Life Extension Factor Klotho Regulates Behavioral Responses to Stress via Modulation of GluN2B Function in the Nucleus Accumbens

Running title: Klotho regulates behavioral responses to stress

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Supplemental Materials and Methods

Chronic Social Defeat Stress (CSDS)

Mice were acclimated to the facility for 3 days before the study and then subjected to CSDS. Male CD1 mice that were prescreened for a threshold level of aggressive behavior were singly housed and used to defeat the C57 mice. A male C57 mouse was placed in a cage with perforated clear dividers containing an aggressive resident male mouse (CD1). The C57 mouse was physically attacked by a resident for 10 min and then housed opposite the resident on the other side of the divider for 24 h of sensory interaction. This process was repeated daily for 10 consecutive days with a different CD1 aggressor each day. On Day 11, the defeated mouse was subjected to a social interaction test and sorted into either a susceptible or unsusceptible (resilient) phenotype based on the social interaction score.

Sucrose Preference Test (SPT)

The SPT was carried out as previously described [1]. During the first 2 days, mice were individually exposed to two bottles, one containing pure water and one containing 1% sucrose solution. To prevent the potential influence of bottle location, the bottles were counterbalanced across the right and left sides of the home cage during the two-day procedure. On the 3rd day, the mice were deprived of both the food and the two bottles. On the 4th day, the test was conducted and lasted for 6 h, with the two bottles weighed before and after the test. The preference for the sucrose solution was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed. The relative amount of sucrose consumed was calculated using the following formula: sucrose preference rate (%) = sucrose consumption/(water consumption + sucrose consumption).

Forced Swimming Test (FST)

In this test, each mouse was placed in a cylinder (diameter 23 cm; height 70 cm) filled with water at a constant temperature of 25 ± 1 °C at 30 cm of height and had to remain in the cylinder for 6 min. The duration of immobility was recorded during the last 4 min of the 6-min test period by an investigator blinded to the study. The

immobility time of the mouse was defined as the time spent floating in the water without struggling, except for those movements necessary to keep its head above the water.

Tail Suspension Test (TST)

In this test, the mouse was suspended 60 cm above the floor using adhesive tape, placed approximately 1 cm from the tip of the tail. All of the mice were suspended for 6 min, and the immobility time during the last 4 min was recorded by an investigator blinded to the study. The mice were regarded as immobile only when they hung passively and were completely motionless. Any mice that did climb their tails were removed from the experimental analysis.

Open Field Test (OFT)

The open field was made of white wood (50×50×25 cm). The central zone was defined as the 25×25 cm interior portion of the box. Mice were individually placed in the center of the apparatus to freely explore for 5 min. Movement was recorded and analyzed by ANY-maze tracking software (ANY-maze, Wood Dale, IL). The open field arena was thoroughly cleaned with 75% ethanol after each trial.

Viral Vectors and Stereotaxic Surgery

Alpha-Klotho was knocked down or overexpressed using an adeno-associated virus (AAV) vector delivery system. Vectors carrying a short hairpin RNA targeting Klotho (AAV9-shRNA) or Klotho (AAV9-Klotho) were designed and chemically synthesized by GeneChem Co., Ltd. (Shanghai, China). A pAAV-U6-shRNA-CMV bGlobin-EGFP-3Flag and an hSyn promoter-EGFP-MCS-SV40 PolyA vector were used for AAV-shRNA and AAV-Klotho packaging, respectively. For AAV9-shRNA packaging, the sequence of the shRNA primer was 5’-GCATCACAGTAAGAGGCTT-3’. A scrambled sequence (5’-CGCTGAGTACTTCGAAATGTC-3’) was used as a sham control. These vectors also expressed enhanced green fluorescent protein (EGFP). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then placed in a stereotaxic frame (RWD Co, China). The body temperature was kept constant (37 °C) with an insulating blanket during surgery. A midline skin incision was made to

expose the dorsal surface of the skull and a 1.0-mm hole was drilled in the skull. Then, 1.0 μl of virus (1.45*10^12 vg/mL AAV9-shRNA or 1.16*10^12 vg/mL AAV9-Klotho) was delivered to each hemisphere of the NAc (AP: +1.2 mm, ML: ±1.0 mm, DV: -4.6 mm) at a rate of 0.5 μl/min. The needle was left in the NAc for 10 min before withdrawal to ensure complete diffusion of the virus. Finally, the incision was closed with nylon sutures. Mice were kept warm with a heat lamp for at least 24 h after surgery. Behavioral experiments were conducted 2 weeks after viral injection to allow gene expression.

