**Supplementary Materials**

**Klotho regulated by estrogen plays a key role in sex differences in stress resilience**

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**Summary of Supplementary Results**

1. The specificity of Klotho (KL) antibody was validated in KL knockout mice (**Fig. S1**). This antibody was used for immunostaining in this study.
2. Expression of Klotho was regulated by endogenous estrogen (E2) in cultured hippocampal neurons. Inhibition of endogenous E2 synthesis with letrozole caused a decrease in KL protein levels, KL positive clusters and Vglut1 positive clusters along MAP2 positive dendrites in the cultured hippocampal neurons (**Fig. S2**).
3. Exogenous Estrogen (E2) increased the levels of Klotho protein expression in the hippocampus of male rats (**Fig. S3**).
4. CUMS induced a deficit in spatial learning and memory in the Barnes maze test in male but not female rats, showing females are resilient to stress (**Fig. S4**).
5. Summary of two-way ANOVA, three-way ANOVA (**Table S1-S2)**

**Supplementary methods**

Wildtype and KL knockout mice were fixed with 4% paraformaldehyde by perfusion. Fixation detail, immunostaining and western blot analysis were performed as described in the method section of the main text.

Hippocampal cultures prepared from embryonic day 18 rats of both sexes and quantification of KL positive and Vglut1 positive clusters were performed as described in the main text.

Chronic unpredictable mild stress (CUMS) in male and female rats was performed as in the main text.

The Barnes maze test was performed in an apparatus for rat as shown in our previous study [[1](#_ENREF_1), [2](#_ENREF_2)] with a minor modification. This test is generally thought to be less stressful than the [Morris water maze](https://health.uconn.edu/neuroscience/research/service-center-for-neurobehavior/#collapsepanel-4306-0-0-10) test according to plasma corticosterone levels, and more sensitive to early cognitive deficits [[3](#_ENREF_3), [4](#_ENREF_4)]. The BMT consisted of three phases: (1) habituation, (2) Training, and (3) Test. Rats were habituated to this maze by allowing to freely explore the apparatus for 4 min or until find an escape hole, once the exploration period was over, the rat was gently guided to the escape hole compartment, where it remained for one minute. The first training session was performed immediately after the habituation. During training phase, rats were trained to find an escape hole (target) for another 3 days (4 min/trial, 2 trials/day, 15-20 min apart between 2 trails). When a rat failed to find the escape hole within 4 min, a gloved hand was used to guide the rat to it. Once in the escape hole compartment, the rat was left there for 1 min before returning its home cage. The probe test (with escape box removed) was conducted on day 7 after a 72h break and carried out the same as the training trials. The latency to find the target hole, time spent in the target quadrant and the number of errors to locate the target were used to assess learning (during training) and memory (test on day 7) capabilities. Between trials, urface was cleaned with 10% alcohol and then wiped dry. All trials were recorded on a video camera.

Supplementary figure 2.tif

**Figure S1. KL antibody that was used for immunostaining in this study is specific.** Representative confocal images of a 2-month old wild-type (WT) mouse show that KL staining is localized (red) in the dendrites (white arrows) and soma of hippocampal CA1 pyramidal neurons (**A**), and KL staining in the same CA1 area of a KL knockout (KO) mouse is absent (**B**). Primary KL antibody was visualized with Cy3-labeled donkey anti-goat IgG (The Jackson Laboratory). Western blot shows that only specific KL protein bands are detected in the hippocampus of a 2-month old wild-type (WT) mouse, but not Klotho KO mouse (**C**). Western blot shows that only specific full length KL (130 kDa) band and cleavage KL band (62 kDa) are detected in the hippocampus of a 2-month old male rat (**D**). Scale bars = 20 µm.

