Article

Klotho Regulated by Estrogen Plays a Key Role in Sex Differences in Stress Resilience

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Abstract: Klotho (KL) is a glycosyl hydrolase and aging-suppressor gene. Stress is a risk factor for depression and anxiety that are highly comorbid with each other. The aim of this study was to determine KL is regulated by estrogen and plays an important role in sex differences in stress resilience. Our results showed that KL was regulated by estrogen in rat hippocampal neurons in vivo and in vitro and was essential for estrogen-mediated increase in the number of presynaptic vesicular glutamate transporter 1 (Vglut1) positive clusters on the dendrites of hippocampal neurons. The role of KL in sex differences in stress responses was examined in rats using three-week chronic unpredictable mild stress (**CUMS**). CUMS produced a deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors in male but not female rats, which was accompanied by a reduction in KL protein levels in the hippocampus of male, but not female rats. This demonstrated the resilience of female rats to CUMS. Interestingly, knockdown of KL protein levels in the rat hippocampus of both sexes caused a decrease in stress resilience in both sexes, especially in female rats. These results suggest that regulation of KL by estrogen plays an important role in estrogen-mediated synapse formation, and KL plays a critical role in the sex differences in cognitive deficit, anhedonic-like and anxiety-like behaviors induced by chronic stress in rats, highlighting an important role of KL in sex differences in stress resilience.

Keywords: klotho; estrogen; hippocampus; chronic stress; sex difference; stress resilience

1. Introduction

The hippocampus is a primary target of estrogens, and plays a key role in learning, memory, and stress responses [1]. 17β-estradiol (E2), a major form of estrogen, plays an important role in spine formation, synaptic plasticity, learning and memory [1-3]. However, the underlying mechanism is largely unknown. Our RNA-seq study showed that Klotho (KL) mRNA levels in the hippocampus are positively correlated with the levels of circulating E2 during the estrous cycle, and the OVX-mediated decrease in hippocampal KL mRNA levels is reversed by E2 replacement [4]. These results spurred an interest in further studying the regulation of KL by estrogen and function of KL protein in the rat hippocampus.

KL is expressed in the hippocampus [5] and exists in three different forms, a full length, transmembrane protein (130 kDa), shed form (cleavage form) and a secreted protein (70 kDa) in humans and mice [6-9]. Since secreted KL is not expressed in rats [10, 11], the KL we investigated in the rat hippocampus is the full length form of KL. KL-deficiency shortens lifespan, while KL overexpression slows the aging process and extends lifespan [8, 12]. KL is localized in both pre and post-synaptic compartments of CA1 pyramidal neurons and regulates synaptic plasticity [5]. KL mutant mice show a decrease in both synapse number and the level of synaptophysin in the hippocampus [13] and cognitive impairment [14]. KL-overexpressing mice show enhanced cognitive function [15]. In vitro studies show the neuroprotective properties of KL on hippocampal

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neurons [16]. These studies exemplify a role of KL in cognition and neuroprotection in mice. However, the functions of KL in the rat brain are not well understood.

The clinical evidence shows that low level of circulating KL is related to depression [17-19]. Chronic psychological stress causes a decrease in serum KL in human, and KL is positively correlated with the severity of depression symptoms [17]. Patients with major depression have decreased KL in the cerebrospinal fluid [18]. These studies suggest an important role of KL in depression. Preclinical studies show that chronic unpredictable stress results in a decrease in KL mRNA levels in the rat choroid plexus [20], and KL in the mouse nucleus accumbens regulates behavioral response to social defeat stress [21]. These studies suggest an important role of KL in stress response. Stress is a risk factor for depression and anxiety [22] in which there is a sex difference [23]. Depression and anxiety are often present as comorbid disorders [24]. Chronic unpredictable mild stress (CUMS), an established animal model of depression, induces anhedonic-like and anxiety-like behaviors [25]. There are sex differences in stress resilience in rodents in which females generally show resilience to the detrimental effects of chronic stress on dendritic spines, synaptic plasticity, and cognition compared with males [26-29]. However, little is known about the underlying mechanisms. These studies motivated us to determine the role of KL in sex differences in stress resilience. Understanding of stress resilience should result in a better understanding of the mechanisms underlying the stress-related disorders. Our hypothesis is that KL protein level in the rat hippocampus is regulated by estrogen, which contributes to the mechanisms underlying estrogen-mediated synapse formation, and KL plays a key role in CUMS-induced sex differences in cognitive deficit, anhedonic-like and anxiety-like behaviors in rats. Cognitive deficit, anhedonia, the core future of depression and anxiety are aligned with important Research Domain Criterion (RDoC) [30]. Our results confirm our hypothesis and will most likely enhance our understanding of estrogen-mediated synapse formation, sex differences in stress resilience.

2. Results

2.1. E2 regulated the KL protein levels in the hippocampal neurons

Our RNA-seq study shows that E2 up-regulates KL mRNA levels in rat the hippocampus [4]. To confirm this result, OVX rats received vehicle or E2 treatments for 48h and 7 days **(Fig. 1A)**. Immunostaining results showed that KL is localized in the soma and dendrites of the hippocampal CA1 pyramidal neurons of rats, as expected (**Fig. S1**) [5, 31] and the antibody specificity is validated in KL knockout mice (**Fig. S1**). E2 treatment for 48h significantly increased the levels of KL staining intensity in the hippocampal CA1 pyramidal neurons of the OVX+E2 group compared to the vehicle-treated OVX group ($t_{12} = 3.78$, $P = 0.003$, **Fig. 1B-D**). Western blot analysis showed that 48h E2 treatment had a significant effect on KL protein levels in the hippocampus ($F_{2, 15} = 9.18$, $P = 0.003$, **Fig. 1E**). Similarly, 7-day E2 treatment caused a significant increase in the levels of KL staining intensity in the hippocampal CA1 pyramidal neurons ($F_{2, 18} = 9.5$, $P = 0.002$, **Fig. 1F-I**) and KL protein levels in the hippocampus ($F_{2, 15} = 22.21$, $P < 0.001$, **Fig. 1J**). These results confirmed that OVX-induced decrease in KL expression was reversed by E2 treatment. To determine the effects of E2 on KL and Vglut1 expression in cultured hippocampal neurons, primary cultures were treated with vehicle (**Fig. 1L1-L3, M1-M3**), E2 (**Fig. 1L4-L6, M4-M6**) or E2 plus ICI 182,780 (**Fig. 1L7-L9, M7-M9**) at Div13 for 48h as described in our previous study [32]. Vglut1, a marker for excitatory presynaptic terminals, was up-regulated by E2 treatment and Vglut1 positive cluster numbers were used to evaluate the number of excitatory presynaptic terminals along MAP2 positive dendrites (a positive control for KL) [32, 33]. E2 treatment resulted in a significant increase in KL protein levels ($F_{2, 6} = 38.86$, $P = 0.007$, **Fig. 1K**) evaluated by western blot and the number of KL positive clusters along MAP2 positive dendrites ($F_{2, 21} = 10.65$, $P = 0.004$, **Fig. 1N**). E2-induced increase in both the KL protein levels and the

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number of KL positive clusters was reversed by ICI182, 780 treatments (**Fig. 1K-N**).