Surgeries for Intra-NAc Injection with GluN2B Antagonist

Mice were anesthetized and placed in a stereotaxic frame. Two 22-gauge guide cannulas (stainless steel) were bilaterally implanted into the NAc. Mice were allowed to recover for 14 days before behavioral testing. On the day of the experiment, the injection cannula was connected via PE20 tubing to a 5 μl microsyringe driven by a microinjection pump. A GluN2B antagonist was infused into the NAc in freely moving mice, with a volume of 0.5 μl per side. Following injection, the injection cannula was left for an additional 3 min before withdrawal to minimize dragging of injected liquid along the injection track. Standard histological examination was conducted after the experiments to verify the injection sites.

Western Blot Analysis

The experiments were carried out according to protocols described in a previous study [2]. Briefly, NAc tissue was homogenized in homogenization buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 1% NP-40, 20 mM NaF, 10 mM EDTA, 1 mM PMSF, 3 mM Na_3VO_4, and a protease inhibitor mixture (BOSTER; PROTP17931). The protein concentrations were determined using a BCA assay. The samples were boiled for 5 min after mixing with sample buffer and then stored at -80 °C. The surface expression of NMDAR subunits was detected using a bis (sulfosuccinimidyl) suberate (BS^3) protein cross-linking assay. Briefly, the NAc tissues were added to Eppendorf tubes that were filled with artificial cerebrospinal fluid (ACSF) and immediately spiked with BS^3 (2 mM). The reaction was stopped by addition of glycine (100 mM) to tubes after the tissue was cross-linked for 60 min at 4 °C. The

tissue was collected after brief centrifugation and rapidly homogenized via sonication for 5 s in ice-cold lysis buffer containing protease and phosphatase inhibitors. Then, the tubes were centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatant fraction was stored at -80 °C before electrophoresis.

For western blotting, the proteins were transferred onto nitrocellulose membranes after they were separated via 8% SDS-PAGE. The membrane was blocked in 5% BSA in TBST for 1 h at room temperature and sequentially incubated with anti-GluN1 (Abcam; ab109182), anti-GluN2A (Abcam; ab124913), anti-GluN2B (Abcam; ab65783), anti-PSD-95 (Abcam; ab238135), anti-Klotho (Abcam; ab181373) or anti-β-actin (Santa Cruz; sc-8432) antibody overnight at 4 °C. After washing with TBST, the immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by detection via enhanced chemiluminescence. The protein bands were quantitated using ImageJ software.

Real-Time Quantitative RT-PCR

Twenty-four hours after the last behavior tests, the mice were sacrificed by decapitation, and total RNA was extracted from NAc tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Using a PrimeScript First Strand cDNA Synthesis Kit (Takara Biotechnology, China), we performed cDNA synthesis. The cDNA was amplified via PCR using standard methods. The following specific primers were used: klotho primers (forward: 5-CAGCGATAGTTACAACAACGTC-3, reverse: 5-GATATGGAGAAGCGGTAGTGG-3); GluN2A primers (forward: 5-GAACGCGAACTTCGAAATCTG-3, reverse: 5-GTCAGTGCGGTTCATCAATAAC-3); GluN2B primers (forward: 5-AAGAAGAATCGGAACAAACTGC-3, reverse: 5-CAGCTGGCATCTCAAACATATG-3); PSD-95 primers (forward: 5-ATGTGCTTCATGTAATTGACGC-3, reverse: 5-TTTAACCTTGACCACTCTCGTC-3); and β-actin primers (forward: 5-TTCCTTCCTGGGTATGGAAT-3, reverse:

5-GAGGAGCAATGATCTTGATC-3). Each group was analyzed in triplicate, and β-actin was used as an internal reference. The fluorescence signals were collected during the extension stage, Ct values of the sample were calculated, and data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Electrophysiological Recordings