Supplementary figure 1.tif

**Figure S2. Expression of Klotho was regulated by endogenous estrogen (E2) in cultured hippocampal neurons.** Hippocampal cultures prepared from embryonic *day* 18 rats of both sexes were treated withvehicle (control, Con), 0.1 μM letrozole (LET, aromatase inhibitor) and LET + E2 at Div 13 for 48 hours.Immunostaining of cultured neurons with antibodies specific to KL and MAP2 in Con, LET and LET+E2 treated neurons (**A**). Quantification of fluorescence intensity of KL staining in cultured hippocampal neurons (**B**).Immunostaining of cultured neurons with antibodies specific to Vglut1 and MAP2 in Con, LET and LET+E2 treated neurons(**C**).Thenumber of Vglut1 positive clusters on MAP2 positive dendrites of Con, LET and LET+E2 treated neurons (**D**)**.** Western blot analysis of KL protein in Con, LET and LET+E2 treated neurons (**E**). Data were shown as mean±SEM. One-way ANOVA followed by Tukey’s test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. Scale bars = 5 µm.

Male-klotho作图.tif

**Figure S3. Estrogen (E2) increased the relative Klotho protein expression in hippocampus of male rats.** After seven days of adaption to the laboratory conditions, male rats received subcutaneous injection of 10µg of β-Estradiol 3-benzoate dissolved in 100µl sesame oil (E2) or 100μl sesame oil only (Con) for seven consecutive days. Western blot result revealed that seven days of E2 treatment significantly increased the level of klotho protein in male hippocampus. Data were shown as mean±SEM. T-test, \**P* < 0.05, n= 6.

Supplementary figure 4.tif

**Figure S4. CUMS induced spatial learning and memory impairment in the Barnes maze test.** The escape latency in rats of both sexes on day 1-4 during training phase in Barnes maze test (**A**) and the escape latency (**B**), the time spent in the target quadrant (**C**) and number of errors (**D**) to find target hole during probe trial on day 7. Data were shown as mean±SEM. Two-way ANOVA followed by Tukey’s test. \**P* < 0.05, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001, n=7.

**Table S1.** Summary ofTwo-way ANOVA

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| Fig 2 | Parameter | shRNA x treatment | | shRNA | | treatment | |
| Fig 2E | Vglut1 positive clusters | *F*1, 21 = 4.71 | *P* =0.04 | *F*1, 21 = 324.8 | *P* <0.001 | *F*1, 21 = 24.76 | *P* <0.001 |

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| Fig 3 | Parameter | sex x stress | | sex | | stress | |
| Fig 3B | Escape latency | *F*1, 28 = 1.47 | *P* =0.2355 | *F*1, 28 = 5.88 | *P* =0.022 | *F*1, 28 = 23.51 | *P* <0.0001 |
| Fig 3C | Platform area crossings | *F*1, 28 = 10.94 | *P* =0.003 | *F*1, 28 = 3.29 | *P* =0.08 | *F*1, 28 = 3.28 | *P* =0.08 |
| Fig 3D | Time spent in target quadrant | *F*1, 28 = 9.29 | *P* =0.005 | *F*1, 28 = 4.03 | *P* =0.05 | *F*1, 28 = 12.97 | *P* =0.001 |
| Fig 3E | Sucrose preference | *F*1, 36 = 1.76 | *P* =0.19 | *F*1, 36 = 6.14 | *P* =0.02 | *F*1, 36 = 9.15 | *P* =0.005 |
| Fig 3F | Time in center | *F*1, 28 = 3.56 | *P* = 0.069 | *F*1, 28 = 32.05 | *P* <0.0001 | *F*1, 28 = 8.89 | *P* =0.006 |
| Fig 3G | Rearing | *F*1, 28 = 1.61 | *P* =0.21 | *F*1, 28 = 6.31 | *P* =0.02 | *F*1, 28 = 11.39 | *P* =0.002 |
| Fig 3H | Grooming | *F*1, 28 = 0.04 | *P* =0.84 | *F*1, 28 = 0.11 | *P* =0.74 | *F*1, 28 = 4.25 | *P* =0.05 |
| Fig3I | Total distance | *F*1, 28 = 1.72 | *P* =0.20 | *F*1, 28 = 5.96 | *P* =0.02 | *F*1, 28 = 9.98 | *P* =0.004 |
| Fig 3N | Integrated intensity of KL | *F*1, 16 = 1.71 | *P* =0.21 | *F*1, 16 = 0.34 | *P* =0.57 | *F*1, 16 = 17.83 | *P* =0.006 |
| Fig 3P | KL protein expression | *F*1, 24 = 7.13 | *P* =0.01 | *F*1, 24 = 7.13 | *P* =0.01 | *F*1, 24 = 18.16 | *P* =0.0003 |

