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Chronic unpredictable stress induces depression/anxiety-related behaviors and alterations of hippocampal monoamine receptor mRNA expression in female mice at different ages

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ABSTRACT

Depression and anxiety are the most common mental health disorders. Though they affect people at any age and occur more often in females, the pathophysiological changes under these conditions are less investigated. In the present study, we examined the effects of age and stress on depression- and anxiety-related behaviors in female mice. Saccharin preference and the open field test were carried out before and after chronic unpredictable stress in 4-, 14- and 25-month-old female mice. After behavioral tests, mRNA levels of monoamine receptors in the hippocampus were measured by real-time RT-PCR. Chronic unpredictable stress decreased saccharin preference in 4-, 14- and 25-month-old mice and the time spent in the center in the open field test in 25-month-old mice. For monoamine receptors, analysis of variance revealed significant effects of age on mRNA levels of Htr1a, Htr2a, Htr6, Adra1a, Adrb2, and Adrb3, significant effects of stress on mRNA levels of Htr4, Adra2c, Adrb1, and Adrb2, and interactions of age × stress on mRNA levels of Htr1a, Htr5b, Adra1d, Adra2a, Adra2c, and Adrb1. Chronic unpredictable stress decreased mRNA levels of Htr4, Htr5b, Adra2c, and Adrb1 in 4-month-old female mice. Correlations were observed between saccharin preference and mRNA levels of Htr4, Htr5b, Htr6, Adra1d, Adra2a, and Adra2c in 4-month-old mice and between the time spent in the center in the open field test and mRNA levels of Htr1b in 4-month-old mice, Htr3a, Htr7, and Adrb2 in 14-month-old mice, and Drd2 in 4- and 14-month-old mice. Our findings support that stress induces depression- and anxiety-related behaviors and the expression of hippocampal monoamine receptors in an age-dependent manner in female mice.

1. Introduction

Depression and anxiety are the most common and debilitating mental health disorders [1,2]. These two disorders are highly

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Abbreviations

5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
CUS	chronic unpredictable stress
OFT	open field test
SEM	standard error of the mean

comorbid, their symptoms are sometimes inseparable and they share some common pathophysiological mechanisms [3–6]. For years, hypotheses have been proposed involving multiple systems including neurotransmitter, endocrine, neurotrophin, inflammation, and others to explain the mechanisms of depression and anxiety [7–12], while medications used for the treatment mainly target the neurotransmitter systems, especially the monoaminergic system. These medications have both antidepressant and anxiolytic effects and mainly work by increasing extracellular monoamine neurotransmitters [13–16]. There is a conflict between the delayed onset of efficacy and the quickly increased neurotransmitter levels by these medications, which suggests that not only the decreased levels of monoamine neurotransmitters but also the dysfunctions of the monoamine receptors are involved in the pathophysiology of depression and anxiety [17,18]. Monoamine receptors, including fifteen serotonin receptors, nine adrenoceptors, and five dopamine receptors, have been identified and functionally studied [19–21]. Increasing evidence suggests that the monoamine receptors play important roles in mood regulation [22–27]. These receptors are abundant in mood-regulating brain regions including the hippocampus [28–30], which receives serotonergic, noradrenergic, and dopaminergic projections from the raphe nucleus, the locus coeruleus, and the ventral tegmental area, respectively [31–34] and integrates input information to regulate multiple physiopathological processes including depression, anxiety, stress and aging [35–40].

In animal studies of depression and anxiety, two conditions have been underestimated and less investigated: female and elderly. Though depression and anxiety occur about twice as commonly in females than in males [41,42], animal studies have tended to use males for a long time, which might result from the mistaken bias that females were more variable in behavior due to hormone levels and other reasons [43,44]. In recent years, more studies using females have been reported. Increasing evidence suggests the existence of sex differences in the underlying mechanisms of depression and anxiety and indicates the importance and necessity of using females in research [41,43,45]. Similarly, the factor of age also affects the symptom, treatment, and pathology of depression and anxiety. Classic diagnostic criteria for depression and anxiety reflect the symptoms most commonly in younger adults, while different ages are associated with different symptoms, such as irritability in younger age and sleep change in older age [46,47]. Antidepressants are less effective for late-life depression [48–51], indicating an age-related variation of the monoaminergic system, while the changes in the monoaminergic system with depression and anxiety regarding different ages are far from well characterized. In this study, using chronic unpredictable stress, a stress paradigm widely used in the study of depression and anxiety [52,53], we examined stress-induced changes in behaviors and mRNA levels of hippocampal monoamine receptors in adult female mice at the ages of 4, 14 and 25 months.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 J mice (Stock No. 000664) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Breeding cages were set up with one male and two female mice per cage. Female offspring at the ages of 4, 14, and 25 months were used. The mice were housed in groups of 3–5 per cage under a 12-h light/dark cycle (lights on at 7:00 a.m.) with *ad libitum* access to food and water. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. All animal experiments were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University Hospital.

2.2. Behavioral procedures

2.2.1. Chronic unpredictable stress (CUS)

Mice in CUS group were singly housed in a separate procedure room and subjected to different stressors daily for 15 consecutive days [54]. The stressors include 2-h restraint, 15-min tail pinch, 24-h constant light, 24-h wet bedding and 45° cage tilt, 10-min inescapable foot shocks, and 30-min elevated platform. Control mice were group housed and briefly handled daily in the housing room.

2.2.2. Saccharin preference

Depression-related behavior was evaluated by saccharin preference, which is a paradigm similar to sucrose preference and rules out the potential confounding effect of caloric intake because saccharin is a noncaloric sweetener [55–57]. Mice were habituated with two bottles of water for at least 4 days before the test until the end of the study. Saccharin preference was tested before and one day after CUS. On the test day, both CUS and control mice were singly housed, water-deprived for 6 h, and then provided with a free choice between a bottle containing 0.01% saccharin solution and a bottle of plain water. Fluid intake for 2 h was measured from the start of the dark cycle. After the test, control mice were put back into their home cage and all 0.01% saccharin solution was changed back to

plain water. Saccharin preference was calculated as the percentage of the mass of 0.01% saccharin solution consumed over the total mass of fluid consumed.

