily for14 days; the start time for exposure was changed daily to prevent adaptation. The terrified sound was broadcasted through a loudspeaker (Panda CD-100) located 50 cm above the animal cages, and the intensity was 45-60 dB measured by a sound level meter (Landtek SL-5800). The terrified sound was recorded in advance as follows: C57BL/6 mice were subjected to an electric foot shock (1-mA scramble shock, variable-interval schedule, mean intershock interval of 30 s and shock duration of 30 s) for 30 min in a chamber with a grid floor. The terrified sound produced by the mice was simultaneously recorded on CD-ROM in a professional recording room (Xi'an Yin Zhi Xuan). Digital audio production systems, including the ProTools HD audio workstation, a Yamaha digital mixer, Genelec monitors, a Neumann U87 AI condenser microphone, and NEVE1 were used to record the terrified sound. The mice in the control group were exposed only to the background noise of the animal room. The level of the background noise produced by the ventilation system inside the room and the eating or fighting activities of the mice was 40-45 dB (18-20)

Sucrose preference test (SPT)

The sucrose preference test was conducted as described literature with minor modifications (22). In the adaption phase, mice were placed individually in cages with two bottles of sucrose solution (1%, w/v) for the first 24-h period. In the second 24h period, one bottle of sucrose solution was then replaced with a bottle containing tap water. In the test phase, rats were deprived of water and food for 24 h and then permitted access to the two bottles for 24 h. The sucrose preference was defined as sucrose consumption/ [water consumption+ sucrose consumption] × 100% during the 24h test.

The Morris Water Maze (MWM)

The testing lasted for 6 days. Mice were trained twice a day at 10:00 ~ 12:00 and at 16:00 ~ 18:00 respectively for 5 days. In each training trial, mice was placed in one of the sectors facing the wall of the pool and allowed to search for the hidden platform for 2 min, the time when the mice found the platform was used as the first recording point, namely the escape incubation period. On day 6, a probe trial was

administered: remove the platform and remember the number of crossing the platform and the time spent in each sector within 2 min in the pool (23).

Open field tests（OFT）

OFT were performed 3 weeks after stress. The chamber 100 cm (length) x 100 cm (width) x 38 cm (height), the center of the box (60cm ×60 cm), the movement of mice was recorded and analyzed. Wipe the chamber with a 75% Ethanol prior to use and before subsequent tests to remove any scent clues left by the previous subject mouse. Mice were placed in the center of the open box and allowed to explore for 1min to eliminate the mice's sensitivity to the new environment. The next 5 minutes, the total distance traveled and the time spent in the center were recorded(24).

ELISA Assay

Serum levels of cortisol (CORT), GnRH, CRH, ACTH, FSH, LH and T were measured by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (Elisa Biotech, Shanghai, China). Briefly, standards with known hormone concentrations and plasma samples were added to wells pre-coated with plasma serine protease inhibitor, followed by addition of the target antibody and HRP-conjugated secondary antibody. After incubation and washing, HRP substrate was added, followed by stopping solution. The optical density (OD) at 450 nm was measured within 15 min using a microtiter plate reader (FLUO star Omega, BMG LABTECH GmbH, Germany).

MS analysis: WCX fractionation and MALDI-TOF MS analysis

In the process of mass spectrometry, we used serum mixes, three mice serums for one mix. The stress group and the control group each had three mixed serum samples. Serum samples were separated by magnetic bead-based weak cation-exchange chromatography (MB-WCX) using ClinProt™ purification reagent sets from Bruker Daltonics. MB-WCX purifications were performed using the Bruker Magnetic Separator (eight-well, #65554) according to the manufacturer’s protocol. The details of this experiment were reported previously(25). Matrix suppression up to 1000 Da, with a mass range of 1000-10,000 Da was set as the default. Data analyses were performed using the programs Flex analysis v3.0 and ClinProTools v2.2 (Bruker Daltonics, Germany).

Statistics

Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software). All data were expressed as the mean ± SEM. The results were considered statistically significant if $P<0.05$.