The brain of the mouse was quickly removed, and NAc slices (400 μm) were made with a Vibrate tissue slicer (VT 1000S; Leica) in ice-cold ACSF containing (mM) 119 NaCl, 3.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 141 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, bubbled continuously with 95% O_2/5% CO_2 (pH 7.4). These slices were allowed to recover for at least 1.5 h in oxygenated ACSF at 30 °C. Then, a single slice was transferred to the perfusion-type recording chamber and continuously superfused with oxygenated ACSF at 30 °C at a rate of 3 ml/min. Picrotoxin (50 μM) was included to block the inhibitory postsynaptic currents mediated by $GABA_A$ receptors during all recordings. Cortico-accumbal afferents were stimulated by delivering stimuli through a bipolar tungsten stimulation electrode placed at the prefrontal cortex (PFC) near the PFC-NAc border 0.5-3 mm dorsal to the recording electrode in the NAc [1]. Field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass pipette filled with 3 M NaCl. The frequency of stimulation to evoke fEPSPs was 0.03 Hz. LTD was induced by a stimulating protocol that consisted of one train of stimulus at 1 Hz (15 min) after 10 min of stable baseline recording. The input-output (I/O) relationship for synaptic transmission was recorded by varying the intensity of the single-pulse stimulation. Paired stimuli (25, 50, 75, and 100 ms intervals) were delivered, and the paired-pulse ratio (PPR) was calculated as the ratio of the second fEPSP over the first fEPSP.

Whole-cell currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA). Data were filtered at 2 kHz and digitized at 5 Hz using a Digidata 1440A DAC unit. Electrodes (4-6 M) were filled with 140 mM CsF, 10 mM HEPES, 11 mM EGTA, 2 mM TEA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 4 mM K_2ATP (pH 7.3; 290 mOsm). During each experiment, a voltage step of -10 mV from the

holding potential was applied periodically to monitor cell capacitance and access resistance. Recordings in which access resistance or capacitance changed by > 10% were not included in the data analysis. Medium spiny neurons were identified by their morphology and high resting membrane potential (-75 to -85 mV). EPSCs were evoked at 0.05 Hz. NMDAR-mediated EPSCs were pharmacologically isolated at +40 mV in the presence of 10 μM NBQX and blocked with 50 μM AP5.

Golgi-Cox Staining and Spine Density Analysis

Six mice were randomly chosen from each group and used for Golgi-Cox staining after behavioral tests. Golgi-Cox staining is an established method for studying the morphology of neuronal dendrites and dendritic spines [4]. The protocol consists of three major steps: preprocessing, staining and slide preparation. For preprocessing, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then intracardially perfused with 0.9% saline. After perfusion, the brain was fixed in bottles filled with 4% paraformaldehyde (PFA) for 1 h at room temperature. Following fixation, the brain was transferred to Golgi-Cox staining solution for 48 h at 37 °C. The solution was changed every 24 h. Then, the brain was placed in protecting solution (300 g/l sucrose in ddH_2O) at 4 °C in the dark for 4-6 days to protect brain tissues. Tissues were mounted with 1.5-2% gelatin and cut into 150 μm sections with a Vibrate tissue slicer (VT 1000S; Leica). After washing twice with ddH_2O for 5 min each, the brain slices were incubated with 25% ammonia solution in the dark for 30 min with gentle shaking. Then, the slices were washed again with ddH_2O and kept in 5% sodium thiosulfate in the dark for 30 min. Finally, the slices were dehydrated with 70%, 90% and 100% ethanol for 6 min each and defatted with xylene for 6 min to reduce the background.

Measurements of spines were performed on apical and basal dendrites in each region, at least 50 μm away from the soma for the apical dendrites and at least 30 μm away for the basal dendrites, on secondary and tertiary branches. These distances allowed us to exclude dendritic segments near the soma that are essentially devoid of spines. For each neuron, 20-μm long segments were randomly selected on apical and basal dendrites within a distance of at most 100 μm from the limit of the exclusion

zone. Counting was performed under 1000 × magnification using an oil immersion objective. Spines were counted manually by a researcher blinded to the experimental conditions. For quantification of total spines, 24 dendritic segments (20 μm) were analyzed (4 neurons per mouse, n = 6 mice per group).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) and SPSS 25 (IBM, Armonk, NY, USA) software. Data are expressed as the means ± SEM. We applied the PauTa criterion, which regarded data out of the mean ± 3 SD as outliers, to identify and exclude outliers. A Shapiro-Wilk test was used to assess the normality of the data and the test revealed that all data were normally distributed ($p > 0.05$). Thus, the data were statistically analyzed using a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA). *Post hoc* comparisons were performed using Bonferroni's or Dunnett's T3 tests based on the presence of equal or unequal variance in the groups, as shown by Levene's test. When $p < 0.05$, the difference was considered statistically significant.