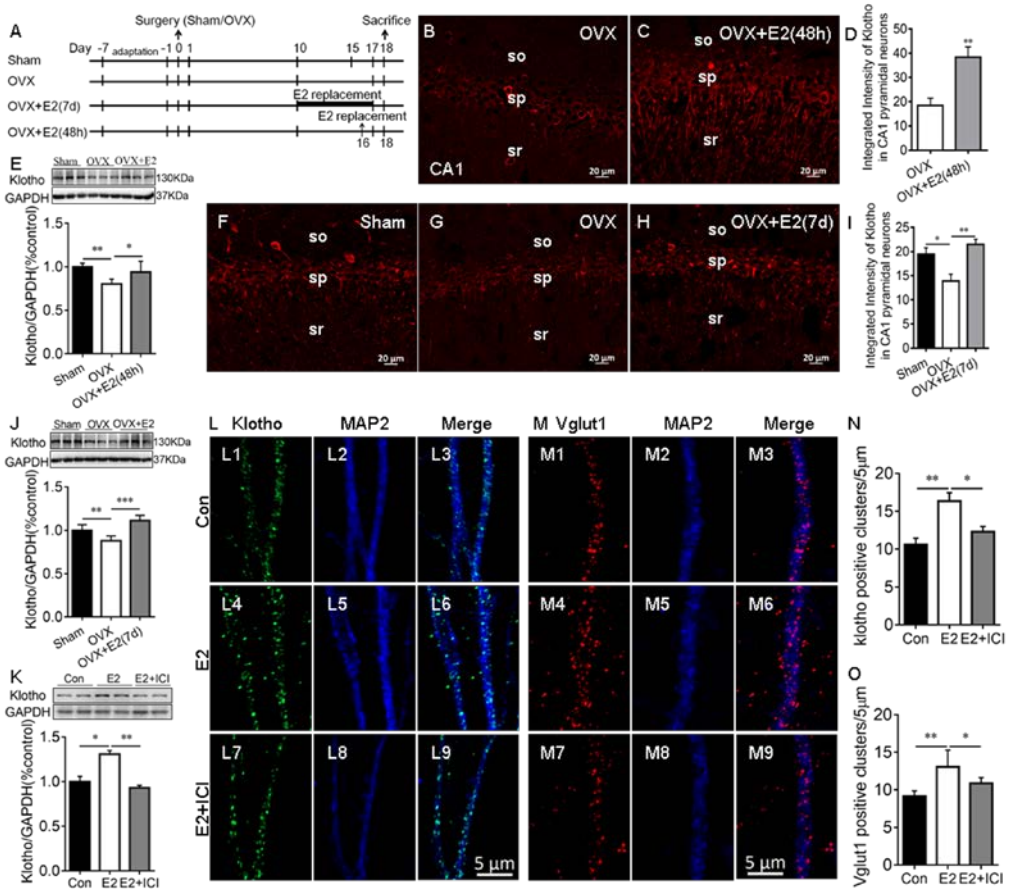

Figure 1. E2 increased Klotho expression in hippocampus of ovariectomized (OVX) rats and the cultured hippocampal neurons. Experimental design (**A**). Representative images of KL immunostaining (red) in the hippocampal CA1 area of sham, OVX and E2-treated OVX (OVX+E2) rats (48h after vehicle or E2 injection, **B-C**; daily vehicle or E2 injection for 7 days, **F-H**). Quantification of fluorescence intensity of KL staining in the hippocampal CA1 area (48h, **D**; 7d, **I**). Western blot showing the relative KL protein levels in hippocampus of sham, OVX and OVX+E2 rats (48h, **E**; 7d, J, n = 6). The cultures were treated with vehicle (Con), 10nM E2 and E2+1 μM ICI (ICI182,780, estrogen receptor inhibitor) at Div 13 for 48 hours. Western blot result of hippocampal lysate prepared from primary hippocampal culture (**K**). Immunostaining of cultured hippocampal neurons with antibodies specific to KL and MAP2 in Con, E2 and E2+ICI neurons (**L**). KL staining (green, L1, L4, L7); MAP2 staining (blue, L2, L5, L8); and merge of KL and MAP2 (L3, L6, L9). Quantification of KL positive clusters in cultured hippocampal neurons (**N**). Immunostaining of cultured hippocampal neurons with antibodies specific to Vglut1 and MAP2 in Con, E2 and E2+ICI neurons (**M**). Vglut1 staining (red, M1, M4, M7), MAP2 staining (blue, M2, M5, M8), and merge of Vglut1 and MAP2 (M3, M6, M9). The number of Vglut1 positive clusters in cultured hippocampal neurons (**O**). SO, stratum oriens of CA1 area; SP, stratum pyramidale of CA1 area; SR, stratum radiatum of CA1 area. Con, control. Data were shown as mean±SEM. D with T-test, others with one-way ANOVA followed by Tukey' test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

As expected, E2 had a significant effect on the number of Vglut1 positive clusters along MAP2 positive dendrites ($F_{2, 15} = 11.15$, $P = 0.001$, **Fig. 1O**). E2-induced increased in the number of Vglut1 positive clusters ($P < 0.01$, **Fig. 1M4-M6, O**) was reversed by ICI 182,780 ($P < 0.05$, **Fig. 1M7-M9, O**). In addition, inhibition of endogenous E2 synthesis with letrozole also caused a decrease in KL protein levels, KL positive clusters and Vglut1 positive clusters along MAP2 positive dendrites in the hippocampal neurons (**Fig. S2**) [34]. Overall, these results showed that E2 regulated the levels of KL protein in the hippocampal neurons. To determine whether E2 regulates KL expression in the hippocampus of male rats, male rats received vehicle or E2 treatment for 7 days, and

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western blot analysis showed that E2 treatment increased KL protein levels in the male hippocampus (**Fig. S3**).

2.2. E2 did not affect the number of Vglut1-positive excitatory presynaptic terminal when the

This experiment was to determine whether E2 affects the number of Vglut1 positive clusters along dendrites when endogenous KL protein levels are reduced. Firstly, we demonstrated that expression of KL-shRNA1 caused a significant decrease in both the intensity of KL staining (t_{12} = 3.91, P = 0.002, **Fig. 2A, B**), and the levels of KL protein evaluated in cultured hippocampal neurons ($F_{2,9}$ = 275.8, P < 0.001, **Fig. 2C**).