2.2.3. Open field test (OFT)

Anxiety-related behavior was evaluated in OFT before and two days after CUS. Mice were put individually in the SuperFlex Fusion open field cage (40 × 40 × 30 cm, Omnitech Electronics Inc., Columbus, OH) and monitored for 30 min. The light intensity was 250 lux. The time spent in the center of the arena (20 × 20 cm) and the total distance moved in the arena for the first 5 min were analyzed by Fusion software [54]. The total distance moved in the arena for 30 min was analyzed and used for the evaluation of the locomotor activity.

2.3. Real-time RT-PCR

Three days after CUS, mice were decapitated and the hippocampus was immediately dissected on ice. Total RNA was extracted with the RNeasy Mini kit (Qiagen, Germantown, MD, USA) and 1 μg of total RNA was reversely transcripted to cDNA with RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA). The cDNA was used for quantitative real-time PCR with AceQ qPCR SYBR green master mix (Vazyme, Nanjing, China) on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The relative amount of mRNA for the target gene was normalized to Gapdh using formula $2^{(CT[\text{target gene}]-CT[Gapdh])}$ and presented as the ratio to the average level (set as 1) of 4-month control mice. The primer sequences are listed in Table 1.

2.4. Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). Shapiro–Wilk test was used to test data normality. For normally distributed data, two-way analyses of variance (ANOVA) followed by Tukey's multiple comparisons was used. For non-normally distributed data, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. The linear relationships between two variables were determined by calculating Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CUS induces depression- and anxiety-related behaviors in female mice at different ages

Female mice at the ages of 4, 14, and 25 months were divided into control and CUS groups. The body weight, saccharin preference, and OFT were examined before and after CUS. The data before CUS was only used for grouping purpose. Two-way ANOVA revealed

Table 1
List of primers used for real-time RT-PCR.

Gene	Forward Primer Sequence (5' - 3')	Reverse Primer Sequence (5' - 3')	NCBI reference	Size (bp)
Htr1a	CTGTTTATCGCCCTGGATGT	ATGAGCCAAGTGAGCGAGAT	NM_008308.4	158
Htr1b	CACCAACCTCTCCCACAACT	CCAGAGAGGCGATCAGGTAG	NM_010482.2	197
Htr1d	GCATCCTAGAACGCAAGAGG	AAAGAAAGGCAACCAGCAGA	NM_001285482.1	104
Htr1f	GCAGCAAGGACACTGTACCA	TCCGATCTGGGACTTTTCAC	NM_008310.3	194
Htr2a	TTCGGGCTACAGGATGATTC	TGATGGTTAGGGGGATGAAA	NM_172812.3	112
Htr2b	GGAGAAAAGGCTGCAGTACG	ATAACCAGGCAGGACACAGG	NM_008311.3	155
Htr2c	GCCACGTCGAAAGAAGAAAG	GTTACAGGCCTTCCCACAAA	NM_008312.4	169
Htr3a	CATGTATGCCATCCTCAACG	CCACGTCCACAAACTCATTG	NM_001099644.1	188
Htr3b	AGCATCTTCCTGATGCTCGT	AGACCCCAATCAGAGGTGTG	NM_020274.4	166
Htr4	TGCTATCACCTGCTCTGTGG	CTGCCTTGGTCTCTGTCCTC	NM_001364958.1	197
Htr5a	TGAGCCTGGTACATGAGCTG	GCGGGTACGGAGTGTGTACT	NM_008314.2	176
Htr5b	GGAGCCTTCTACCTGCCTCT	CTCTGGAGGTGCTTCCTTTG	NM_010483.3	141
Htr6	TTCTTCCTGGTGTCGCTCTT	GGCAGAGGTTGAGAATGGAG	NM_001377096.1	169
Htr7	GCTGGGCTATGCAAACTCTC	GCCTCTCAGCAAGTTTCAGG	NM_001360297.1	161
Adra1a	ACCATTGTCACCCAGAGGAG	ATGATGGTCAGTGGCACGTA	NM_001271761.1	194
Adra1b	GAGGCTGCGCTTACACCTAC	CTGCCACTGTCATCCAGAGA	NM_007416.4	91
Adra1d	TCCGTAAGGCTGCTCAAGTT	GGACGAAGAAAAAGGGGAAC	NM_013460.5	103
Adra2a	CAGGCCATCGAGTACAACCT	TCTGGTCGTTGATCTTGCAG	NM_007417.5	172
Adra2b	CCCTGCCTCATCATGATTCT	GTCCATTAGCCTCTCCGACA	NM_009633.4	199
Adra2c	TCATCGTTTTCACCGTGGTA	GAAAAGGGCATGACCAGTGT	NM_007418.3	142
Adrb1	GCTGATCTGGTCATGGGATT	AAGTCCAGAGCTCGCAGAAG	NM_007419.3	100
Adrb2	GAGCACAAAGCCCTCAAGAC	GTTGACGTAGCCCAACCAGT	NM_007420.3	153
Adrb3	GGCAACCTGCTGGTAATCAT	CTGGTGGCATTACGAGGAGT	NM_013462.3	121
Drd1	CTGCTGGCTCCCTTTCTTCA	GGGGTTCAGGGAGGAATTCG	NM_010076.3	136
Drd2	AGTGAACAGGCGGAGAATGG	GAGAGTGAGCTGGTGGTGAC	NM_010077.3	103
Drd3	ACTCGACAGAACAGCCAGTG	ATGTGCTCCATTTGTCCCGT	NM_007877.2	137
Drd4	CGCCTCCATCTTCAACCTGT	TAGTCTCGGTTCTCCAGGCA	NM_007878.3	210
Drd5	GAACCTACGCCATCTCCTCG	TCTAGGGAGGAGATACGGCG	NM_013503.3	121
Gapdh	TCCCACTCTTCCACCTTCGA	TAGGGCCTCTCTTGCTCAGT	NM_008084.3	192