References

1. Jiang B, Wang W, Wang F, Hu ZL, Xiao JL, Yang S *et al.* The stability of NR2B in the nucleus accumbens controls behavioral and synaptic adaptations to chronic stress. *Biol Psychiatry* 2013; **74**(2)**:** 145-155.

2. Boudreau AC, Wolf ME. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci* 2005; **25**(40)**:** 9144-9151.

3. Liu XY, Chu XP, Mao LM, Wang M, Lan HX, Li MH *et al.* Modulation of D2R-NR2B interactions in response to cocaine. *Neuron* 2006; **52**(5)**:** 897-909.

4. Yang M, Luo CH, Zhu YQ, Liu YC, An YJ, Iqbal J *et al.* 7, 8-Dihydroxy-4-methylcoumarin reverses depression model-induced depression-like behaviors and alteration of dendritic spines in the mood circuits. *Psychoneuroendocrinology* 2020; **119:** 104767.

Supplemental Data

Supplemental Figure 1

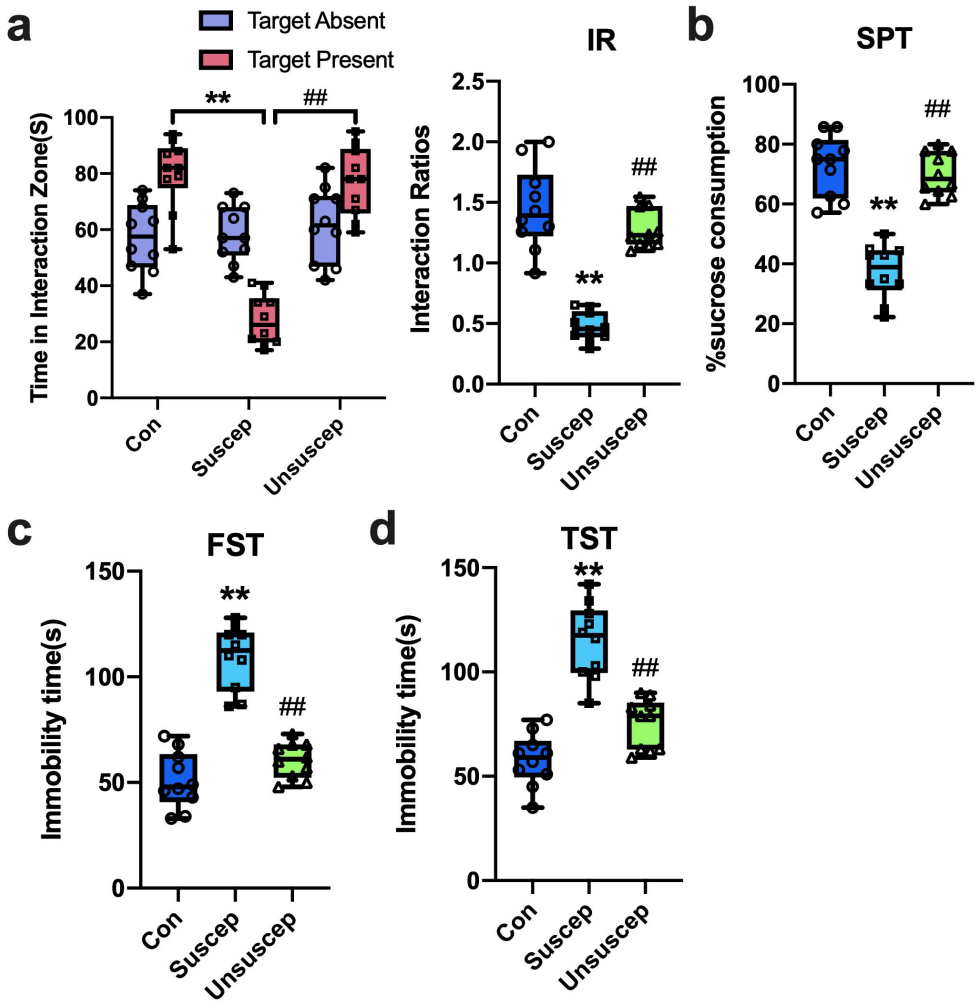

Figure S1. Chronic social defeat stress （CSDS） induces behavioral alterations relevant to depression in mice. (a) Mice exposed to CSDS were designated as susceptible or unsusceptible group based on time in interaction zone and social interaction ratios. (b) Susceptible mice displayed obvious behavioral alterations relevant to depression, manifested as anhedonia in SPT (n = 10 mice per group). (c, d) Susceptible mice displayed obvious behavioral alterations