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| Fig 4 | Parameter | sex x shRNA | | sex | | shRNA | |
| Fig 4E | Escape latency | *F*1, 28 = 0.11 | *P* =0.74 | *F*1, 28 = 5.60 | *P* =0.03 | *F*1, 28 = 9.25 | *P* =0.005 |
| Fig 4F | Platform area crossings | *F*1, 28 = 0.001 | *P* =0.97 | *F*1, 28 = 1.28 | *P* =0.27 | *F*1, 28 = 0.98 | *P* =0.33 |
| Fig 4G | Time spent in target quadrant | *F*1, 28 = 0.16 | *P* =0.69 | *F*1, 28 = 0.97 | *P* =0.33 | *F*1, 28 = 7.11 | *P* =0.01 |
| Fig 4H | Sucrose preference | *F*1, 45 = 0.04 | *P* =0.84 | *F*1, 45 = 0.23 | *P* =0.64 | *F*1, 45 = 1.67 | *P* =0.204 |
| Fig 4I | Time in center | *F*1, 45 = 0.028 | *P* = 0.87 | *F*1, 45 = 9.38 | *P* =0.0048 | *F*1, 45 = 3.18 | *P* =0.086 |
| Fig 4J | Rearing | *F*1, 45 = 0.77 | *P* =0.38 | *F*1, 45 = 6.03 | *P* =0.02 | *F*1, 45 = 0.03 | *P* =0.86 |
| Fig4K | Grooming | *F*1, 45 = 3.67 | *P* =0.06 | *F*1, 45 = 3.18 | *P* =0.08 | *F*1, 45 = 0.36 | *P* =0.55 |
| Fig4L | Total distance | *F*1, 45 = 2.45 | *P* =0.12 | *F*1, 45 = 3.14 | *P* =0.08 | *F*1, 45 = 0.01 | *P* =0.92 |

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| Fig S4 | Parameter | sex x stress | | sex | | stress | |
| Fig S4A | Escape latency | *F*1, 24 = 9.67 | *P* =0.0048 | *F*1, 24 = 15.99 | *P* =0.0005 | *F*1, 24 = 33.37 | *P* <0.0001 |
| Fig S4B | Escape latency  (Day 7) | *F*1, 24 = 8.71 | *P* =0.007 | *F*1, 24 = 0.004 | *P* =0.9515 | *F*1, 24 = 22.99 | *P* <0.0001 |
| Fig S4C | Time spent in target quadrant | *F*1, 24 = 1.53 | *P* =0.23 | *F*1, 24 = 0.68 | *P* =0.42 | *F*1, 24 = 8.32 | *P* =0.0082 |
| Fig S4D | Number of errors | *F*1, 24 = 3.294 | *P* =0.082 | *F*1, 24 = 0 | *P* >0.99 | *F*1, 24 = 20.59 | *P* =0.0001 |