Results

Terrified sound stress induced depression-like behavior in adult female mice

To investigate the effects of chronic psychological stress on adult female mice, adult female C57 mice were exposed to a ‘terrified sound’ stress in 6h sessions for 14 consecutive days. During the modeling process, the mice exhibited nervousness and irritability. Sucrose preference is a test for anhedonia, a form of emotional disorders, including depression. It was conducted on the 7th and 14th days of stress, and found that the sucrose preference of the stress group decreased compared with the control group, a significant difference on the 14th days($P<0.05$) (Figure 2a). The sucrose preference tests revealed significant anhedonia in stress group, suggesting the onset of a stress-induced depression-like phenotype. The open field test was used to test the free exploration ability and anxiety of mice in a new environment. The activity ability of mice was measured by the total distance in the open field; Anxiety was measured by the time spent in the center. The open field test showed that the total distance of mice in the stress group was higher and the time spent in the center was lower than control group, but no statistical difference, suggesting the activity of the stress group increased and appearing the depression-like phenotype (Figure 2b,c).

Terrified sound stress induced changes in endocrine levels

We performed serological tests by ELISA. Serum concentrations of the neuroendocrine hormones CRH GC ACTH and CORT were normal on day 14(Figure 3e-h). However, stress leads to lower levels of the reproductive endocrine hormone GnRH ($P<0.05$), LH, FSH, and T ($P<0.05$) are reduced on 14 d (Figure 3a-d)

Terrified sound stress affected the learning and memory ability of adult female mice

After exposure to terrified sound stress for 21 days, the MWM task was used to examine the changes of subsequent spatial learning and memory. In the 5-day platform learning phase, with the increase of training time, the latency of escape and total movement distance of each group decreased gradually (Figure 4a,b).The stress group took longer and moved farther to find the platform($P<0.05$) (Figure 4a,b).The 6th day of spatial memory testing, the memory ability of the mice was characterization by the number of crossings, the percentage of residence time in the target quadrant and velocity (Figure 4c,d,e). There was no significant difference in the indicators of memory. The swimming tracks during training and probe trials were recorded for each day and each group (Figure 4f).These results indicated that terrified sound stress reduced the spatial learning and no significant effect on memory of adult female mice.

Terrified sound stress affected the serum protein level of mice.

To assess the effects of terrified sound stress on the expression of proteins or peptides in the sera of mice, we performed proteomics analysis of serum samples of stressed and control mice. Prefractionation of serum samples by MB-WCX and MALDI-TOF MS identified up to 36 peaks, of which 7 showed relatively large multiples of difference between the stressed and control groups m/z peaks, difference is greater than 1.2 times or less than 0.8 times (Figure 5d). Evaluation of the controls (green) and stressed group (red) (Figure 5a). Overall, the stressed groups and controls showed protein profiles ranging from 1

to 10 kDa. Within this mass range, a significant number of differentially expressed proteins or peptides could be detected (Figure 5a, b). In the component analysis, a bivariate plot showed few overlapping regions between the stressed (red) and control (green) groups (Figure 5c).

LC-ESI-MS/MS and the Mascot database were used for MS/MS fragmentation of peptides, and one of the peaks was sequence identified (peak m/z: 3511.13), Figure 5e is identification map of the protein. Sequence information on the peak identified is as follows: K.VLVSDATESVPAAAGAATSDNTFSGESKQQQLAEK.E. The peptide was identified as CEP350-centrosomal protein 350, associated with mitotic spindles. It presents in the pericentrosomal area and plays an essential role in centriole growth.

Discussion

People in every occupation face various kinds of stress and health risk. Mental health has also become a social issue. Genetic and environmental factors have a synergistic effect on psychological problems. Stress, as an important environmental factor of mental health, cannot be ignored. The great physiological difference between males and females implies that the effects of stress may differ by gender. The current study, a terrified sound stress model has been used in female adult mice, from the behavior, endocrine hormone, serum protein differences to comprehensive assessment the influence of stress on the adult female mice, found that terrified sound stress can induce depression female mice behavior, damage the ability of learning and memory, affect the endocrine level.