significant effects of age and stress on body weight (age: $F_{(2,38)} = 69.74$, $P < 0.0001$; stress: $F_{(1,38)} = 13.97$, $P = 0.0006$). The body weight of mice increased gradually with age but decreased after CUS (Fig. 1B). Depression-related behavior was examined by saccharin preference, which has been used to measure hedonic response to natural reward in mice. CUS decreased saccharin preference in female mice at all ages tested ($H = 26.46$, $P < 0.0001$; Fig. 1C). A significant effect of stress ($F_{(1,38)} = 11.75$, $P = 0.0015$) on total liquid consumption was also found (Fig. 1C). The preferences for saccharin solution after CUS were similar at different ages, which suggests that CUS induced anhedonia at a consistent level in adult female mice at different ages. Anxiety-related behavior was examined by OFT, in which anxious mice tend to spend less time in the center. Significant effects of age and stress on the time spent in the center for the first 5 min were found (age: $F_{(2,38)} = 5.818$, $P = 0.0062$; stress: $F_{(1,38)} = 6.990$, $P = 0.0118$). Multiple comparisons showed that CUS decreased the time spent in the center only in 25-month-old mice (Fig. 1D). No difference was found in the total distance moved in the arena for the first 5 min (Fig. 1D). Locomotor activity in the arena for total 30 min was not affected by CUS (Fig. 1E). A positive correlation between saccharin preference and the time spent in the center in OFT was observed in 25-month-old mice (Fig. 1F), indicating comorbidity of depression- and anxiety-related behaviors induced by CUS. These results suggest that CUS consistently induces depression-related anhedonia in adult female mice at different ages but is more sensitive in older female mice to induce anxiety-related behavior.

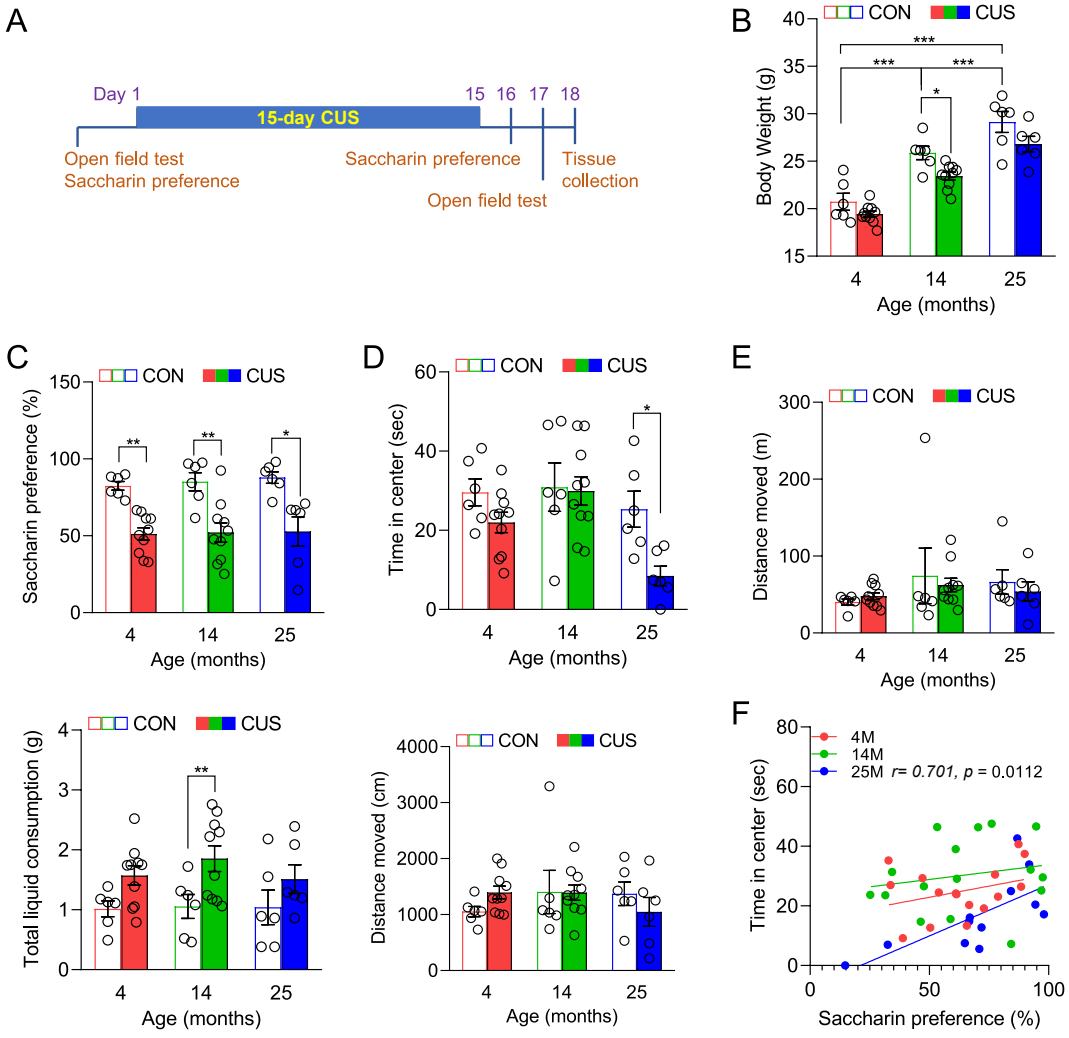

Fig. 1. Effect of CUS on depression- and anxiety-related behaviors in female mice at different ages. (A) Timeline of experimental design. Female mice at the ages of 4, 14, and 25 months were divided into Control and CUS groups. Behavioral tests were carried out before and after CUS. 4-Month Control: n = 6; 4-month CUS: n = 10; 14-month Control: n = 6; 14-month CUS: n = 10; 25-month Control: n = 6; 25-month CUS: n = 6. (B) Body weight. (C) Saccharin preference. Top, saccharin preference. Bottom, total liquid consumption. (D) OFT. Top, time spent in the center for the first 5 min. Bottom, total distance moved in the arena for the first 5 min. (E) Locomotor activity. Total distance moved in the arena for 30 min $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are shown as mean ± SEM. (F) Correlation between saccharin preference and the time spent in the center after CUS.

