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Preventive effect of a Kampo medicine, kososan, on recurrent depression in a mouse model of repeated social defeat stress

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ABSTRACT

Depression is deemed a mood disorder characterized by a high rate of relapse. Therefore, overcoming of the recurrent depression is globally expecting. Kososan, a traditional Japanese herbal medicine, has been clinically used for mild depressive mood, and our previous studies have shown some evidence for its antidepressive-like efficacy in experimental animal models of depression. However, it remains unclear whether kososan has beneficial effects on recurrent depression. Here, we examined its effect using a mouse model of modified repeated social defeat stress (SDS) paradigm. Male BALB/c mice were exposed to a 5-min SDS from unfamiliar aggressive CD-1 mice for 5 days. Kososan extract (1.0 kg/kg/day) or an antidepressant milnacipran (60 mg/kg/day) was administered orally for 26 days (days 7–32) to depression-like mice with social avoidant behaviors on day 6. Single 5 min of SDS was subjected to mice recovered from the social avoidance on day 31, and then the recurrence of depression-like behaviors was evaluated on day 32. Hippocampal gene expression patterns were also assayed by DNA microarray analysis. Water- or milnacipran-administered mice resulted in a recurrence of depression-like behaviors by re-exposure of single SDS, whereas kososan-administered mice did not recur depression-like behaviors. Distinct gene expression patterns were also found for treating kososan and milnacipran. Collectively, this finding suggests that kososan exerts a preventive effect on recurrent depression-like behaviors in mice. Pretreatment of kososan is more useful for recurrent depression than that of milnacipran.

1. Introduction

Depression is a highly prevalent psychiatric disorder characterized by various symptoms such as apathy, loss of interests, anhedonia, anorexia, and insomnia. Long-lasting depression causes many harmful events including suicide (Kuo et al., 2015), exacerbation of basic diseases (Barnard et al., 2013), and temporary retirement (Paunio et al., 2014). It has been concerned that these events would lead to lower the quality of life and vast economic loss. Therefore, overcoming depression is a major task for public mental health in the world. Numerous studies have shown that antidepressants exert their therapeutic benefits via modulation of multiple neurotransmitters (Delgado and Moreno, 2000; Harmer et al., 2017). Notably, ketamine, a N-methyl-D-aspartate glutamate receptor antagonist, is expected as a rapid and sustained