**Table S2.** Summary ofThree-way ANOVA

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| ANOVA table | F | P value |
| **Fig 5B Escape latency on day5** | | |
| shRNA | *F*1, 56 = 10.99 | P = 0.0016 |
| sex | *F*1, 56 = 1.096 | P = 0.2996 |
| stress | *F*1, 56 = 25.61 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 0.1218 | P = 0.7284 |
| shRNA x stress | *F*1, 56 = 2.467 | P = 0.1219 |
| sex x stress | *F*1, 56 = 0 | P > 0.9999 |
| shRNA x sex x stress | *F*1, 56 = 0 | P > 0.9999 |
| **Fig 5C Platform area crossings** | | |
| shRNA | *F*1, 56 = 18.17 | P < 0.0001 |
| sex | *F*1, 56 = 0.08642 | P = 0.7699 |
| stress | *F*1, 56 = 26.47 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 0.0216 | P = 0.8837 |
| shRNA x stress | *F*1, 56 = 5.531 | P = 0.0222 |
| sex x stress | *F*1, 56 = 0.0216 | P = 0.8837 |
| shRNA x sex x stress | *F*1, 56 = 0.08642 | P = 0.7699 |
| **Fig 5D Time spent in the target quadrant** | | |
| shRNA | *F*1, 56 = 21.56 | P < 0.0001 |
| sex | *F*1, 56 = 0.7195 | P = 0.3999 |
| stress | *F*1, 56 = 18.75 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 0.001993 | P = 0.9645 |
| shRNA x stress | *F*1, 56 = 4.036 | P = 0.0494 |
| sex x stress | *F*1, 56 = 0.007973 | P = 0.9292 |
| shRNA x sex x stress | *F*1, 56 = 0.007973 | P = 0.9292 |
| **Fig 5E Sucrose consumption in the SPT** | | |
| shRNA | *F*1, 56 = 7.683 | P = 0.0084 |
| sex | *F*1, 56 = 0.4136 | P = 0.5238 |
| stress | *F*1, 56 = 27.09 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 0.06441 | P = 0.8010 |
| shRNA x stress | *F*1, 56 = 4.096 | P = 0.0497 |
| sex x stress | *F*1, 56 = 0.1828 | P = 0.6712 |
| shRNA x sex x stress | *F*1, 56 = 0.909 | P = 0.3461 |
| **Fig 5F Time in center** | | |
| shRNA | *F*1, 56 = 53.66 | P < 0.0001 |
| sex | *F*1, 56 = 16.61 | P = 0.0001 |
| stress | *F*1, 56 = 38.05 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 12.82 | P = 0.0007 |
| shRNA x stress | *F*1, 56 = 6.143 | P = 0.0162 |
| sex x stress | *F*1, 56 = 0 | P > 0.9999 |
| shRNA x sex x stress | *F*1, 56 = 4.153 | P = 0.0463 |
| **Fig 5G Rearing times** | | |
| shRNA | *F*1, 56 = 0.8362 | P = 0.3660 |
| sex | *F*1, 56 = 0.0474 | P = 0.8288 |
| stress | *F*1, 56 = 17.11 | P = 0.0002 |
| shRNA x sex | *F*1, 56 = 1.822 | P = 0.1846 |
| shRNA x stress | *F*1, 56 = 6.6 | P = 0.0140 |
| sex x stress | *F*1, 56 = 0.01706 | P = 0.8967 |
| shRNA x sex x stress | *F*1, 56 = 0.001896 | P = 0.9655 |
| **Fig 5H Grooming** | | |
| shRNA | *F*1, 56 = 0.6906 | P = 0.4109 |
| sex | *F*1, 56 = 0.02762 | P = 0.8688 |
| stress | *F*1, 56 = 26.55 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 0.2486 | P = 0.6208 |
| shRNA x stress | *F*1, 56 = 4.669 | P = 0.0368 |
| sex x stress | *F*1, 56 = 0.02762 | P = 0.8688 |
| shRNA x sex x stress | *F*1, 56 = 0.02762 | P = 0.8688 |
| **Fig 5I Total distance travelled** | | |
| shRNA | *F*1, 56 = 22.3 | P < 0.0001 |
| sex | *F*1, 56 = 15.8 | P = 0.0003 |
| stress | *F*1, 56 = 18.96 | P < 0.0001 |
| shRNA x sex | *F*1, 56 = 3.813 | P = 0.0579 |
| shRNA x stress | *F*1, 56 = 10.57 | P = 0.0023 |
| sex x stress | *F*1, 56 = 0.004789 | P = 0.9452 |
| shRNA x sex x stress | *F*1, 56 = 0.02266 | P = 0.8811 |

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