Stress response is usually accompanied by activation of the HPA axis, and stress causes changes in hormone levels on the HPA axis. During the experiment, serum endocrine hormone levels of CRH, GC, ACTH and CORT were detected by ELISA. After 14 days of stress, the level of CORT increased significantly in the stress group, there was no significant difference of other hormones. We predict that it was due to the time of modeling, which was consistent with the results reported in the literature. In 2019, there was an article that made modeling of terrified sound stress for 21 days, and detected the changes of serum hormone in rats every 7 days during the modeling process. It was found that on the 14th day of stress, only CORT level showed significant differences, GC, ACTH and CORT showed no significant differences. At 21 days of stress, the levels of CRH, GC, ACTH and CORT were significantly different (13). These results suggest that it may take a longer time to establish the effects of chronic psychological stress of terrified sound on the endocrine level of mice, but the effects on the cognitive function of mice have been prominent in the second week of stress, and the impairment of learning and memory may become more serious with the increase of time. As part of the adaptive response to pressure, the HPA axis also regulates the function of the HPG axis. Any component of the HPA axis is likely to inhibit reproduction. Experimental, clinical, and population-based studies have shown the interplay between stress, the immune system, and female reproduction (26). In the hypothalamus, stress activates CRH and GABA, inhibit the secretion of GnRH, leads to LH, FSH inhibition, then reduce the production of T, affecting ovarian follicular maturation and reducing ovulation ability (27).

Serum protein was analyzed by MS and 36 differential peaks were screened out. A differential protein CEP350 was found using our protein library. There are 31 proteins in the CEP family .CEP proteins family is the active component of centrosomes and plays critical roles in the centriole biogenesis and cell cycle progression(28). Centrosome protein CEP350, associated with mitotic spindle involving in tissue binding and anchoring of centrosome microtubules and plays a role in the regulation of nuclear hormone receptor signaling (29-37).

Using the STRING database, we found 10 proteins that interact with CEP350, including LOC683722, Smg1, Atad2b, Caskin1, Cep19, Dctn1, Dctn2, Mapre3, Xrn1, Dctn2 (38). The main biological processes involved are microtubule anchoring at centrosome, protein localization to microtubule cytoskeleton, establishment of vesicle localization, and the assembly of cellular components such as microtubules, centrioles, and dynein complexes. Protein Dctn2 may synergistically affect individual cognitive function with CEP350. Dctn2, binding to an organelle, plays a role in prometaphase chromosome alignment and spindle organization during mitosis and involves in anchoring microtubules to centrosomes. Especially, it may play a role in synapse formation during brain development. The development of neurons and the complexity of synapses are closely related to cognitive function. It has been reported that the hippocampus and amygdala are brain regions closely related to learning and memory. Stress may change the neurotransmitter levels in these regions and even affect the dendrite morphology and connectivity of neurons, affecting individual cognitive function (39). The functions of other proteins are mostly related to centrosomes and spindles: LOC683722 is required for anchoring microtubules to the centrosomes; Cep19 involves in the early steps in cilia formation; Dctn1 plays a key role in dynein-mediated retrograde transport of vesicles and organelles along microtubules; Mapre3 promotes microtubule growth and may be involved in spindle function by stabilizing microtubules and anchoring them at centrosomes.

CEP350 contains the Cap-Gly domain, which is mainly involved in mediating tubulin interactions and assisting protein recruitment mechanisms. It has been proposed that CEP350 can specifically stabilize the Golgi apparatus during interphase and participate in the maintenance of the Golgi band around the centrosome(40).CEP350 is associated with disease. As a novel tumor suppressor gene in human melanoma, CEP350 is mutated in 7.2% of the human melanoma genome, and these mutations are predicted to have a destructive effect on the expression of CEP350 (41). There are multiple significant associations between 3 UTR methylation of CEP350 gene and sensitivity to chemotherapy in small cell lung cancer (SCLC)(42). Primary cilia are antennae-like sensory organs that play a key role in coordinating human development and tissue homeostasis signaling pathways (43) . CEP350, together with other molecules, directs the highly conserved intra flagellar transport (IFT) protein complex into the cilia, participating in primary cilia assembly and signaling molecule transport (44).