relevant to depression, manifested as helplessness in FST (c) and TST (d) (n = 10 mice per group). For a-d, all box and whisker plot displays the median, first and third quartiles (boxes), and the min-max (whiskers). $^{**}p < 0.01$ *vs.* control. $^{\#\#}p < 0.01$ *vs.* susceptible group.

Supplemental Figure 2

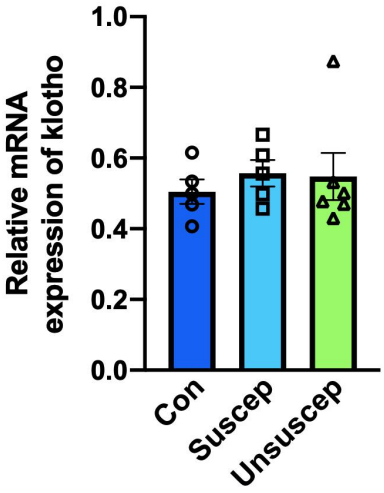

Figure S2. The level of klotho mRNA in the NAc of mice from each group. The expression of klotho mRNA in the NAc of susceptible mice did not differ from that in control or unsusceptible mice. Data were presented as normalized mean ± SEM.

Supplemental Figure 3

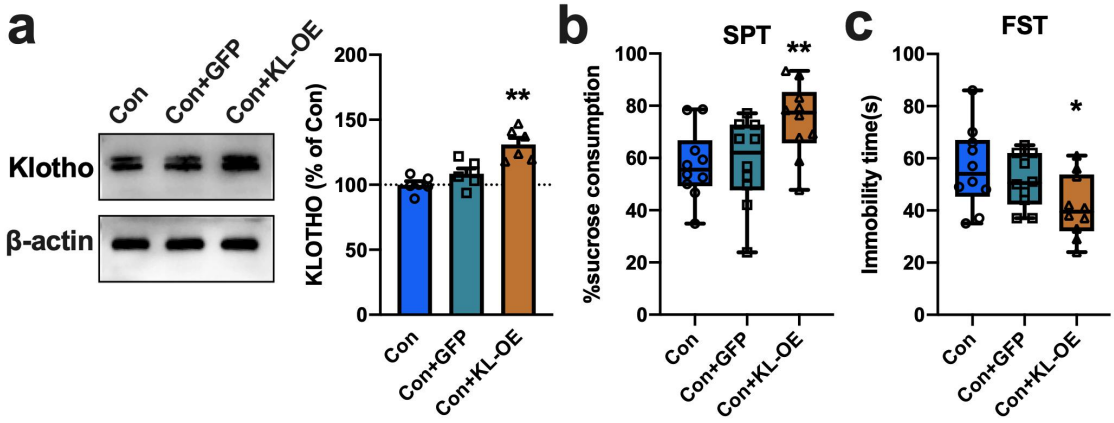

Figure S3. Genetic overexpression of klotho in the NAc exerts an antidepressive effect in normal mice. (a) Representative western blotting images and histograms showing the protein expression of klotho in the NAc in each group (n = 6 mice per group). (b, c) KL-OE in the NAc significantly increased the sucrose preference (b) and decreased the immobility time in FST (c) in mice. For a, data were presented as normalized mean ± SEM. For b and c, all box and whisker plot display the median,

first and third quartiles (boxes), and the min-max (whiskers) (n = 8 mice per group). $^{*}p < 0.05$ *vs*. control group; $^{**}p < 0.01$ *vs*. control group.