3.2. CUS on mRNA levels of serotonin receptors in the hippocampus of female mice at different ages

Hippocampal mRNA levels of monoamine receptors were examined by Real-time RT-PCR at the end of the behavioral tests. The housekeeping gene Gapdh has been used as the reference gene for analyzing gene expression in mouse hippocampus under aging and stress conditions [58,59]. We also chose Gapdh as the internal control for its expression was similar in different groups (average Ct values from 18.75 to 19.18, $p > 0.05$ between groups, data not shown). There are 15 subtypes of serotonin (5-hydroxytryptamine, 5-HT) receptors, including 5-HT1 (1 A, 1 B, 1D, 1 E and 1 F), 5-HT2 (2 A, 2 B and 2C), 5-HT3 (3 A and 3 B), 5-HT4, 5-HT5 (5 A and 5 B), 5-HT6 and 5-HT7 receptors. All subtypes are expressed in mice except 5-HT1E, which was identified in humans but not rodents [60]. ANOVA revealed significant effects of age on mRNA levels of Htr1a (Fig. 2A), Htr2a (Fig. 2D), and Htr6 (Fig. 2J), significant effect of stress on mRNA level of Htr4 (Fig. 2G), and interactions of age × stress on mRNA levels of Htr1a (Fig. 2A) and Htr5b (Fig. 2I). ANOVA statistical results are summarized in Table 2. No effect was found on mRNA levels of Htr1b, Htr1f, Htr2c, Htr3a, Htr5a, or Htr7 (Fig. 2B, C, 2E, 2F, 2H, 2K). Multiple comparisons showed that mRNA levels of Htr1a and Htr5b significantly decreased with age in control mice (Fig. 2A and I). CUS significantly decreased mRNA levels of Htr4 and Htr5b in 4-month-old mice (Fig. 2G and I). The mRNA levels of Htr1d, Htr2b, and Htr3b were either too low or undetectable in the samples to get consistent data for analysis.

3.3. CUS on mRNA levels of adrenoceptors in the hippocampus of female mice at different ages

There are nine subtypes of adrenoceptors, including alpha1a, alpha1b, alpha1d, alpha2a, alpha2b, alpha2c, beta 1, beta 2 and beta 3 adrenoceptors. ANOVA revealed significant effects of age on mRNA levels of Adra1a (Fig. 3A), Adrb2 (Fig. 3G) and Adrb3 (Fig. 3H), significant effects of stress on mRNA levels of Adra2c (Fig. 3E), Adrb1 (Fig. 3F) and Adrb2 (Fig. 3G), and interactions of age × stress on mRNA levels of Adra1d (Fig. 3C), Adra2a (Fig. 3D), Adra2c (Fig. 3E) and Adrb1 (Fig. 3F). ANOVA statistical results are summarized in Table 2. Multiple comparisons showed significant decrease in Adra2c mRNA level in 14-month-old mice compared with that in 4-month-old mice in control groups (Fig. 3E), while significant increase in Adra1a, Adra2a, Adrb2, and Adrb3 mRNA levels were