Gender is one of the factors that might affect individual responses to stressful events. First, due to differences in gene and hormone levels, female respond to stress more negatively (45, 46). Second, the traditional cultivation concept raises the expectation that males should demonstrate greater individualism and assertiveness while females are emotional and sensitive. Therefore, in such a

parenting context, when faced with stressful events, males are more likely to develop problem behaviors such as alcoholism and drug abuse, while females are more likely to develop internalization disorders(46). The research reported that gender and regional differences are still potential risk factors for depression among Chinese adolescent students(47). Internationally, taking the global COVID-19 pandemic as an example, studies show that with the occurrence of COVID-19, people's depressive symptoms, anxiety, sleep disorders and psychological distress increase, and women experience more severe depression and anxiety than men (48-50). A population-based cross-sectional survey in Germany found that women's demand for alcohol, nicotine and illicit drugs has increased during the COVID-19 pandemic, twenty percent reported major depressive and 23.4% symptoms of generalized anxiety(51). Some researchers assessed gender differences in the prevalence of stress disorder (PTSD) and other common psychiatric disorder symptoms among Iraqi internally displaced persons (IDPs) after the 2014 terrorist attacks. No gender differences were found in the incidence of PTSD among IDPs in Iraq, but women showed higher levels of physical and depressive/anxiety symptoms(52).

Historically, female rodents have been challenging to study because of their short estrus cycle and ovarian hormones to induce neuronal plasticity(53, 54).Therefore, female rodents were excluded from most studies, including basic neuroscience studies and stress response studies(55). But excluding female subjects due to increased variability is not an effective approach. Conversely, in some cases, comparing men and women can reveal risk factors for disease. It is necessary to compare the different response patterns and manifestations of stress between men and women, and these differences can provide new ideas for treating stress-related diseases. It also helps to formulate appropriate prevention strategies for different genders, reduce the occurrence of mental diseases and improve the quality of life.

Conclusions

In the current study, we developed a pure psychological stress model by using a terrified sound as the stressor, to assess the influence of stress on the adult female mice, found that terrified sound stress can induce depression female mice behavior, damage the ability of learning and memory, affect the endocrine level.

Abbreviations

WHO: World Health Organization

T: testosterone

FSH: follicle-stimulating hormone

GnRH: gonadotropin-releasing hormone

LH: Luteinizing hormone

CRF: Corticotropin Releasing Factor

CORT: corticosterone

SPT: Sucrose preference test

MWM: The Morris Water Maze

OFT: Open field test

MB-WCX: magnetic bead-based weak cation-exchange chromatography

SCLC: small cell lung cancer

IFT: conserved intra flagellar transport

PTSD: posttraumatic stress disorder

IDPs: Iraqi internally displaced persons

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, The animal experimental protocols were approved specifically by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University of China (Permission No. 2011-0110)

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published paper.

Competing interests

The authors declare that they have no competing interests

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Author Contributions

SFG, LYZ and JY conceived and designed the study. SFG, LYZ, XW, LH and XX collected the data, performed the statistical analyses, and drafted the manuscript. XFX and RFL revised the manuscript critically for important intellectual content. and LN helped revise the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Figures