Supplemental Figure 4

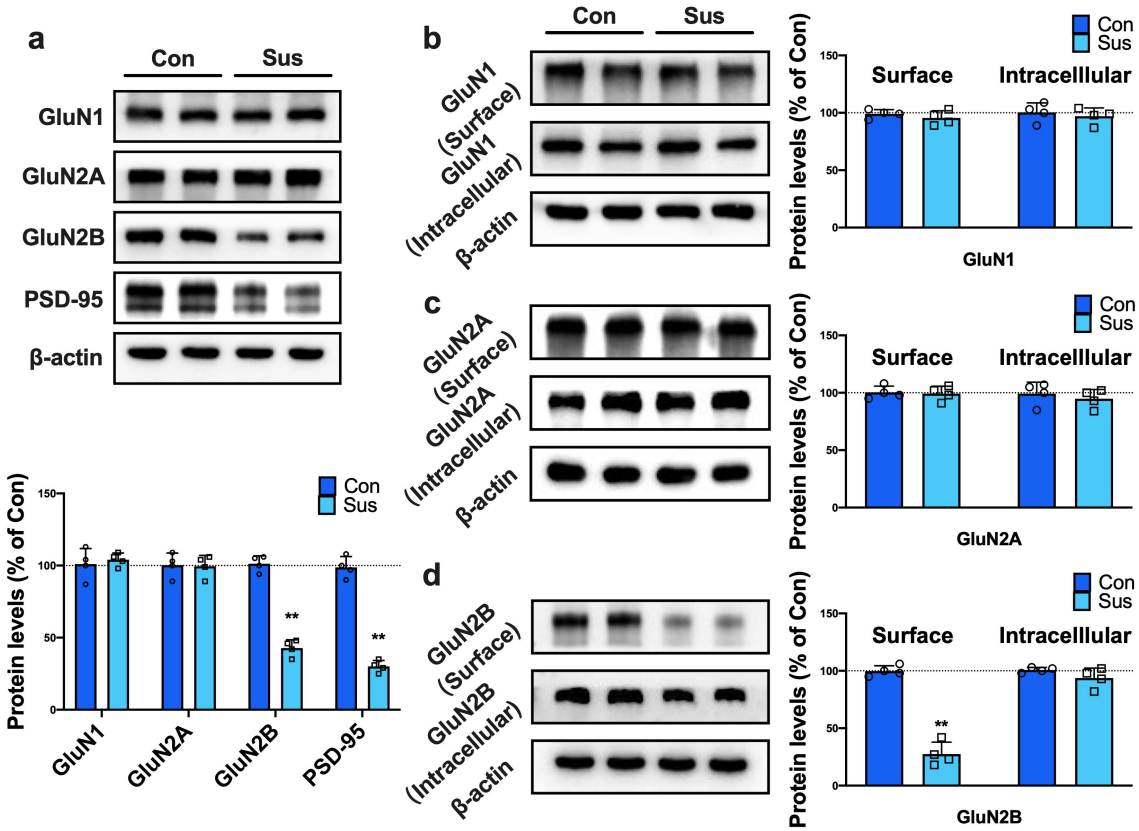

Figure S4. CSDS decreases the total and surface GluN2B expressions in NAc of susceptible mice. (a) Representative images of western blotting. Protein extracts from the NAc of susceptible mice were analyzed by western blotting 14 days after CSDS. There was no difference in the total proteins of GluN1 and GluN2A between control and susceptible group, whereas the total GluN2B and PSD-95 proteins in NAc of susceptible mice were significantly decreased. (b-c) Western blotting analysis revealed that CSDS did no affect the expressions of GluN1 (b) and GluN2A (c) in both the surface and intracellular pools in NAc of susceptible mice. (d) CSDS significantly reduced the expression of GluN2B in the surface pool but not in the intracellular pool. All data were presented as normalized mean ± SEM. n = 4 mice per group. $^{**}p < 0.01$ vs. control.