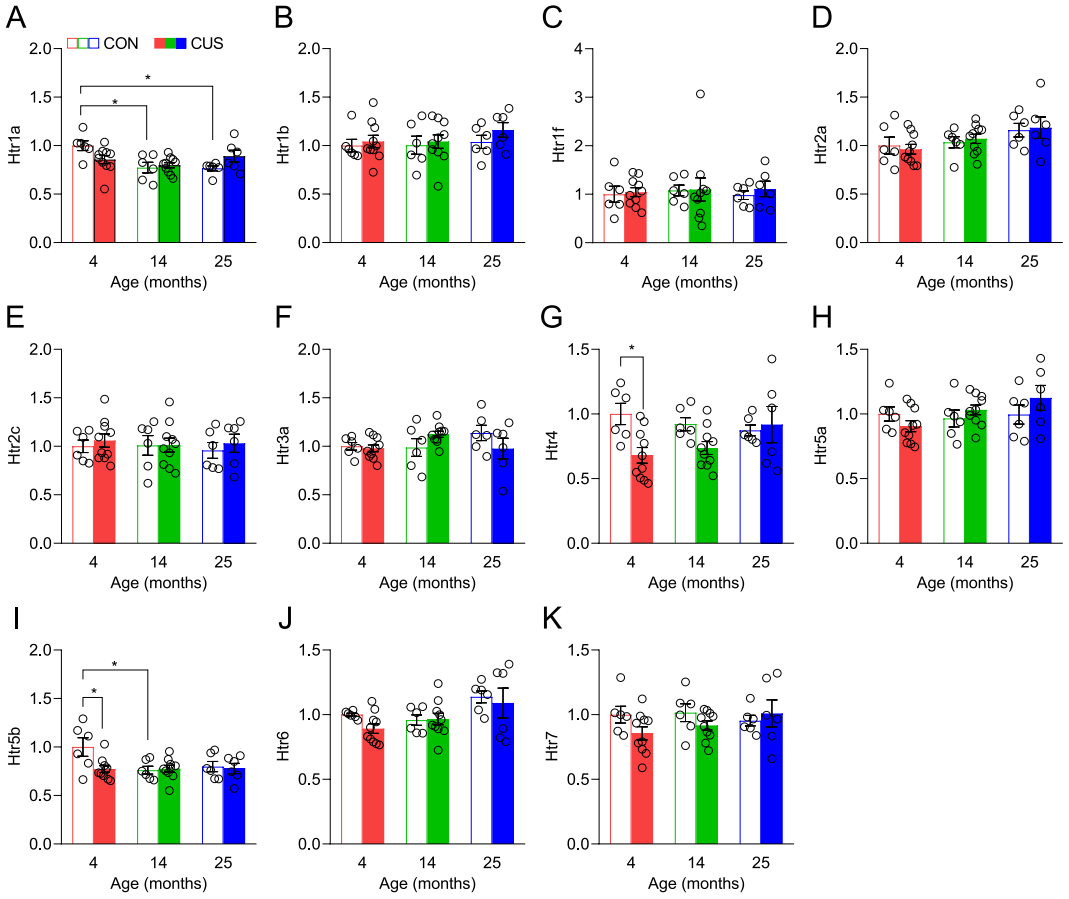

Fig. 2. Effect of CUS on mRNA levels of serotonin receptors in the hippocampus of female mice at different ages. The mRNA levels of serotonin receptors in the hippocampus of Control and CUS female mice at the ages of 4, 14, and 25 months were examined by real-time RT-PCR. (A) Htr1a. (B) Htr1b. (C) Htr1f. (D) Htr2a. (E) Htr2c. (F) Htr3a. (G) Htr4. (H) Htr5a. (I) Htr5b. (J) Htr6. (K) Htr7. 4-Month Control: n = 6; 4-month CUS: n = 10; 14-month Control: n = 6; 14-month CUS: n = 10; 25-month Control: n = 6; 25-month CUS: n = 6. *$P < 0.05$. Data are shown as mean ± SEM.

Table 2
ANOVA results summary for monoamine receptor mRNA levels in the hippocampus.

Gene	age	stress	age × stress
Htr1a	$F_{(2,38)} = 5.911$, $P = 0.0058$, **	$F_{(1,38)} = 0.003646$, $P = 0.9522$	$F_{(2,38)} = 4.708$, $P = 0.0149$, *
Htr2a	$F_{(2,38)} = 3.647$, $P = 0.0356$, *	$F_{(1,38)} = 0.01912$, $P = 0.8908$	$F_{(2,38)} = 0.1764$, $P = 0.8405$
Htr4	$F_{(2,38)} = 0.4245$, $P = 0.6572$	$F_{(1,38)} = 6.261$, $P = 0.0168$, *	$F_{(2,38)} = 2.879$, $P = 0.0685$
Htr5b	$F_{(2,38)} = 3.193$, $P = 0.0523$	$F_{(1,38)} = 3.350$, $P = 0.0751$	$F_{(2,38)} = 3.453$, $P = 0.0419$, *
Htr6	$F_{(2,38)} = 5,141$, $P = 0.0106$, *	$F_{(1,38)} = 1.165$, $P = 0.2873$	$F_{(2,38)} = 0.5846$, $P = 0.5622$
Adra1a	$F_{(2,38)} = 5.190$, $P = 0.0102$, *	$F_{(1,38)} = 1.085$, $P = 0.3041$	$F_{(2,38)} = 1.691$, $P = 0.1978$
Adra1d	$F_{(2,38)} = 1.857$, $P = 0.1700$	$F_{(1,38)} = 0.6326$, $P = 0.4313$	$F_{(2,38)} = 4.261$, $P = 0.0214$, *
Adra2a	$F_{(2,38)} = 2.371$, $P = 0.1071$	$F_{(1,38)} = 0.08470$, $P = 0.7726$	$F_{(2,38)} = 4.044$, $P = 0.0256$, *
Adra2c	$F_{(2,38)} = 2.786$, $P = 0.0743$	$F_{(1,38)} = 11.70$, $P = 0.0015$, **	$F_{(2,38)} = 6.580$, $P = 0.0035$, **
Adrb1	$F_{(2,38)} = 0.8978$, $P = 0.4159$	$F_{(1,38)} = 4.503$, $P = 0.0404$, *	$F_{(2,38)} = 5.048$, $P = 0.0114$, *
Adrb2	$F_{(2,38)} = 8.167$, $P = 0.0011$, **	$F_{(1,38)} = 4.326$, $P = 0.0443$, *	$F_{(2,38)} = 0.4909$, $P = 0.6159$
Adrb3	$F_{(2,38)} = 6.833$, $P = 0.0029$, **	$F_{(1,38)} = 1.234$, $P = 0.2736$	$F_{(2,38)} = 2.929$, $P = 0.0656$

*$P < 0.05$, **$P < 0.01$.

found in 25-month-old mice compared with 4-month-old mice in CUS groups (Fig. 3A, D, 3G, 3H). In 4-month-old mice, CUS significantly decreased mRNA levels of Adra2c and Adrb1 (Fig. 3E and F). The mRNA level of Adra2b was too low in the samples to get consistent data for analysis.

3.4. CUS on mRNA levels of dopamine receptors in the hippocampus of female mice at different ages

Dopamine receptors include D1-like and D2-like families. The D1-like family includes D1 and D5 receptors, and the D2-like family includes D2, D3, and D4 receptors. ANOVA showed no significant effect of age, stress, or interaction on mRNA levels of Drd1 (Fig. 4A), Drd2 (one sample from 25-month CUS group was excluded due to extremely high value; Fig. 4B) or Drd5 (Fig. 4C). The mRNA levels of D3 and D4 were too low in the samples to get consistent data for analysis.