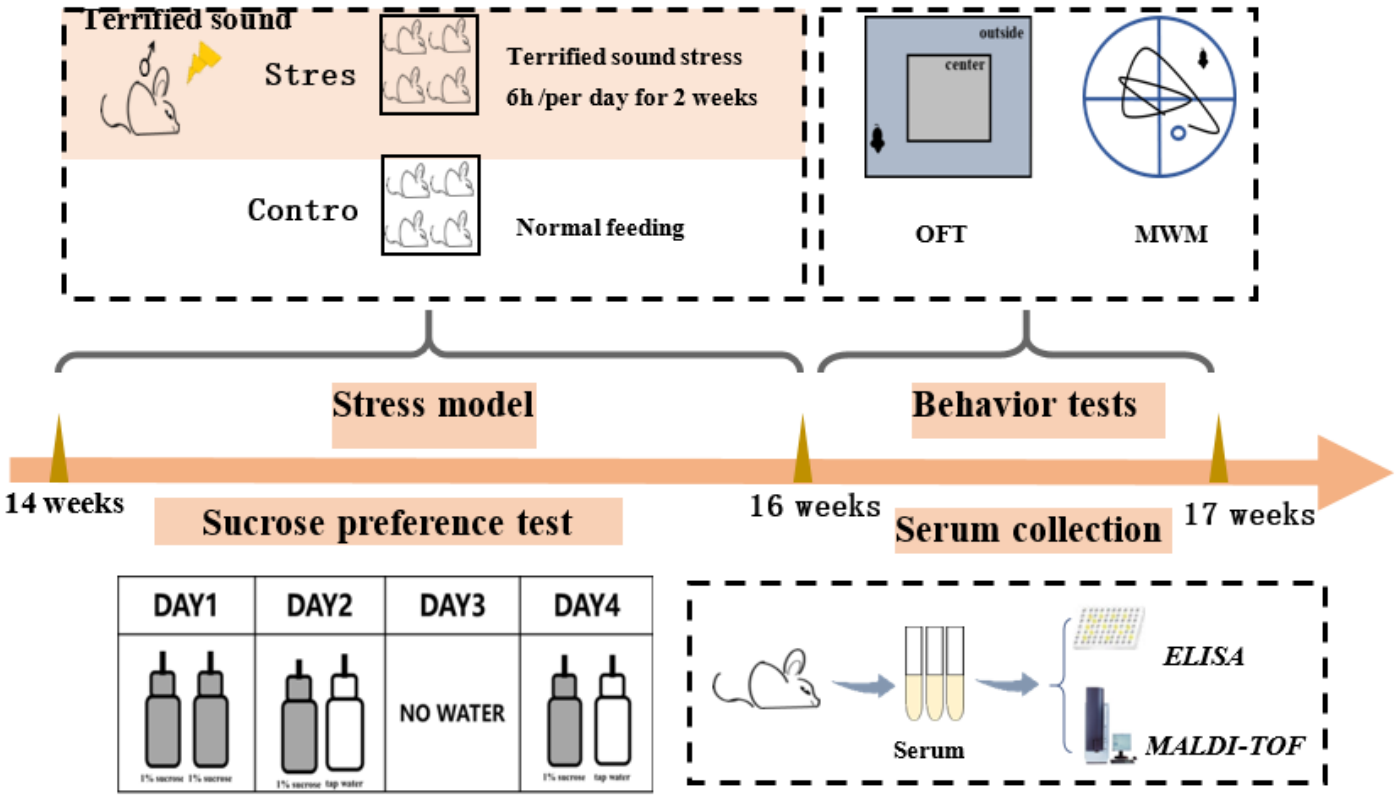

Figure 1

Timeline and methods for experiments

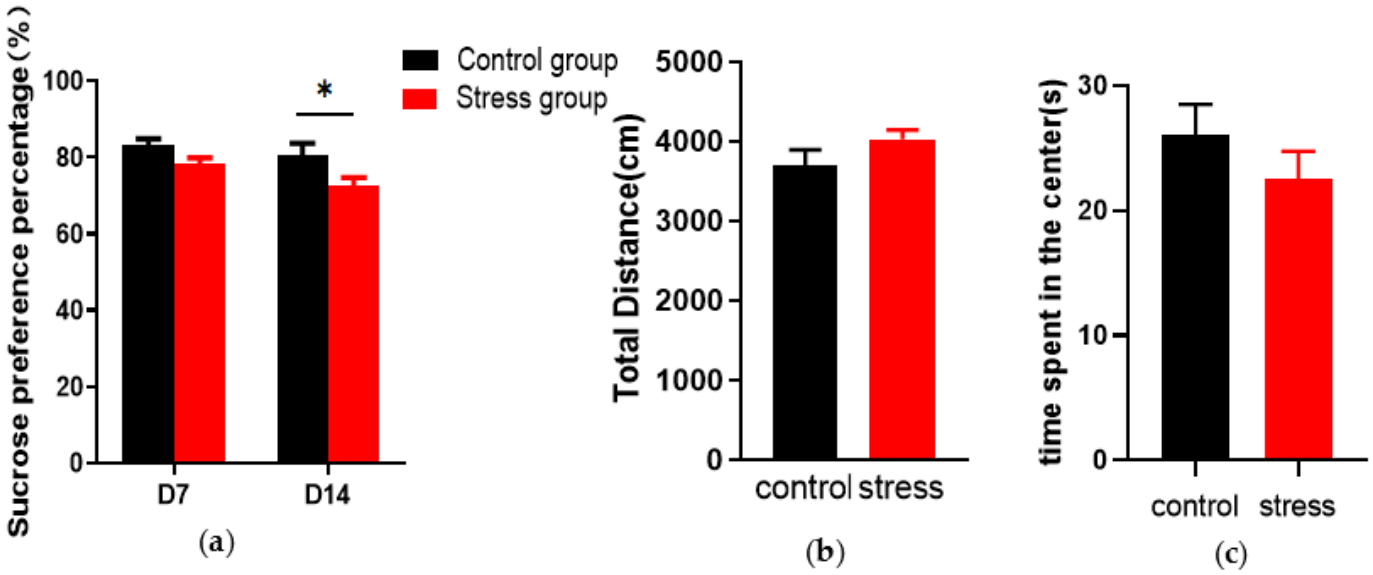

Figure 2