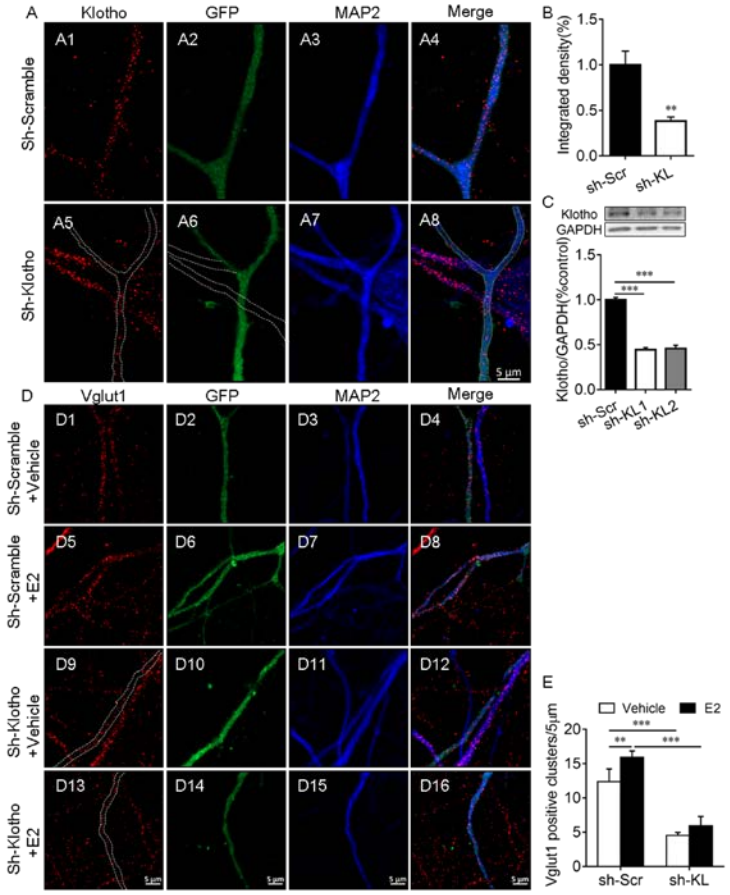

Figure 2. Klotho played an essential role in E2-mediated increase in the number of Vglut1 positive clusters in hippocampal neurons. Primary cultured hippocampal neurons were transfected with a vector encoding scrambled control shRNA (sh-Scr, A1-A4) or Klotho shRNA (sh-KL, A5-A8) at Div 10, and the neurons were fixed for double immunostaining with anti-KL (red) and anti-MAP2 (blue) antibodies at Div14. The dashed lines in A6 showed the dendrites of the non-transfected neuron which did not express Klotho shRNA-GFP (**A**). Quantification of KL expression in cultured hippocampal neurons (**B**). Designed two different Klotho shRNA, sh-KL #1 and sh-KL# 2. Vectors encoding sh-Scr and sh-KL were introduced into hippocampal neurons by electroporation at the time of plating and the western blot result (**C**) showed that expression of shRNA #1 and #2 reduced klotho (130kDa) expression effectively. We used sh-KL #1 in the following experiments. Cultured hippocampal neurons were transfected with a vector encoding sh-Scr (D1-D8) and sh-KL (D9-D16) at Div 10, and the cultures at Div 13 were treated with vehicle (D1-D4, D9-D12) or 10 nM E2 (D5-D8, D13-D16) for 48h before fixing for double immunostaining with antibodies specific to Vglut1 (red) and MAP2 (blue) (**D**). Quantification of Vglut1 positive clusters (**E**). One-way and two-way ANOVA followed by Tukey's test. Data were shown as mean±SEM. **P < 0.01, ***P < 0.001.

To determine whether E2-mediated up-regulation of KL expression is required for E2-induced increase in the number of Vglut1 positive clusters, the cultured hippocampal neurons were transfected with a vector encoding KL-shRNA-GFP or

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scrambled-shRNA-GFP at Div 10. At Div 13, the cultures were treated with vehicle or E2 for 48h (**Fig. 2D**) as described [32]. Two-way ANOVA analysis showed that KL-shRNA and E2 treatment had a significant effect on the number of Vglut1-positive clusters (interaction: $F_{1,21} = 4.71$, $P = 0.04$; shRNA: $F_{1,21} = 324.8$, $P < 0.001$; E2: $F_{1,21} = 24.76$, $P < 0.001$; **Fig. 2D, E**)). *Post hoc* Tukey's test showed that in KL-shRNA-expressing neurons, Vglut1-positive clusters were decreased in comparison to scrambled-shRNA-expressing neurons ($P < 0.001$, Fig. 2D9-D12 vs D1-D4, **Fig. 2E**). In scrambled-shRNA-expressing neurons, E2 treatment caused a significant increase in the number of Vglut1-positive clusters versus vehicle-treated scrambled-shRNA-expressing neurons ($P < 0.01$, Fig. 2D5-D8 vs D1-D4, **Fig. 2E**). However, this effect of E2 was eliminated in KL-shRNA-expressing neurons ($P > 0.05$, Fig. 2D13-D16 vs D9-D12, **Fig. 2E**). E2 no longer increased the number of Vglut1 positive clusters in hippocampal neurons after KL expression was decreased by KL-shRNA.

2.3. CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors were accompanied by a decrease in KL protein levels in male rats only

To determine the effects of CUMS on KL protein expression in the rat hippocampus, rats of both sexes received CUMS for 3 weeks (**Fig. 3A**).

Spatial learning and memory. The Morris water maze (**MWM**) test was used to evaluate spatial learning and memory. On day 5 during the 5-day learning phase, we detected a significant main effect of stress ($F_{1,28} = 23.51$, $P < 0.0001$) and sex ($F_{1,28} = 5.88$, $P = 0.022$) on escape latency to the platform, where only male stressed rats took significantly longer time to find the platform than unstressed males ($P < 0.01$; **Fig. 3B**).

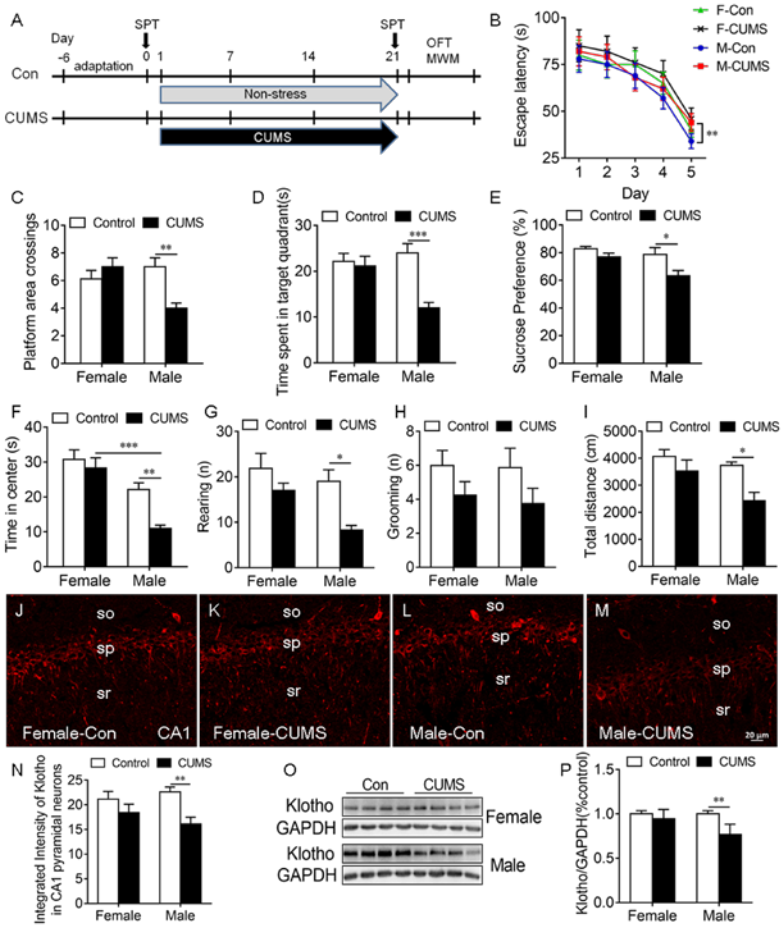

Figure 3. CUMS-induced spatial learning and memory impairment, anhedonic-like and anxiety-like behaviors in male rats were accompanied by a decrease in KL protein levels in hippocampus of male but not female rats. Experimental design. SPT (sucrose preference test), OFT (open field test) and MWM (Morris water maze) test (A). The latency to platform in rats of both

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sexes during 5-day training in the MWM test. CUMS decrease the latency on day 5 during training in male, but not female rats (**B**). CUMS decreased the number of platform crossings (**C**) and time spent in the target quadrant (**D**) in males only on day 6 during the probe trial. Only male rats showed a decrease in sucrose consumption in the SPT after CUMS (**E**). Time spent in the center of the open field (**F**), the number of rearing (**G**), the number of grooming (**H**) and the total distance travelled (**I**) in the OFT. Representative confocal images of KL staining (red) in the hippocampal CA1 area in female control (**J**), female CUMS (**K**), male control (**L**) and male CUMS (**M**) rats. Quantification of fluorescence intensity of KL staining in hippocampal CA1 area in male and female rats (**N**). Western blot analysis of KL protein (130kDa) in hippocampus of male and female rats (**O**, **P**). Data were shown as mean±SEM. Two-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ n = 8-10.

During the probe trial on day 6, stress and sex had a significant effect on platform area crossings (Interaction: $F_{1, 28} = 10.94$, $P = 0.003$, **Fig. 3C, Table S1**) and time spent in the target quadrant (interaction: $F_{1, 28} = 9.29$, $P = 0.005$; stress: $F_{1, 28} = 12.97$, $P = 0.001$; sex: $F_{1, 28} = 4.03$, $P = 0.05$; **Fig. 3D**). Tukey's test showed that stressed male, but not stressed female rats exhibited a decrease in platform area crossings ($P < 0.01$, **Fig .3C**) and time spent in the target quadrant ($P < 0.001$, **Fig. 3D**) compared to unstressed controls, respectively (**Fig. 3B-D**).