Abbreviations: SDS, social defeat stress; IFN-α, interferon-α; mRSDS, modified repeated social defeat stress; SAT, social avoidance test; SI, social interaction; DAVID, Database for Annotation, Visualization and Integrated Discovery; ANOVA, analysis of variance; Ttr, transthyretin; Zic1, Zinc finger protein of the cerebellum 1; Enpp2, Ectonucleotide pyrophosphatase/phosphodiesterase 2; Tnnt1, Troponin T1, skeletal, slow; Homer1, Homer homolog 1; Scai, Suppressor of cancer cell invasion; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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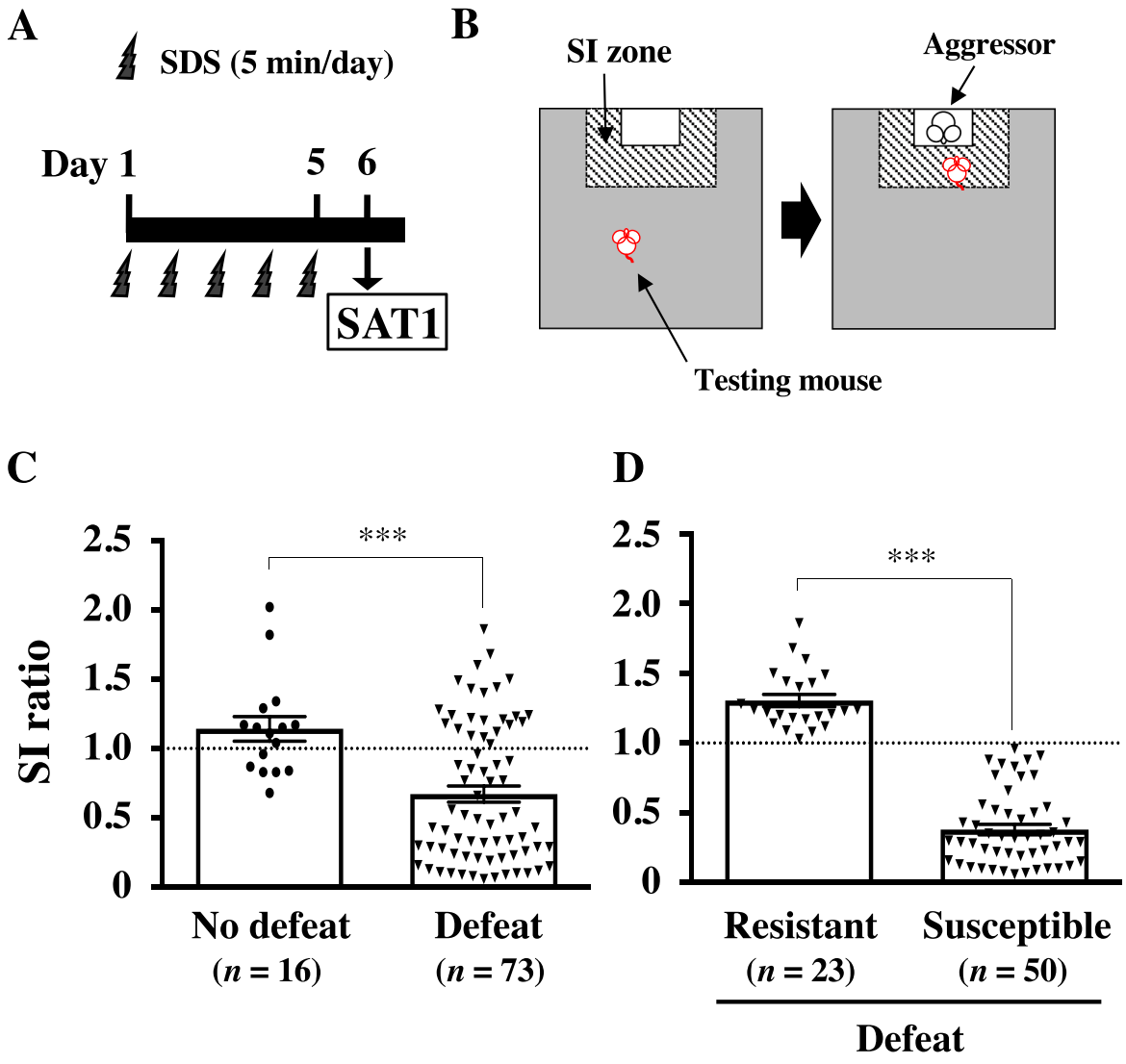


Fig. 1. Selection of mice susceptible to social defeat stress. (A) A schematic diagram of the experimental schedule for mRSDS exposure and 1st SAT. (B) A schematic procedure of the SAT. (C) SI ratio of undefeated and defeated mice in the 1st SI. (D) SI ratio of mice resistant and susceptible to mRSDS among the defeated mice. Each column represents the mean ± SEM. Dots depict individual data of mice. ***$P < 0.001$ (unpaired *t*-test). mRSDS, modified repeated social defeat stress; SAT, social avoidance test; SI, social interaction.

antidepressant against patients with depression (Berman et al., 2000; Berton and Nestler, 2006) and treatment-resistant depression (Fava et al., 2020; Singh et al., 2016; Zarate et al., 2006).

One of the issues to overcome in the pathology of depression is that depression is easy to relapse and recur (Nahas et al., 2005; Schlaepfer et al., 2008). A large proportion of patients with depression experience recurring episodes after remission (Holtzheimer and Mayberg, 2011; Liu et al., 2019). Indeed, high relapse rates (34–83%) were seen over six months in patients with depression (Rush et al., 2006), which has been recognized as an urgent issue that should be resolved. Although long-lasting antidepressant medications (Gelenberg et al., 2003; Keller et al., 2007) and cognitive therapy (Hollon et al., 2005) may be effective for decreasing relapse rates, it remains difficult to treat recurrent depression.