Psychological stress induced depression-like behavior in adult female mice.

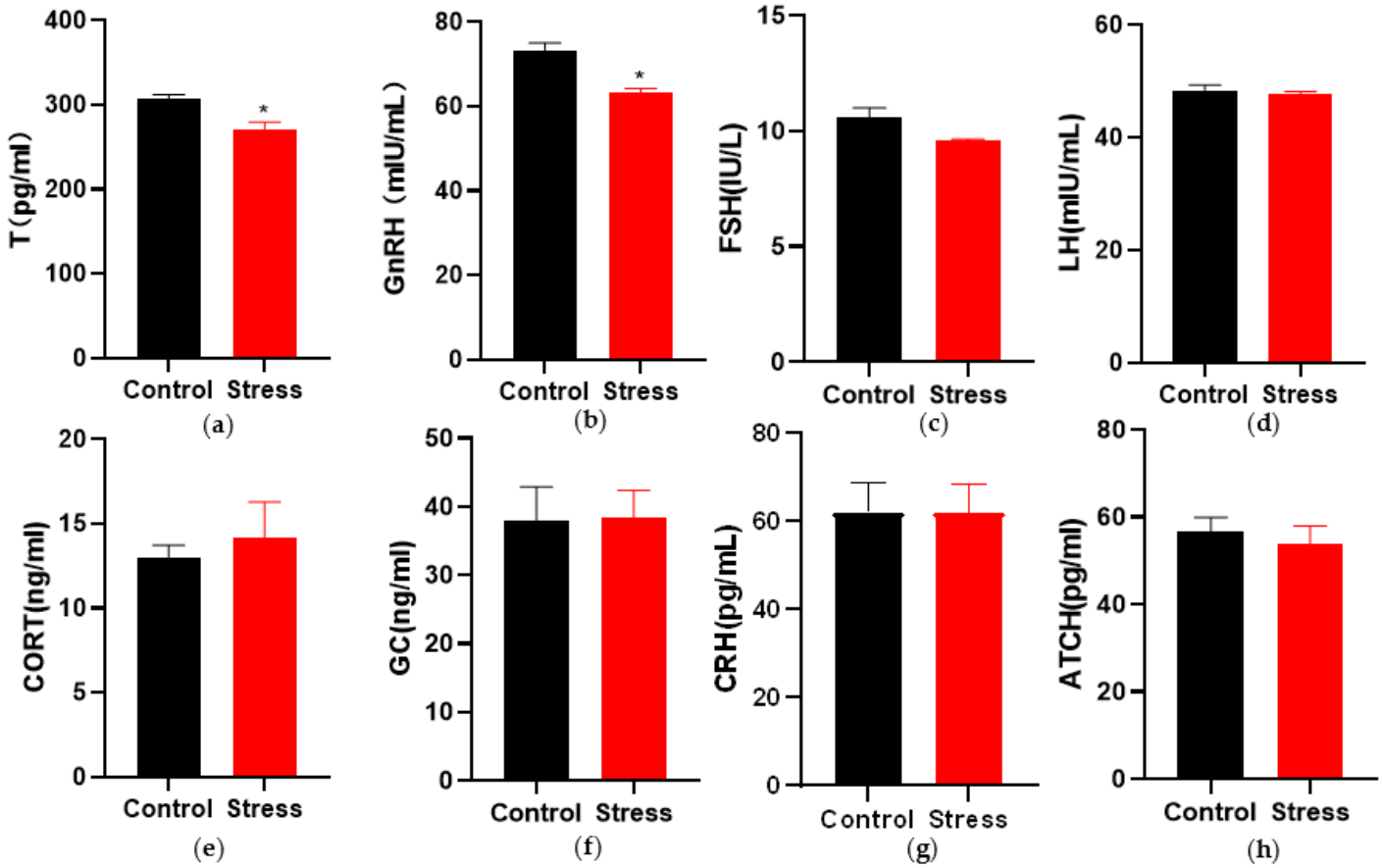

Figure 3

Psychological stress induced changes in endocrine levels.