Supplemental Figure 5

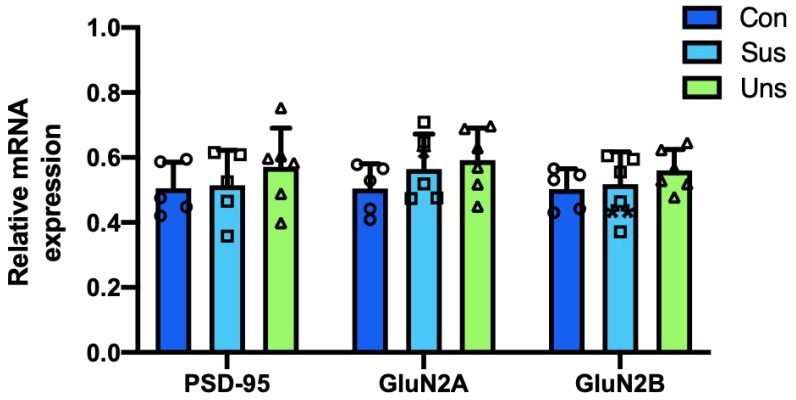

Figure S5. The level of GluN2A, GluN2B and PSD-95 mRNA in the NAc of mice from each group. The mRNA level of GluN2A, GluN2B or PSD-95 in the NAc of susceptible mice was not different from that in control or unsusceptible mice. Data were presented as normalized mean ± SEM.