Although the MWM has been widely used to evaluate CUMS-induced deficit in spatial learning and memory [35-38], swimming in the MWM which may be a stressor to animals and affect the CUMS-mediated results. The Barnes maze test (**BMT**) is generally thought to be less stressful than the Morris water maze according to plasma corticosterone levels, and more sensitive to early cognitive deficits [39, 40]. To determine whether the swimming in the MWM test affects the results of the MWM, another cohort of rats of both sexes receiving the same CUMS exposure, the BMT was used to verify the results of the MWM test. Our results showed that swimming in the MWM did not affect the results of the MWM test since the results of MWM and BMT are identical (**Fig. S4**), which may result from the fact that swimming that was included in our CUMS protocol is a novel stress to rats in the MWM test.

In the Barnes Maze test, stress and sex displayed a significant effect on escape latency on day 4 during 4-day training (interaction: $F_{1, 24} = 9.67$, $P = 0.005$; sex: $F_{1, 24} = 15.99$, $P = 0.0005$; stress: $F_{1, 24} = 33.37$, $P < 0.0001$; **Fig. S4A**). Tukey's test showed that only stressed males exhibited an increase in escape latency compared to unstressed males ($P < 0.0001$). On day 7 during the probe trial, there was a significant effect of sex and/or stress on the escape latency (interaction: $F_{1, 24} = 8.71$, $P = 0.007$; stress: $F_{1, 24} = 22.99$, $P < 0.0001$ **Fig.S4B, Table S1**); Only stress had a significant effect on number of errors to find the target hole ($F_{1, 24} = 20.59$, $P = 0.0001$**)** and time spent in the target quadrant ($F_{1, 24} = 8.32$, $P = 0.008$**)**. Tukey's test showed that on day 7 during the probe trail, stressed males, but not stressed females showed an increase in escape latency ($P < 0.001$, **Fig.S4B**), a decrease in time spent in the target quadrant ($P < 0.05$, **Fig.S4C**) and an increase in number of errors to locate the target hole ($P < 0.001$, **Fig.S4D**) compared to unstressed controls. These results showed that CUMS caused a deficit in spatial learning and memory in males only, and females were resilient to CUMS.

Anhedonic-like behavior. Sucrose consumption in the SPT was used to evaluate anhedonic-like behavior. Stressed rats that did not like sweet by drinking less sucrose solution showed anhendoic-like behavior. Two-way ANOVA analysis revealed a significant main effect of sex ($F_{1, 36} = 6.14$, $P = 0.02$) and stress ($F_{1, 36} = 9.15$, $P = 0.005$) on sucrose consumption (**Table S1**). CUMS caused a significant decrease in sucrose consumption in males compared with male control ($P < 0.05$), but CUMS did not alter sucrose consumption in females (**Fig. 3E)**. CUMS-induced anhedonic-like behavior is sex dependent.

Anxiety-like behavior: Stress had a significant effect on time spent in the center ($F_{1, 36} = 8.89$, $P = 0.006$), rearing ($F_{1, 28} = 11.39$, $P = 0.002$), and the distance travelled ($F_{1, 28} = 9.98$, $P = 0.004$) in the OFT. Sex also had a significant effect on time spent in the center ($F_{1, 28} = 32.05$, $P < 0.0001$), rearing ($F_{1, 28} = 6.31$, $P = 0.002$), and the distance travelled ($F_{1, 28} = 5.96$ P

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= 0.02) in the OFT (**Table S1**). Tukey's test showed that CUMS caused a significant decrease in time spent in the center ($P < 0.01$, **Fig. 3F**), the number of rearing ($P < 0.05$, **Fig. 3G**), and the distance travelled ($P < 0.05$, **Fig. 3I**) in the OFT in males compared to unstressed male controls. CUMS did not alter grooming in rats of both sexes (**Fig. 3H**). CUMS induced anxiety-like behaviors in male, but not female rats.

Since KL is associated with cognition and stress response, the next experiment was to determine whether the CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behavior,in male rats was accompanied by an alteration in KL expression in the hippocampus. The results showed that there was a significant main effect of stress on the integrated intensity of KL staining in CA1 pyramidal neurons ($F_{1, 16} = 17.83$, $P = 0.006$; **Table S1**). CUMS induced a significant decrease in the integrated intensity of KL staining in CA1 pyramidal neurons in male, but not female rats compared to unstressed controls ($P < 0.01$, **Fig. 3J-N**). Western blot results showed that stress and sex had a significant effect on the levels of KL protein in the hippocampus (interaction: $F_{1, 24} = 7.13$, $P = 0.01$; sex: $F_{1, 24} = 7.13$, $P = 0.01$; stress: $F_{1, 24} = 18.16$, $P = 0.0003$, **Fig. 3O, P**). Tukey's test showed that stressed male, but not female rats displayed a significant decrease in the levels of KL protein in the hippocampus compared with unstressed controls ($P < 0.01$). Females showed a significantly higher degree of resilience to stress than males. Sex differences in CUMS-mediated decrease in KL expression may contribute to the sex difference in resilience to stress.

2.4. Reduction of endogenous KL in hippocampus did not alter spatial learning and memory, anhedonic-like and anxiety-like behavior in rats of both sexes

CUMS-induced decrease in KL expression in the hippocampus was accompanied by a deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors in male, but not female rats, which raised the possibility that endogenous KL plays a key role in the sex differences in stress resilience. To address this question, endogenous KL expression in the rat hippocampus of both sexes was knocked down (**KL-KD**) by expressing AAV vector encoding KL-shRNA for 3 weeks compared to control rats of both sexes expressing scrambled shRNA (**KL-CO**) (female: $t_{10} = 29.9$, $P < 0.001$; male: $t_{10} = 6.46$, $P < 0.001$; Fig. **4A-D**).

Spatial learning and memory. The MWM test showed that KL-KD did not have a significant effect on the latency to platform during training on day 1-5, the number of platform area crossings and time spent in the target quadrant on day 6 during the probe trail (**Fig.4E-G, Table S1**).

Anhedonic-like and anxiety-like behaviors. KL-KD did not have a significant effect on sucrose preference in sucrose preference test (**Fig. 4H**), time spent in the center of the open filed (**Fig. 4I**) number of rearing (**Fig. 4J**), number of grooming (**Fig. 4K**) and the total distance travelled in the OFT (**Fig. 4L**) in rats of both sexes (**Table S1**). These results showed that reduction of hippocampal KL did not impair spatial learning and memory, induce anhedonic-like and anxiety-like behaviors in rats of both sexes. The next experiment was to determine whether reduction of endogenous KL levels alter sex difference in resilience to stress.