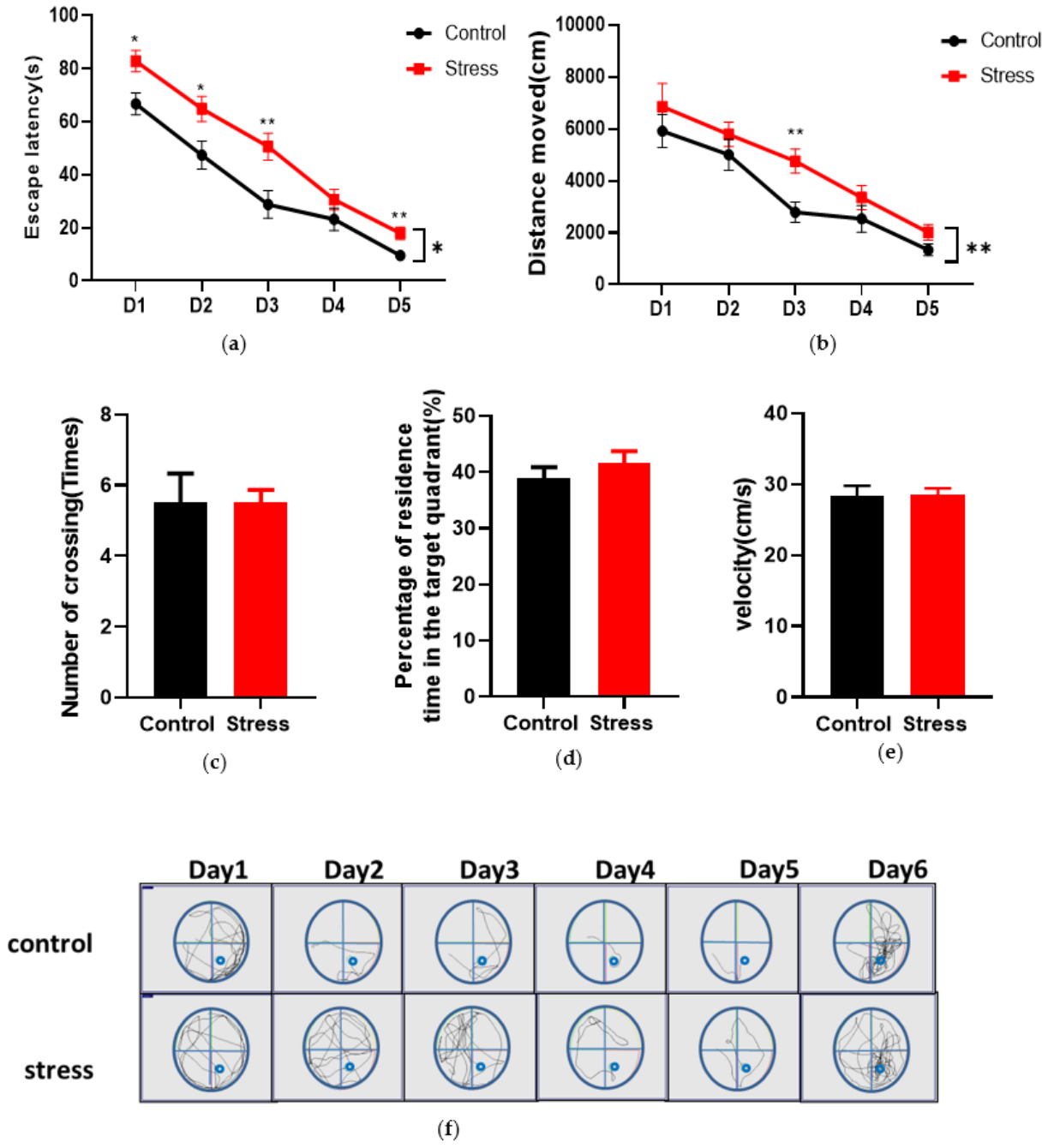

Figure 4

Psychological stress affects the learning and memory of adult female mice.