3.5. Correlations of mRNA levels of monoamine receptors with depression- and anxiety-related behaviors

The linear regression between behavior and mRNA level was determined. For depression-related behavior, positive correlations

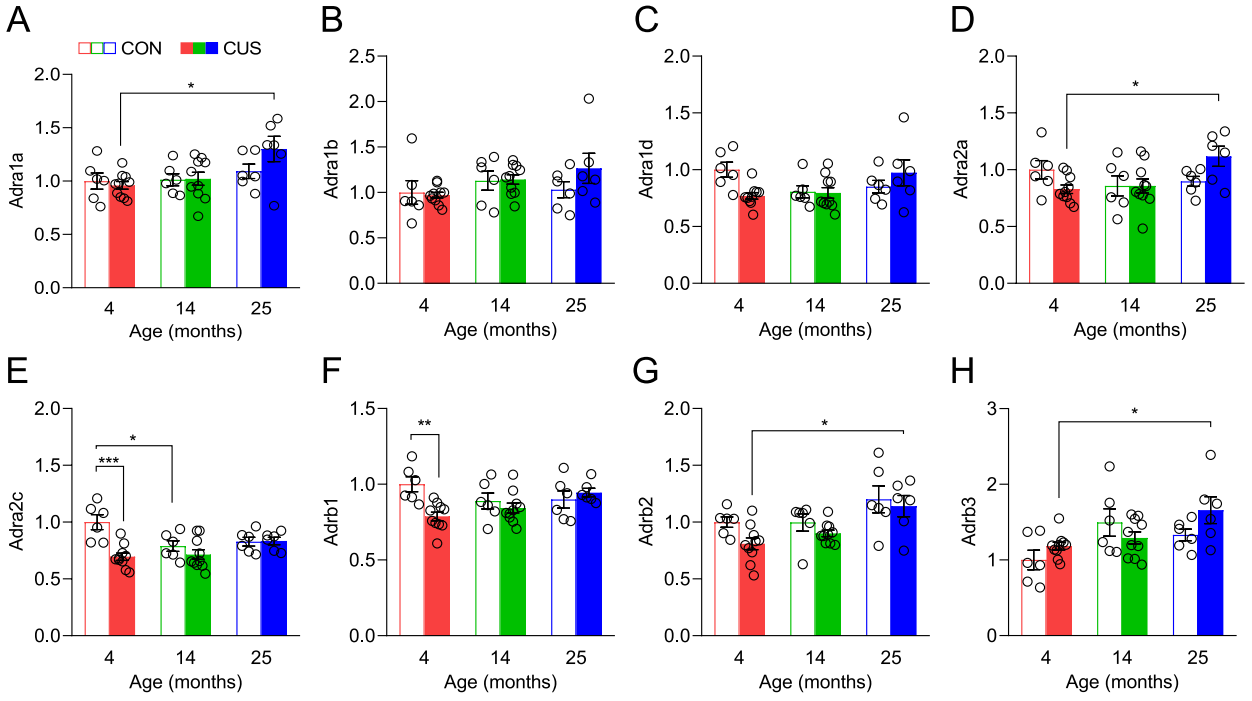

Fig. 3. Effect of CUS on mRNA levels of adrenoceptors in the hippocampus of female mice at different ages. The mRNA levels of adrenoceptors in the hippocampus of Control and CUS female mice at the ages of 4, 14, and 25 months were examined by real-time RT-PCR. (A) Adra1a. (B) Adra1b. (C) Adra1d. (D) Adra2a. (E) Adra2c. (F) Adrb1. (G) Adrb2. (H) Adrb3. 4-Month Control: n = 6; 4-month CUS: n = 10; 14-month Control: n = 6; 14-month CUS: n = 10; 25-month Control: n = 6; 25-month CUS: n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are shown as mean ± SEM.

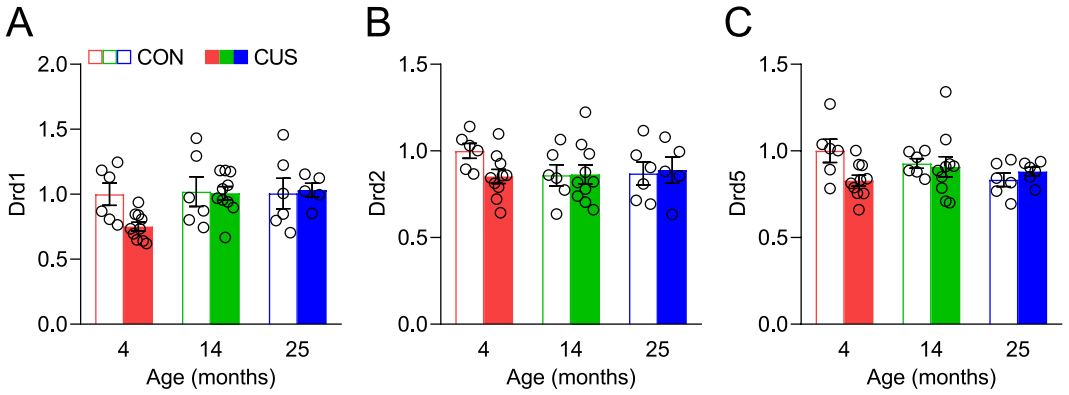

Fig. 4. Effect of CUS on mRNA levels of dopamine receptors in the hippocampus of female mice at different ages. The mRNA levels of dopamine receptors in the hippocampus of Control and CUS female mice at the ages of 4, 14, and 25 months were examined by real-time RT-PCR. (A) Drd1. (B) Drd2. (C) Drd5. 4-Month Control: n = 6; 4-month CUS: n = 10; 14-month Control: n = 6; 14-month CUS: n = 10; 25-month Control: n = 6; 25-month CUS: n = 6. Data are shown as mean ± SEM.

were observed between saccharin preference and mRNA levels of Htr4, Htr5b, Htr6, Adra1d, Adra2a, and Adra2c in 4-month-old mice (Fig. 5A). For anxiety-related behavior, although a significant behavioral change was observed in 25-month-old mice, no correlation was found at this age. A negative correlation was observed between the time spent in the center in OFT and the mRNA level of Htr1b in 4-month-old mice (Fig. 5B). Positive correlations were observed between the time spent in the center and mRNA levels of Htr3a, Htr7, Adrb2 in 14-month-old mice and the mRNA levels of Drd2 in 4- and 14-month-old mice (Fig. 5B). No correlation was observed between behavior and mRNA level of Adrb1 though CUS reduced its level in 4-month-old mice. These results suggest a functional role of monoamine receptors in depression- and anxiety-related behaviors in young and middle-aged females.