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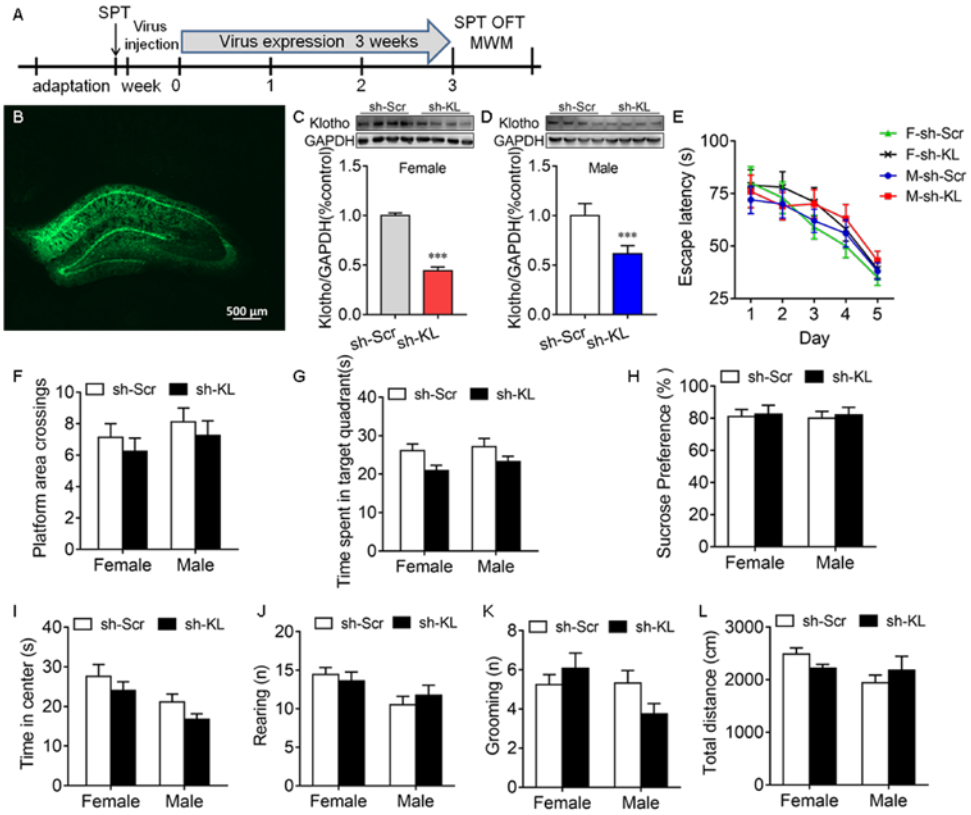

Figure 4. AAV-mediated decrease in the expression of hippocampal klotho (KL) protein did not impair spatial learning and memory in Morris water maze test, and induce anhedonic-like behaviors. Experimental design. SPT (sucrose preference test), OFT (open field test) and MWM (Morris water maze) test (**A**). Representative image of AAV-KLshRNA-GFP expression (**B**). Western blot analysis showed a decrease in the levels of hippocampal KL protein (130kDa) by Klotho shRNA (sh-KL) compared with scrambled shRNA (sh-Scr) in female (**C**) and male rats (**D**). In the MWM, decreased expression of KL did not alter the latency to platform on day 1-5 during training in the MWM (**E**), the number of platform area crossings (**F**) and time spent in target quadrant on day 6 (**G**) during probe trial in male and female rats. No significant difference between sh-KL and sh-Scr groups in sucrose consumption was found in the SPT (**H**). Time spent in the center of the open field (**I**), the number of rearing (**J**), the number of grooming (**K**) and total distance travelled (**L**) in the OFT. C-D, T-test; E, two-way repeated measures ANOVA; F-L, two-way ANOVA followed by Tukey's test. Data were shown as mean±SEM. ***$P < 0.001$, n = 8-13.

2.5. Endogenous KL plays an essential role in sex differences in CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors

To determine whether KL-KD altered stress resilience in female rats, rats of both sexes which had KL-KD or KL-CO were subjected to CUMS for 2 weeks (**Fig. 5A**). The rational for using 2-week CUMS is based on our time course of sucrose preference test in which 2-week, but not 1-week CUMS induced a significant decrease in sucrose consumption in KL shRNA-expressing rats, but not scrambled shRNA-expressing rats of both sexes. Three-week CUMS induced behavioral phenotypes in normal male rats only **(Fig. 3).**

Spatial learning and memory. Three-way ANOVA analysis revealed a significant main effect of shRNA ($F_{1, 56}$ = 10.99, P =0.002) and stress ($F_{1, 56}$ = 25.61, $P < 0.0001$) on escape latency on day 5 during training (**Table S2)**. **Tukey's** test showed that two-week CUMS caused a significant decrease in escape latency in KL-KD female ($P < 0.05$) and male ($P < 0.05$) rats compared to unstressed KL-CO controls (**Fig. 5B**). However, CUMS did not alter escape latency in normal rats of both sexes. These results showed that

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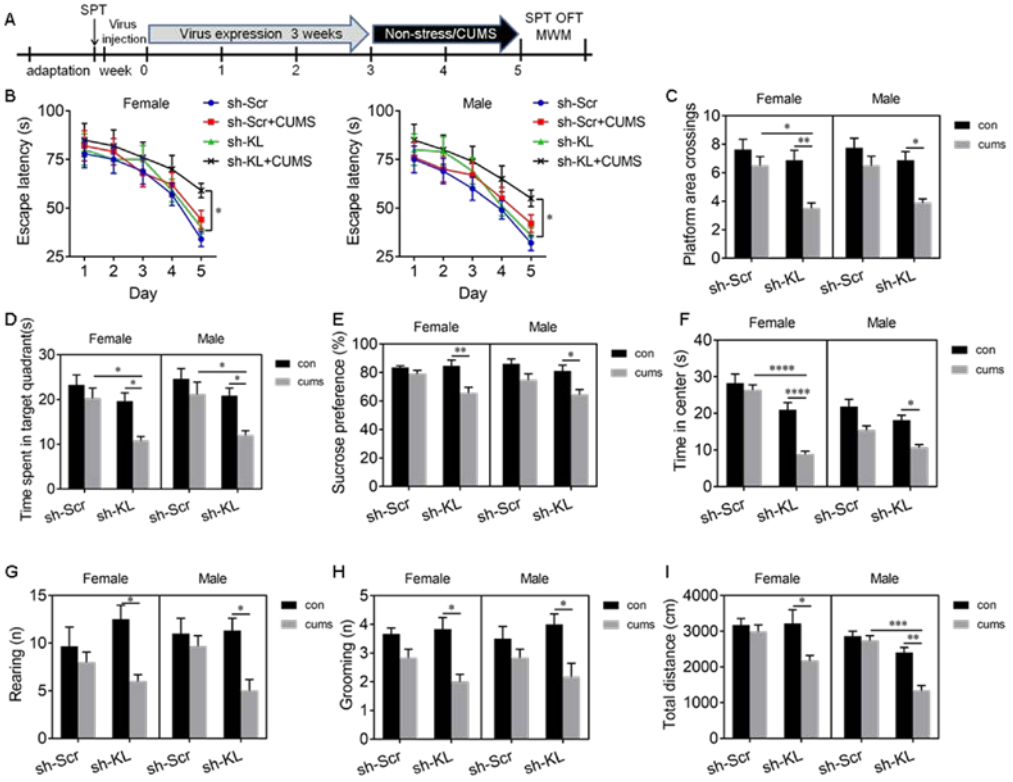

Figure 5. AAV-mediated decrease in KL protein levels increased stress susceptibility and diminished sex differences in spatial learning and memory deficit, anhedonic-like and anxiety-like behaviors induced by 2-week CUMS. Experimental design. SPT (sucrose preference test), OFT (open field test) and MWM (Morris water maze) test (**A**). In the MWM, CUMS did not alter the latency to platform in KL normal rats of both sexes during 5-day training, but CUMS caused a decrease in the latency to platform in KL-KD (knockdown) rats of both sexes on day 5 during training (**B**). CUMS did not alter the number of platform area crossings (**C**) and time spent in the target quadrant (**D**) in KL normal rats of both sexes. In contrast, in KL-KD rats of both sexes, CUMS decreased the number of platform area crossings (**C**) and time spent in the target quadrant (**D**) in the MWM test. CUMS caused a decrease in sucrose consumption in the SPT (**E**), a decrease in time spent in the center of open field (**F**), a decrease in rearing times (**G**), a decrease in grooming times (**H**) and total distance travelled (**I**) in the OFT in KL-KD rats of both sexes. Data were shown as mean±SEM. B, three-way repeated measures ANOVA; C-I, three-way ANOVA followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ***P* < 0.001, ***P* < 0.0001, n=6-8. sh-KL, KL-shRNA; sh-Scr, control scrambled shRNA.