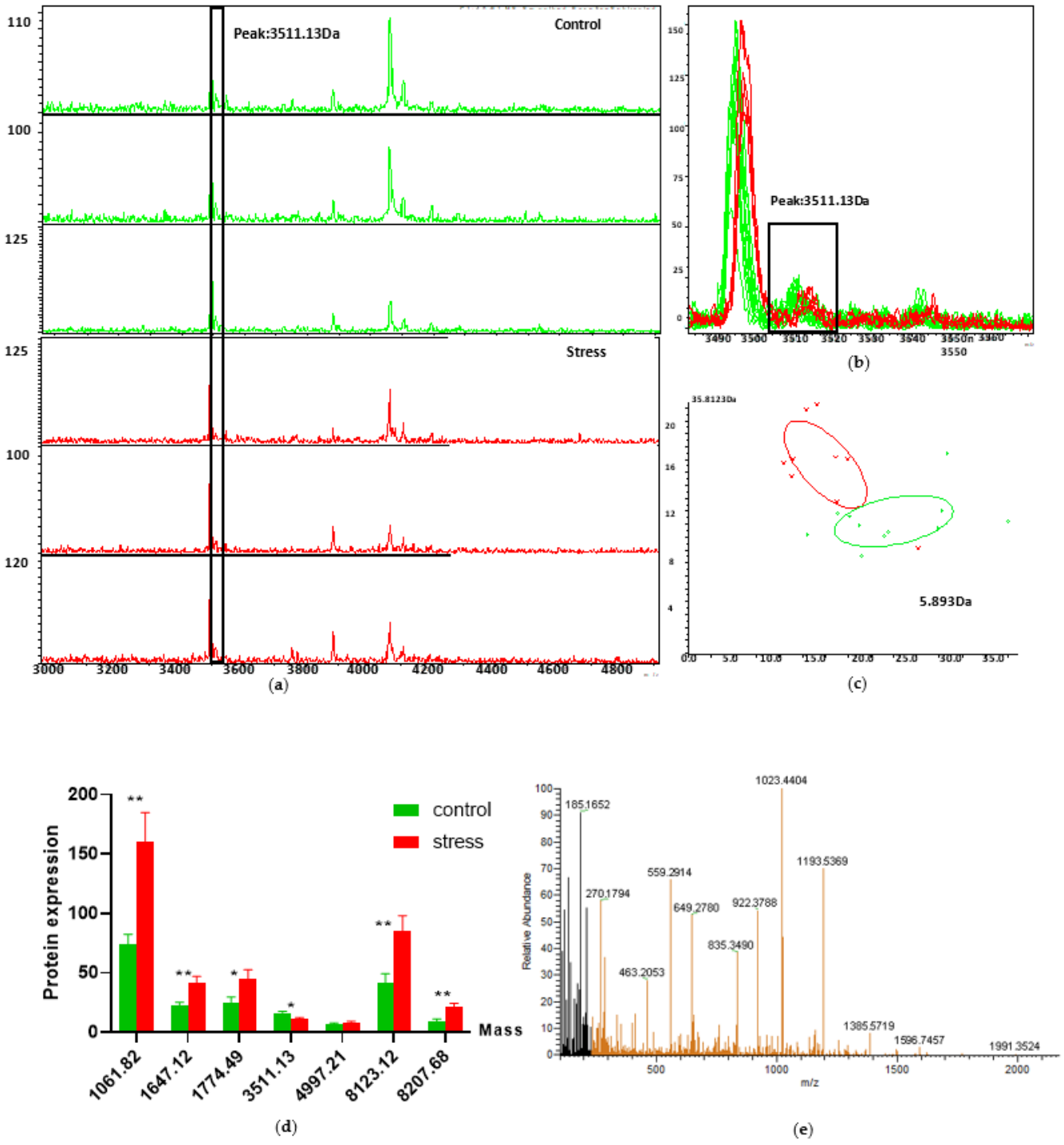

Figure 5

Comparative analysis of serum protein in female mice.