Kososan (Xiang-Su-San in Chinese), a Kampo (traditional Japanese herbal) medicine, is clinically used to treat depressive mood disorders in addition to the initial stage of the common cold, allergic urticaria due to the ingestion of food, irritable bowel syndrome, chronic fatigue syndrome, insomnia, and autonomic imbalance in Japan. Clinical evidence has also shown that kososan attenuates depressive mood triggered by interferon-α (IFN-α) therapy for hepatitis C (Hanawa, 1995). In our previous animal studies, kososan treatment attenuated the depression-like behaviors of chronic mild stress-exposed or IFN-α-injected mice by regulating the dysfunction of the hypothalamic–pituitaryadrenal axis, a region strongly associated with the pathogenesis of depression (Ito et al., 2006; Nagai et al., 2008), mediating the orexin/neuropeptide Y signaling system (Ito et al., 2012; Ito et al., 2009), and modulating metabotropic glutamate receptor 2 and 2′,3′-cyclic nucleotide 3′-phosphodiesterase 1 (Nagai et al., 2015). Psychological stress-induced depression-like behaviors in mice were also mitigated by treatment with kososan, but not antidepressant milnacipran (Hori et al., 2015), a serotonin noradrenaline reuptake inhibitor. Furthermore, we reported that kososan prevented social avoidant behaviors, presumably through alleviating neuroinflammation in mice exposed to chronic social defeat stress (SDS) (Ito et al., 2017). These findings propose a promising efficacy of kososan on depression, but little is known whether kososan exhibits beneficial effects on recurrent depression. Additionally, animal models available for assessing recurrent depression is lacking.

In this study, a mouse model for evaluating the recurrent depression was established using modified repeated SDS (mRSDS) (Ito et al., 2020). And then, we assessed whether kososan treatment rescues recurrent depression in the mRSDS model. Moreover, since the hippocampus in

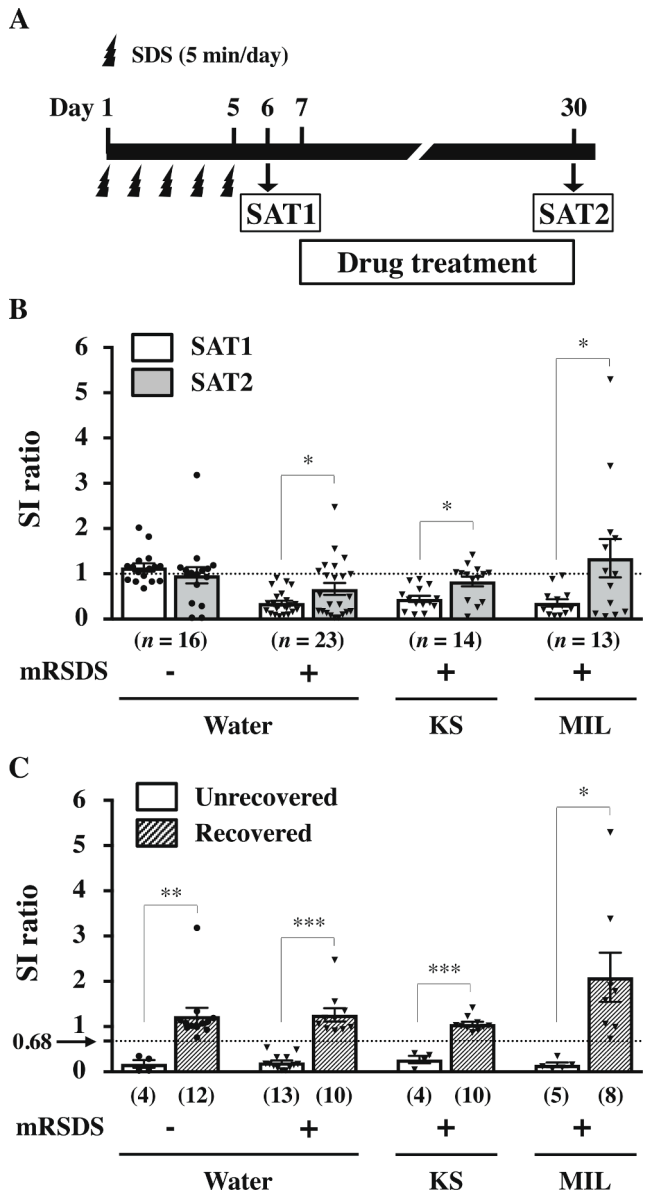


Fig. 2. Effects of drug treatment on mRSDS-induced social avoidance and selection of mice recovered from the social avoidant behaviors. (A) A schematic diagram of the experimental schedule for mRSDS exposure, 1st and 2nd SAT, and drug treatment. (B) SI ratio of drug-treated mice susceptible to mRSDS. (C) SI ratio of mice recovered and unrecovered from the social avoidant behaviors in the 2nd SAT. Numbers in the parentheses represent the number of mice. Each column represents the mean ± SEM. Dots depict individual data of mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (B, paired *t*-test; C, unpaired *t*-test). KS, kososan; MIL, milnacipran; mRSDS, modified repeated social defeat stress; SAT, social avoidance test; SI, social interaction.

the brain is well known to be closely linked to pathology of depression, hippocampal genes correlated with kososan's effect were examined by DNA microarray analysis.