Supplemental Figure 6

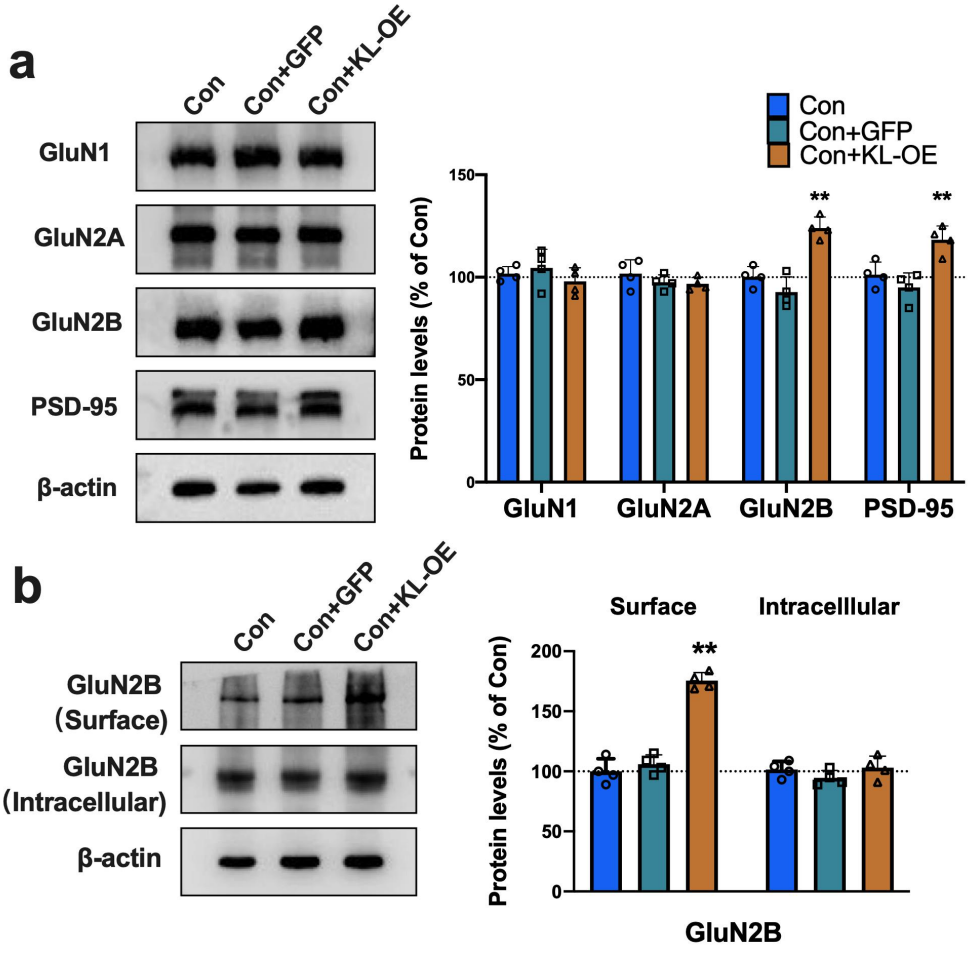

Figure S6. Genetic overexpression of accumbal klotho upregulates the total and surface expression of GluN2B in normal mice. (a) Overexpression of accumbal klotho did not affect the total GluN1 and GluN2A protein levels, while it significantly increased the total GluN2B and PSD-95 protein expression in NAc (n = 4 mice per group). (b) Western blotting analysis revealed that genetic overexpression of accumbal klotho significantly increased GluN2B expression in the surface pool but not in the intracellular pool (n = 4 mice per group). All data were presented as normalized mean ± SEM. $^{**}p < 0.01$ *vs*. control group.

Supplemental Figure 7

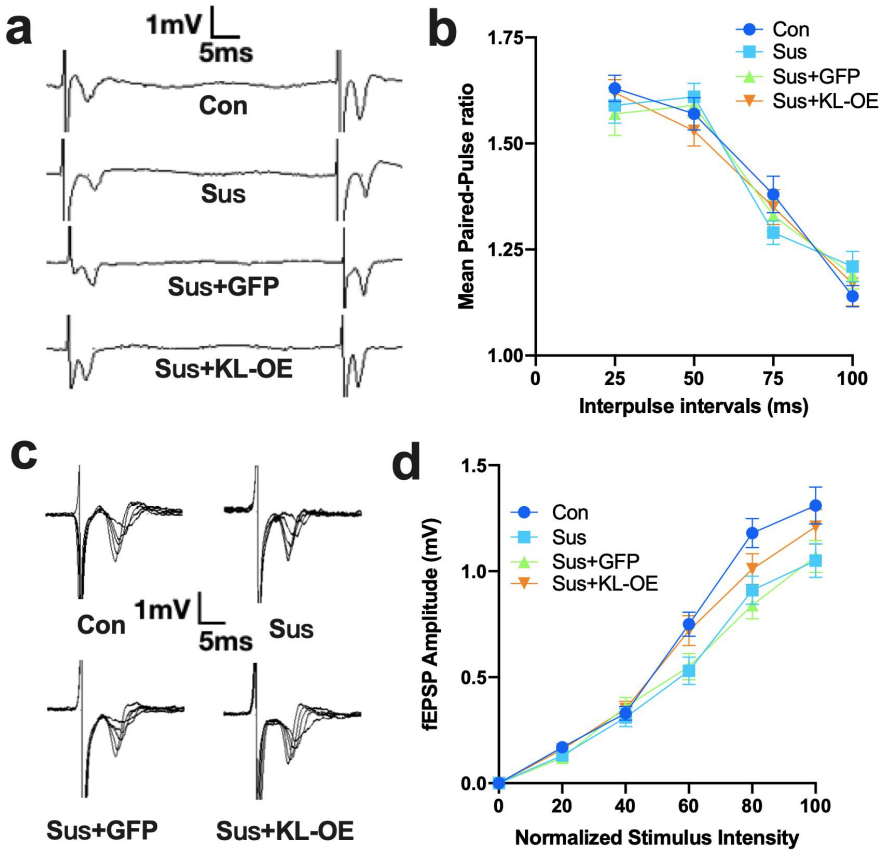

Figure S7. Genetic overexpression of accumbal klotho reverses the decreased basal synapse transmission but had no effect on presynaptic function. (a) The typical field excitatory postsynaptic potentials (fEPSPs) show respective recordings from example experiments at 50 msec interpulse interval. (b) Paired-Pulse ratios (slope of fEPSPs no.2/slope fEPSPs no.1) was measured by varying the intervals between pairs of

stimuli and no difference was found among groups (n = 5 mice per group). (c) Cortico-accumbal field potential recordings of acute brain slices show the typical superimposed fEPSPs by increasing stimulation intensity. (d) Input-output curves illustrating the relationship between the magnitudes of stimulation and evoked response for fEPSPs recorded from each group. Compared to control group, CSDS susceptible mice displayed lower responses to stimulations and overexpression of accumbal klotho reversed the downregulation of input-output curves in these mice (n = 5 per group).