two-week CUMS induced a deficit in spatial learning in male and female rats when endogenous KL expression was reduced. Consistent with these results, there was a significant main effect of shRNA ($F_{1, 56}$ = 18.17, P < 0.0001), a main effect of stress ($F_{1, 56}$ = 26.47, P < 0.0001) and a significant effect of shRNA x stress interaction ($F_{1, 56}$ = 5.53, P = 0.02) on platform area crossings (**Fig. 5C, Table S2). Tukey's** test showed that 2-week CUMS caused a significant decrease in platform area crossings in KL-KD females (P < 0.01) and males (P < 0.05) compared to unstressed KL-KD controls (**Fig. 5C**). Similarly, there was a significant effect of shRNA ($F_{1, 56}$ = 21.56, P < 0.0001) and stress ($F_{1, 56}$ = 18.75, P < 0.0001), and a significant interaction of shRNA x stress ($F_{1, 56}$ = 4.04, P = 0.049) on time spent in the target quadrant (**Fig. 5D, Table S2**). CUMS caused a significant decrease in time spent in the target quadrant in KL-KD females (P < 0.05) and KL-KD males (P < 0.05) compared to unstressed KL-KD controls (**Fig. 5D**). However, CUMS did not alter these behaviors in normal KL-CO rats of both sexes. Two-week CUMS induced a similar deficit in spatial memory in KL-KD rats of both sexes, and reduction of endogenous KL expression diminished the sex differences in stress resilience.

Anhedonic-like behavior. Three-way ANOVA analysis showed that there was a significant main effect of shRNA ($F_{1, 56}$ = 7.68, P = 0.008), stress ($F_{1, 56}$ = 27.09, P < 0.0001)

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and shRNA x stress interaction ($F_{1, 56}$= 4.1, P = 0.049) on sucrose consumption (**Fig. 5E, Table S2**). Tukey's test showed that CUMS caused a significant decrease in sucrose consumption in KL-KD females (P < 0.01) and males (P < 0.05) compared to unstressed KL-KD controls (**Fig. 5E**). Two-week CUMS induced a similar level of anhedonic-like behavior in KL-KD, but not normal male and female rats.

Anxiety-like behavior was evaluated by time spent in the center of the open field. There was a significant main effect of shRNA ($F_{1, 56}$ = 53.66, P < 0.001), stress ($F_{1, 56}$ = 38.05, P < 0.0001) and a significant interaction of shRNA x stress ($F_{1, 56}$ = 6.14, P = 0.02) on time spent in the center (**Fig. 5F, Table S2**). Tukey's test showed that CUMS caused a significant decrease in time spent in the center in KL-KD females (P < 0.0001) and males (P < 0.05) compared to unstressed female and male KL-KD controls, respectively (**Fig. 5F**). These results showed two-week CUMS induced anxiety-like behavior in KL-KD female and male rats with more severe phenotype in females. In addition, there was a significant effect of stress on rearing ($F_{1, 56}$ = 17.11, P = 0.0002, **Fig. 5G**) and grooming ($F_{1, 56}$ = 26.55, P < 0.0001, **Fig. 5H**), and a significant interaction of shRNA x stress on rearing ($F_{1, 56}$= 6.6, P = 0.01, **Fig. 5G**) and grooming ($F_{1, 56}$= 4.67, P = 0.04, **Fig. 5H**) (**Table S2**). *Post hoc* multiple comparison tests revealed that CUMS caused a significant decrease in rearing (P < 0.05) and grooming (P < 0.05) times in KL-KD, but not normal KL-CO rats of both sexes compared to unstressed controls (**Fig. 5G-H**). CUMS induced a similar decrease in rearing and grooming times in male and female rats when endogenous KL expression was reduced. Similarly, there was a significant main effect of shRNA ($F_{1,56}$ = 22.3, P < 0.0001), stress ($F_{1, 56}$ = 18.96, P < 0.0001) and the interaction of shRNA x stress ($F_{1, 56}$ = 10.57, P = 0.002) on total distance travelled (**Fig. 5I, Table S2**). CUMS caused a significant decrease in total distance travelled in KL-KD males (P < 0.05) and females (P < 0.01), but not normal KL-CO males and females compared to unstressed controls (**Fig. 5I**). Two-week CUMS induced a similar decrease in total distance travelled in KL-KD male and KL-KD female rats.

Overall, in KL intact control rats of both sexes, two-week CUMS did not alter cognitive function, induce anhedonic-like and anxiety-like behaviors. Reduction of KL expression in the hippocampus did not alter these behaviors, but decreased resilience, especially female rats, to stress and diminished the sex differences in CUMS-induced deficit in spatial learning and memory, anhedoic-like and anxiety-like behaviors, highlighting an important role of KL in the sex differences in stress resilience.

3. Discussion

3.1. E2 regulated KL expression in hippocampal neurons

Immunostaining showed that KL is localized in the soma and dendrites of hippocampal CA1 pyramidal neurons and MAP2 positive dendrites of cultured hippocampal neurons, in agreement with previous studies [5, 31]. E2 treatments for 48h and 7 days significantly reversed OVX-mediated decrease in KL protein levels in the hippocampus. There are three KL forms with different functions, a full length, transmembrane KL (130 kDa), shed KL and a secreted KL (70 kDa) in humans and mice [6, 7, 41]. Our study focused on full length KL. Secreted KL which is expressed in mice and humans, but not expressed in rats [10, 11] plays an important role in spatial learning and memory in mice [6, 42]. Our western blot results confirmed that E2 replacement reversed OVX-mediated decrease in KL protein (130kDa) level in OVX rat hippocampus. This is in agreement with our previous study [4]. To our knowledge, this is the first report that showed regulation of KL protein by E2 in the rat hippocampus. E2 increases the excitatory synapse number in hippocampal neurons [33]. KL deficient mice show a decrease in synapse number in the hippocampus [13]. These studies suggest an important role of KL in E2-mediated synapse formation.

Our results indicated localization of KL positive clusters on the dendrites of dissociated hippocampal neurons, and E2 treatment caused an increase in the number of both KL positive and Vglut1 positive clusters along MAP2 positive dendrites. Vglut1 is

 doi:10.20944/preprints202212.0211.v1

localized to the presynaptic side of excitatory synapses, and the number of Vglut1 positive clusters on the dendrites reflects the number of excitatory synapses [32]. The E2-mediated increase in the number of Vglut1 clusters is in agreement with our previous study [32] and reports by others [33, 43, 44]. Western blot analysis confirmed upregulation of KL protein by E2 in dissociated hippocampal neurons. Reversal of E2-mediated increase in KL protein levels in hippocampal neurons by ICI 182,780 [33] showed regulation of KL expression by E2 via estrogen receptors, but further study is required to determine which estrogen receptors contribute to this effect.