4. Discussion

In the present study, we examined the effect of 15-day CUS on depression- and anxiety-related behaviors and the mRNA levels of monoamine receptors in the hippocampus of female mice at the ages of 4, 14, and 25 months. CUS decreased saccharin preference at all ages examined and the time spent in the center in OFT at the age of 25 months. Significant effects of age, stress, and age × stress interaction were found on mRNA levels of hippocampal serotonin receptors and adrenoceptors but not dopamine receptors. Correlations between behavioral performance and receptor mRNA levels were found at the ages of 4 and 14 months but not at 25 months.

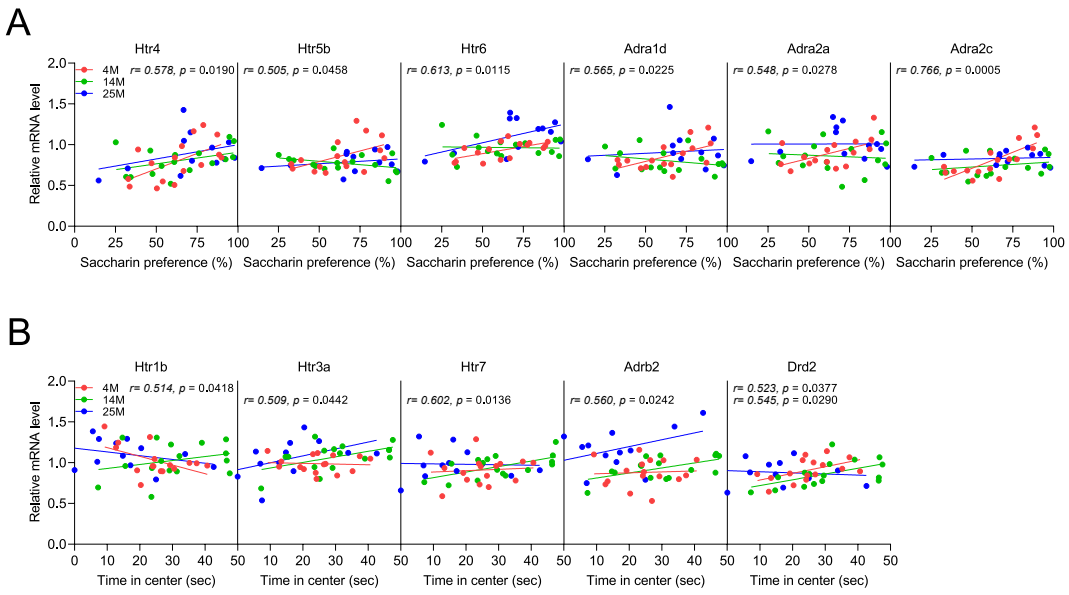

Fig. 5. Correlations of mRNA levels of monoamine receptors with depression- and anxiety-related behaviors at different ages. (A) Correlation between mRNA levels of monoamine receptors and saccharin preference. (B) Correlation between mRNA levels of monoamine receptors and time spent in the center in OFT.

These results suggest that stress induces behavioral changes and mRNA expression of hippocampal monoamine receptors in an age-dependent manner.

The CUS model has been used in thousands of studies and the stressor and duration vary among labs [52,61]. In the present study, we chose 15-day CUS, which was based on our previous work [54,62], preliminary data, and our purpose for investigating the behavioral sensitivity to CUS in female mice at different ages. CUS has been reported to induce depression-related behavior in mice at different ages [63], which is also supported by our results that CUS decreased saccharin preference in female mice at the ages of 4, 14, and 25 months. The effects of CUS on anxiety-related behaviors have been reported inconsistently and different stressors and stress duration could affect the result. In our study, CUS induced anxiety-related behavior in OFT in 25- but not 4- or 14-month-old female mice, which is consistent with the study showing no change in OFT after 3-week CUS in young adult mice [64]. Decreased time spent in the center in OFT after CUS was also reported in young adult mice with 4 weeks of stress [65], in which the duration of stress is longer than what we and Yin et al. used. Different behavioral tests may also affect the results. Sachs et al. reported anxiety-related behavior in female mice after 4-week CUS in EPM but not after 6-week CUS in OFT [66]. For time in the center in the OFT, young adult male mice are more sensitive to CUS than female mice [66]. Lotan et al. reported that CUS for 6 weeks increased time and entries in open arms in EPM in 3- but not 20-month-old female mice, even suggesting a stress-resilient phenotype limited to young adulthood [67]. Taken together, these results indicate that in adult female mice, CUS is effective in inducing depression-related behaviors at different ages, but the induction of anxiety-related behaviors is age dependent.

The monoaminergic system including serotonin, noradrenaline, and dopamine, plays a vital role in mood regulation, stress response, and brain aging [17,68–71]. In the present study, we found a significant effect of age on Htr1a mRNA in the hippocampus with decreased levels in 14- and 25-month-old mice when compared with that in 4-month-old mice, which is consistent with the decreased mRNA and protein levels of 5-HT1A receptor in the hippocampus of aged humans and rodents [72–75]. The effects of age were also found on mRNA levels of Htr2a and Htr6 though no significant difference among ages was observed by multiple comparisons. Studies on the effect of age on Htr2a expression have reported U-shape or no changes [72,76,77], suggesting that the different results of these studies, including ours, might be due to the difference in the age of experimental animals. The effect of stress was only found on the mRNA level of Htr4 with a significant CUS-induced decrease in 4-month-old mice. 5-HT4R has been reported to be involved in the etiology of depression. The 5-HT4R in the hippocampus has been reported to increase, decrease, or not change in different depressed animal models [78–80], which suggests a complex regulatory mechanism of the 5-HT4R system in depression and further studies will be needed to reveal its exact change and function in stress. Interactions of age × stress were found on the mRNA levels of Htr1a and Htr5b. Multiple comparisons showed a significant decrease in the mRNA level of Htr5b in 4-month-old CUS mice and 14-month-old control mice compared with that in 4-month-old control mice. It has been reported that hippocampal 5-HT5b is involved in the regulation of neuroplasticity and anxiety-related behavior [81,82]. Our results suggest further evidence for its involvement in stress and depression.