3.2. KL plays an essential role in E2-medaited synapse formation in hippocampal neurons

Letrozole-mediated decrease in the number of KL positive clusters was accompanied by a decrease in the number of Vglut1 positive clusters in the dendrites of the hippocampal neurons, in agreement with previous report that inhibition of endogenous E2 synthesis by letrozole causes a decrease in synapse number in hippocampal neurons [43, 45], which raised the hypothesis that KL plays an important role in E2-mediated synapse formation. E2 treatment caused a significant increase in the number of Vglut1-positive clusters along the MAP2 positive dendrites of GFP-expressing hippocampal neurons expressing scramble-shRNA. As expected, the ability of E2 to increase the number of Vglut1 positive clusters was eliminated in cultured hippocampal neurons expressing KL shRNA, which showed that E2 treatment no longer significantly increased the number of Vglut1-positive clusters when endogenous KL expression in these neurons was reduced. These results demonstrated that endogenous KL plays an essential role in E2-mediated formation of excitatory synapses in hippocampal neurons. Importantly, our results showed that decreased KL levels by KL-shRNA led to a similar decrease in the number of Vglut1 positive clusters in hippocampal neurons compared with scramble-shRNA-expressing control neurons in which KL levels were intact. These results showed an essential role of KL in the formation of excitatory synapses in hippocampal neurons. The mechanism through which endogenous KL regulates the number of Vglut1 positive clusters and affects E2-mediated formation of Vglut1 positive clusters is one of our future research directions. Estrogen plays a key role in sex differences in cognition, and there is a sex difference in both stress resilience and chronic stress-mediated cognitive deficit in rodents [29]. KL may play a role in sex differences in stress resilience characterized by sex differences in cognitive deficits, anhedonic-like and anxiety-like behaviors induced by CUMS

3.3. Sex differences in CUMS-induced KL expression is associated with sex difference in stress resilience in behaviors

Cognitive deficit, anhedonia and anxiety are often comorbid in depression [46, 47]. CUMS, an established animal model of depression, is widely used to induce cognitive deficits, anhedonic-like and anxiety-like behaviors in rodents [35, 48]. CUMS-induced deficit in spatial learning and memory in male, but not female rats is in agreement with previous studies in which chronic stress generally induced a cognitive deficit in male but did not have an effect on cognition or even enhance cognitive function in female rodents [27, 29]. CUMS-induced decrease in time spent in the center of the open field in males showed increased anxiety-like behavior, in line with previous reports [49, 50]**.** There are conflicting reports about the sex differences in CUMS-induced anhedonic-like behaviors in rats [51-54]. Rearing and grooming behaviors are the indicators of the emotional state of rats [55]. CUMS-induced decrease in time spent in the center and grooming times showed a high level of anxiety-like behavior [56]. However, conflicting reports suggested that rearing and grooming behaviors may not be the best indexes for anxiety-like behavior [57, 58]. The estrous cycle in female rats was not taken into consideration in the current study as CUMS disrupted the estrous cycle [51, 52]. CUMS-mediated decrease in the level of KL protein in the hippocampus is consistent

 doi:10.20944/preprints202212.0211.v1

with previous reports in which chronic stress causes a decrease in the KL mRNA levels in the hippocampus and choroid plexus of male rats [20, 59], but female rats were not included in these studies. However, the mechanisms through which chronic stress induce sex differences in cognitive deficit are largely unknown. In this study, the CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors in male rats was accompanied by a decrease in the levels of KL protein in the hippocampus of male but not female rats. Previous reports show that KL in the brain is required for maintaining a normal cognitive function in mice, since global knock down of KL in the mouse brain causes a cognitive deficit [14]. Global overexpression of KL in the mouse brain [60] and KL overexpression in the bilateral ventricles of mouse brain enhances cognition [61]. Downregulation of hippocampal KL is correlated with impairment in hippocampal-dependent memory in mice [62]. These results generated our hypothesis that the inability to decrease KL expression in the female hippocampus in the presence of chronic stress is responsible for the sex differences in chronic stress-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like beahviors. We tested our hypothesis and discussed the results below.

3.4. Endogenous KL in the hippocampus is essential for sex difference in stress resilience

To test our hypothesis, rats of both sexes were exposed to CUMS after knocking down expression of full length KL in bilateral hippocampus. The rationale for selecting the hippocampus is that the hippocampus is vulnerable to stress, associated with anhedonic-like and anxiety-like behaviors, and plays an important role in learning and memory. Furthermore, KL is highly expressed in the rat hippocampus. To our knowledge, we showed for the first time that specific knockdown of KL in the rat hippocampus did not induce a clear deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors in males and females. KL deficiency-mediated impairment in cognition in mice may contribute to secreted KL [63] because overexpression of the secreted form of KL enhances cognition while reduction of the secreted form of KL impairs cognition in mice [42]. Secreted KL was not expressed in the rat hippocampus [11]. Our results suggest that full length KL in the hippocampus does not play an essential role in maintaining cognitive function in rats, which requires further study, since all previous studies that show a role of KL in spatial memory were performed in mice. Interestingly, three-week CUMS impaired spatial learning and memory in male but not female rats, as expected when endogenous KL expression was not disrupted. However, two-week CUMS induced a deficit in spatial learning memory, anhedonic-like and anxiety-like behaviors in both female and male rats when endogenous KL levels were reduced using KL shRNA. Two-week CUMS did not alter cognitive function, anhedonic-like and anxiety behaviors in rats of both sexes when endogenous KL levels were intact. Furthermore, the sex differences in CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors were diminished when the levels of endogenous KL protein were reduced in the rat hippocampus of both sexes. Estrogen plays a key role in the sex differences in chronic stress-mediated cognitive deficits, because inhibition of estrogen receptors or blocking of endogenous estrogen synthesis impairs cognition in female rats and diminishes the sex differences [27]. Underlying mechanisms are not clear. Regulation of KL by estrogen may contribute to the underlying mechanism. Our results indicated that E2 up-regulated KL expression in the hippocampus of female rats that were resilient to CUMS-induced cognitive deficit, while a reduction in KL expression in the hippocampus decreased this resistance to stress in female rats and diminished the sex differences in CUMS-induced deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors by increasing the susceptibility of female rats to chronic stress. These studies suggest an important role of KL in sex differences in resilience to stress. Estrogen-mediated KL expression plays a crucial role in counteracting the detrimental effects of chronic stress

 doi:10.20944/preprints202212.0211.v1

on cognition in female rats. The underlying mechanism of this effect should be addressed in future studies.

In conclusion, E2 up-regulated KL protein levels in rat hippocampal neurons. E2 cannot increase Vglut1 expression and the number of Vglut1 positive clusters along dendrites of hippocampal neurons when endogenous KL levels are decreased. KL plays an essential role in the E2-mediated formation of excitatory synapses. A CUMS-mediated decrease in the level of KL protein in the hippocampus of male, but not female rats was accompanied by a deficit in spatial learning and memory, anhedonic-like and anxiety-like behaviors in males only. The AAV-KL shRNA-mediated decrease in KL levels in the hippocampus of female rats decreased the resilience of female rats to stress, and diminished sex differences in CUMS-induced cognitive deficit, anhedonic-like and anxiety-like behaviors. These findings support our hypothesis that KL plays a key role in sex differences in stress resilience.

4. Materials and Methods

4.1. Animals and reagents

Ten-week old male and female Sprague–Dawley rats were purchased from the laboratory animal center of Xi'an Jiaotong University (Xi'an, China). Animals were housed in a temperature (21 ± 1℃) and humidity-controlled (60-65%) animal care facility with 12-h light/-12-h dark cycle and unrestricted access to food and tap water. The animal protocol was approved by the Animal Care Committee of Shaanxi Normal University (Xi'an, China). All manipulations were performed in accordance with the ethical principles of animal use and care. All animals were adapted to the laboratory conditions for 7 days before starting the experiment.

The following reagents were used: β-Estradiol 3-benzoate (E8515, Sigma Aldrich), sesame oil (S3547, Sigma Aldrich), β-Estradiol suitable for cell culture (E2257, Sigma Aldrich), ICI182,780 (estrogen receptor antagonist, 1047, TOCRIS), Letrozole (aromatase inhibitor, 4382, TOCRIS), Primary antibodies: Klotho (AF1819, R&D system, immunostaining and Western blot), Klotho (ab203576, Abcam, western blot), vesicular glutamate transporter (Vglut1) (AB5905, Millipore), MAP2 (sc-32791. Santa Cruz).

4.2. Ovariectomy (OVX) and estradiol replacement

Bilateral OVX or sham surgery was performed as described in our previous studies [4, 32]. The animals were randomly divided into 4 groups: sham, OVX+vehicle, OVX+E2 (7days), OVX+E2 (48h). Ten days after surgery, OVX rats received subcutaneous injection of 10μg of β-Estradiol 3-benzoate dissolved in 100μl sesame oil or 100μl sesame oil only (vehicle) [4]. For OVX+E2 (7days) group, the rats received E2 for 7 consecutive days, and for OVX+E2 (48h) group, rats received one subcutaneous injection of E2 and were sacrificed 48 hours after injection. Successful OVX was confirmed by measuring circulating E2 levels.