For adrenoceptors, most studies reported no change in protein levels in the hippocampus with age in humans and rodents [83–87], but these studies used ligand binding assay and did not separate the receptor subtypes. Our study provides further evidence for the effects of age and stress on hippocampal adrenoceptors. We found significant effects of age on Adra1a, Adrb2, and Adrb3, significant effects of stress on Adra2c, Adrb1, and Adrb2, and interactions of age × stress on Adra1d, Adra2a, Adra2c, and Adrb1 in the hippocampus. These results indicate that the hippocampal adrenoceptor system closely interacts with aging and stress. A human study using *in situ* hybridization reported reduced expression of ADRA1D and ADRA2C mRNA, and no altered expression of ADRA1A or ADRA2A mRNA in the hippocampus of postmortem subjects with Alzheimer's disease and dementia with Lewy bodies [88], also suggesting the different responses of adrenoceptor subtypes in age-related disease. In our study, we found a significant decrease in Adra2c mRNA level in the hippocampus in 14-month-old mice when compared with that in 4-month-old mice, suggesting regulation of Adra2c expression by age. We also found that CUS significantly decreased mRNA levels of Adra2c and Adrb1 in 4-month-old mice. Deficiency of norepinephrine in the brain has been reported under depression and stress, while the changes in adrenoceptors are contradictory. It has been reported that CUS decreased the mRNA and protein levels of alpha 2 adrenoceptor and predator scent decreased the protein level of alpha 2c adrenoceptor in rat hippocampus [89,90], which is consistent with our result. While the same study reported decreased mRNA and protein levels of Adrb2 but not Adrb1 [89]. Increases or no change in hippocampal Adrb1 or Abrb2 were also reported under different stress conditions [91,92]. Adrenoceptors are located on pre- and post-synaptic neurons and non-neuronal cells mediating different biological functions, which include regulating neurotransmitter release and activating downstream signaling pathways, and respond differently to stress [93–98]. The mRNA level represents the net value of receptor gene transcription in the hippocampus and the response of these receptors to stress at different cells and locations will need more detailed studies. An interesting finding of the present study is that in CUS mice, mRNA levels of Adra1a, Adra2a, Adrb2, and Adrb3 were significantly higher in 25-month-old mice than that in 4-month-old mice, which suggests that age could affect stress-induced changes in hippocampal adrenoceptors mRNA levels. Binding sites for estrogen have been identified within the promoter region of adrenoceptors [99]. Estrogen has been reported to regulate the expression of adrenoceptors in mood-regulating brain regions including the hippocampus [100–102]. Chronic unpredictable stress decreases the level of estrogen in female mice [103,104]. The evidence suggests that estrogen may mediate CUS-induced changes in adrenoceptor mRNA levels, while how estrogen signaling is involved at different ages will need further study.

Though only 4 genes (Htr4, Htr5b, Adra2c, and Adrb1) were found significantly affected by CUS when analyzed using multiple comparisons, changing trends were observed after CUS for several other genes. To further reveal the relationship between monoamine receptor gene expression and behavior change, linear regression between behavior and the mRNA level of an individual receptor gene was analyzed. Correlations between depression-related behavior and mRNA levels of Htr4, Htr5b, Htr6, Adra1d, Adra2a, and Adra2c were found in 4-month-old mice. Correlations between anxiety-related behavior and mRNA levels of Htr1b, Htr3a, Htr7, Adrb2, and

Drd2 were found in 4- and/or 14-month-old mice. Though CUS induced depression-related behavior in 4-, 14- and 25-month-old mice and anxiety-related behavior in 25-month-old mice, the monoamine receptor expression is correlated with behavior in young but not aged adult mice. These results indicate that the monoamine receptors might be not as sensitive to CUS-induced behavior changes in aged mice as in young adult mice, suggesting that the monoamine system may not be as important in depression in aged adults as in young adults and other systems, such as neurotrophin and inflammation systems [7–12], may play more important roles. This might partially contribute to the declined efficacy of antidepressants in the elderly [48–51].

The present study has several limitations. The numbers of animals are small, which may affect the results of data analysis, especially for the behavioral tests. Male mice were not included in the study for comparison of sex differences. CUS-induced depression- and anxiety-related behaviors have been reported in both young and old adult male mice [63,66,105,106]. We found CUS-induced depression-related behavior in 4-, 14-, and 25-month-old female mice but anxiety-related behavior only in 25-month-old female mice. These results indicate a potential sex difference in CUS-induced anxiety. The usage of the whole hippocampus for monoamine receptor mRNA measurement is another limitation of the present study. Hippocampus is a functionally heterogeneous brain region, in which the dorsal part is involved in learning and memory and the ventral part regulates emotional behaviors [107,108]. The mRNA levels measured in the present study may mask the effect of CUS in hippocampal subregions. Also, the protein level of the monoamine receptor was not examined, which may reflect the functional change better than mRNA. In the present study, we only analyzed the correlation between gene expression and the behavior change induced by CUS, whether there is a causal effect will need further investigation.

5. Conclusions

In summary, our results indicate that female mice are sensitive to CUS-induced depression-related behavior and elderly female mice are more sensitive to CUS-induced anxiety-related behavior than young adult female mice. Age and CUS affect mRNA levels of hippocampal monoamine receptors and these changes are correlated with behavioral changes in younger but not elderly female mice. These data provide an age-dependent manner of interaction between the hippocampal monoamine system and stress-related behaviors. Further studies will be needed for understanding the pathology and improving the treatment of depression and anxiety in the elderly.

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University Hospital (Ethics Approval Number 20221014–52).

Author contribution statement

Han Zhou and Kaixin Wang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Zhicheng Xu, Dunjiang Liu and Yameng Wang: Contributed reagents, materials, analysis tools or data.
Ming Guo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

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

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