4.3. Primary rat hippocampal neurons, drug treatments and transfection

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague Dawley rats of mixed sexes as described [32]. The cultures were treated with 10 nM E2, vehicle or E2 plus 1 μM ICI182, 780 or E2 plus 0.1 μM letrozole at Div 13 for 48h and then cultures were fixed with 4% paraformaldehyde and stained at Div 15 with antibodies specific to KL, Vglut1, and MAP2 as described [34]. To reduce KL expression, two rat KL shRNAs were designed (BLOCK-iT™ RNAi Designer). The shRNAs were annealed and ligated into a pAAV-U6-IRES-hrGFP vector. The inhibition efficiency of KL shRNAs was confirmed in rat hippocampal cultures. One of the rat KL shRNAs (CCTTACTTCGAGAAATGCGGG, nt1772-1792, NM_031336.1) was used based on its higher knockout efficiency, and the specificity of this shRNA was previously confirmed [64]. In addition, a non-target shRNA (Scrambled shRNA)

 doi:10.20944/preprints202212.0211.v1

(GTCTGTCCTGTCGTCTCTTAA) was used as a control. For shRNA knock-down experiments, pAAV-U6-IRES-hrGFP vector encoding KL shRNA or scrambled shRNA (control) was introduced into hippocampal neurons via electroporation at the time of plating at day 0 or using Lipofectamine 2000 Reagent (Invitrogen) at Div 10 as described [32].

4.4. Western blot

Western blot was performed as described [65]. Total protein was extracted from whole hippocampus or primary cultures using RIPA buffer (Solorbio Life Sciences, # R0010). Samples were homogenized using a Pro Homogenizer and the protein concentration was determined using the bicinchoninic acid assay (BCA) with bovine serum albumin as standard. Briefly, 30 μg proteins were loaded per lane on an 8-10% gradient acrylamide gel and proteins were transferred to Immobilon-P transfer membranes (Millipore) and incubated with the following primary antibodies: KL (1:1000, ab203576, Abcam), GAPDH (1:10000, ZSGB-BIO). After incubation with the corresponding secondary antibodies, membranes were visualized using the Luminescent imaging system (Tanon, China). The signal for each target protein was normalized to the GAPDH signal before being analyzed.

4.5. Immunohistochemistry (brain sections)

The immunohistochemistry was performed as described in our previous report [32]. Briefly, coronal sections (12 μm) were cut in a cryostat and mounted on gelatin-coated slides. Sections were blocked in 1% BSA, 5% normal donkey serum and 0.20% Triton X-100 (pH 7.4) for 1 h at room temperature. Then sections were stained with antibody specific to KL at 4°C overnight. Primary antibodies were visualized with Cy3-labeled donkey anti-rabbit IgG (Jackson Lab). Images were captured with a Zeiss LSM800 confocal microscope.

4.6. Immunocytochemistry of cultured hippocampal neurons

Immunostaining of dissociated neurons was performed as described previously [32]. Briefly, neurons were fixed with 4% paraformaldehyde at room temperature for 18 min. After 7 min in 1%BSA, 5% normal donkey serum, 0.20% Triton X-100 followed by 53 min in the same buffer without Triton X-100 at room temperature, neurons were doubly stained with antibodies specific to KL or Vglut1 plus MAP2 overnight at 4°C. Primary antibodies were visualized with appropriate secondary antibodies [32]. Images were taken with a Zeiss LSM800 confocal microscope.

4.7. Chronic Unpredictable Mild Stress (CUMS)

CUMS was performed as described [66] with a slight modification. Each rat was kept in one cage and was subjected to 8 unpredictable stressors including: (1) Inversion of the light/dark cycle for 12/12 h, (2) Swimming for 5 min in 4°C cold water or 45°C hot water, (3) Cage with damp sawdust for 24 h, (4) Cage tilting for 24 h, (5) Shaking for 30 min, (6) Nip tail for 1 min, (7) Fasting and water deprivation (overnight), (8) Lights on overnight. Rats received one unpredictable stressor per day in the first week, two stressors every other day in the second week and two random stressors per day in the third week. The same stressor was not used in two consecutive days. Unstressed controls were simply handled daily.

4.8. Stereotaxic surgery and adeno-associated virus (AAV) microinjection

Stereotaxic surgery was performed as described [67] according to the coordinates: 3.0 mm posterior to bregma, 2.2 mm lateral from midline and 2.4 mm below the skull surface. Two μl AAV9 vectors encoded KL shRNA or scrambled shRNA (packaged and purified by Hanbio Biotechnology, Shanghai, China) were injected into bilateral hippocampus at infusion rates of 0.2μl /min using a micro-infusion pump. To prevent

 doi:10.20944/preprints202212.0211.v1

back flow, the needle was left in the place for an additional 10 minutes before it was slowly removed. After behavioral tests, we verified the accuracy of the injections by examining brain sections; animals were excluded from analysis when the injection site was off target.

4.9. Behavioral assessments

Sucrose preference test (SPT) [66] was used as a measure of anhedonic-like behavior, a key symptom of depression [68]. For the sucrose preference test, controls and CUMS-exposed rats were deprived of food and water for eight hours (9:00–17:00) and subsequently exposed to 1% sucrose solution and water for 4 hours from 17:00 to 21:00. The positions of the bottles were switched midway to prevent bottle preference. Sucrose and water consumption was measured and the sucrose preference was calculated as described [68]: sucrose preference index (%) = (sucrose intake/total fluid intake) × 100.

Open field test (OFT) was conducted as described in our previous study [68]. All rats were placed in the testing room for an hour before performing the OFT. The test was conducted for 5 min in a dimly lit room. Briefly, rats were placed in the center of the open field box (60cm long × 60cm wide × 40 cm deep black wooden box) individually and the rats were allowed to explore the area freely during a 5-min test session. An automated video tracking system was used to record the locomotive activities of each rat during the test (Smart v3.0, RWD, Shenzhen, China). The total distance travelled, grooming and rearing times and time spent in the center of the open field were recorded.

Morris water maze (MWM) test was performed as described with a slight modification [67]. In the acquisition trials, rats received 4 training trials (60 s per trial) per day for 5 consecutive days and were trained to find the platform in one of four quadrants. Rat was placed in a random starting location in the pool and given 60 s to swim to the platform in each trial. The rat was allowed to stay on the platform for 15 s. If the rat failed to reach the platform within 60 s, it was guided to the platform by the experimenter and was allowed to stay on the platform for 15 s. A probe trial was conducted 24 h after the 5-day training on day 6 with the hidden platform being removed. Rats were placed in a random starting location, and allowed to swim to the former location of the hidden platform in the maze for 60 s. The latency to the platform in the acquisition trials, the number of crossings over the platform location, and time spent in the target quadrant were recorded by a computerized video tracking system.

4.10. Data collection and Statistical analysis

All images were taken with identical settings under the same conditions using a Zeiss LSM800 confocal microscope and analyzed as described [69]. Images were calibrated, and thresholds were set to ensure that all structures of interest were included in the analysis before performing analysis and counting synaptic clusters. Quantifications were performed using MetaMorph image analysis system (Molecular Devices, Inc). Data are shown as mean ± SEM. Statistical analyses were performed with T-test, one-way ANOVA, two-way ANOVA or three-way ANOVA followed by Tukey's test. All the computed *F*- and *p*-values of two-way ANOVA and three-way ANOVA are reported in the supplementary material (Table S1-S2). GraphPad Prism 8.0.2 was used for all analyses and drawing the graphs. $P < 0.05$ was defined as a significant difference.

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