2. Materials and methods

2.1. Animals

Adult male BALB/c (7 weeks of age) and CD-1 (retired breeders) mice were purchased from Japan SLC (Hamamatsu, Japan). All animals were allowed to acclimate for at least 1 week after arrival. BALB/c mice were housed for 4 mice per cage, and CD-1 mice were housed individually during acclimation under a constant condition (temperature, 23 ± 2 °C; humidity, 55% ± 10%; 12-h light/dark cycle with lights on at 8:00) with food (CE-2, CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. All cages (22.5 × 33.8 × 14 cm, CLEA Japan, Inc.) were provided with wood bedding material (Japan Laboratory Animals, Inc., Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan. Every effort was made to minimize the number of animals used and their suffering.

2.2. Drugs

The herbs in kososan were as follows: Cyperi Rhizoma (the rhizome of *Cyperus rotundus* L.), 4.0 g (Lot No. AE7951, Tsumura & Co., Tokyo, Japan); Perillae Herba (leaf of *Perilla frutescens* Britton var. *acuta* Kudo), 2.0 g (Lot No. B04401, Tsumura & Co.); Aurantii Nobilis Pericarpium (pericarp of *Citrus unshiu* Markovich), 3.0 g (Lot No. AD7971, Tsumura & Co.); Glycyrrhizae Radix (root of *Glycyrrhiza uralensis* Fisher), 2.0 g (Lot No. 8661621, Uchida Wakan-yaku Co. Ltd., Tokyo, Japan) and Zingiberis Rhizoma (rhizome of *Zingiber officinale* Roscoe), 0.5 g (Lot No. AK8761, Tsumura & Co.). Kososan was decocted with 600 ml of distilled water until the volume was reduced by half. The water extract was immediately filtered, centrifuged at 1000×*g* for 10 min at 4 °C, and the supernatant was lyophilized. Total yield of kososan extract was approximately 28% from the herbal mixture based on dry weight (Hori et al., 2015; Ito et al., 2017; Ito et al., 2012; Ito et al., 2006). Milnacipran hydrochloride was purchased from Ashahi Kasei Pharma Corporation (Tokyo, Japan).

2.3. Drug treatment and measurement of body weight

The kososan extract or milnacipran was suspended in distilled water. Kososan extract (1.0 g/kg), milnacipran (60 mg/kg) or distilled water was administered orally via gastric gavage once daily for 26 days. The dose of kososan extract and milnacipran used in this study was chosen based on our previous findings that these drugs exhibited antidepressant-like effects in stress-induced depression-like model mice (Berton and Nestler, 2006; Ito et al., 2017; Ito et al., 2009).

Body weight was measured on days 1, 6, and 30 prior to stress exposure, drug treatment, and behavioral testing.

2.4. Repeated social defeat stress

Repeated social defeat stress paradigm was performed as previously described (Ito et al., 2020). Briefly, testing BALB/c mice were exposed to unfamiliar resident CD-1 aggressor mice in their home cage for 5 min daily over 5 consecutive days (days 1–5, Fig. 1A). After 5 min of confrontation, testing mice were housed in their home cages for 24 h with free access to food and water. On each stress exposure, testing mice were defeated by novel aggressor mice to avoid acclimation to familiar aggressors. Undefeated control mice were handled every day, but they were never exposed to aggressors.

2.5. Social avoidance test

Social avoidance test was performed as previously described (Golden et al., 2011; Ito et al., 2017; Ito et al., 2020). Briefly, each mouse was introduced into an opaque grey open field box (40 × 40 × 40 cm) with an empty perforated Plexiglas enclosure (7 × 10 × 40 cm) located in the social interaction (SI) zone (13.5 × 24.0 cm) at one end of the box and was allowed to explore freely for 150 s (Fig. 1b). The mouse was then removed from the box, and 1 min later, the mouse was re-introduced into the box with an unfamiliar aggressor and was allowed to explore

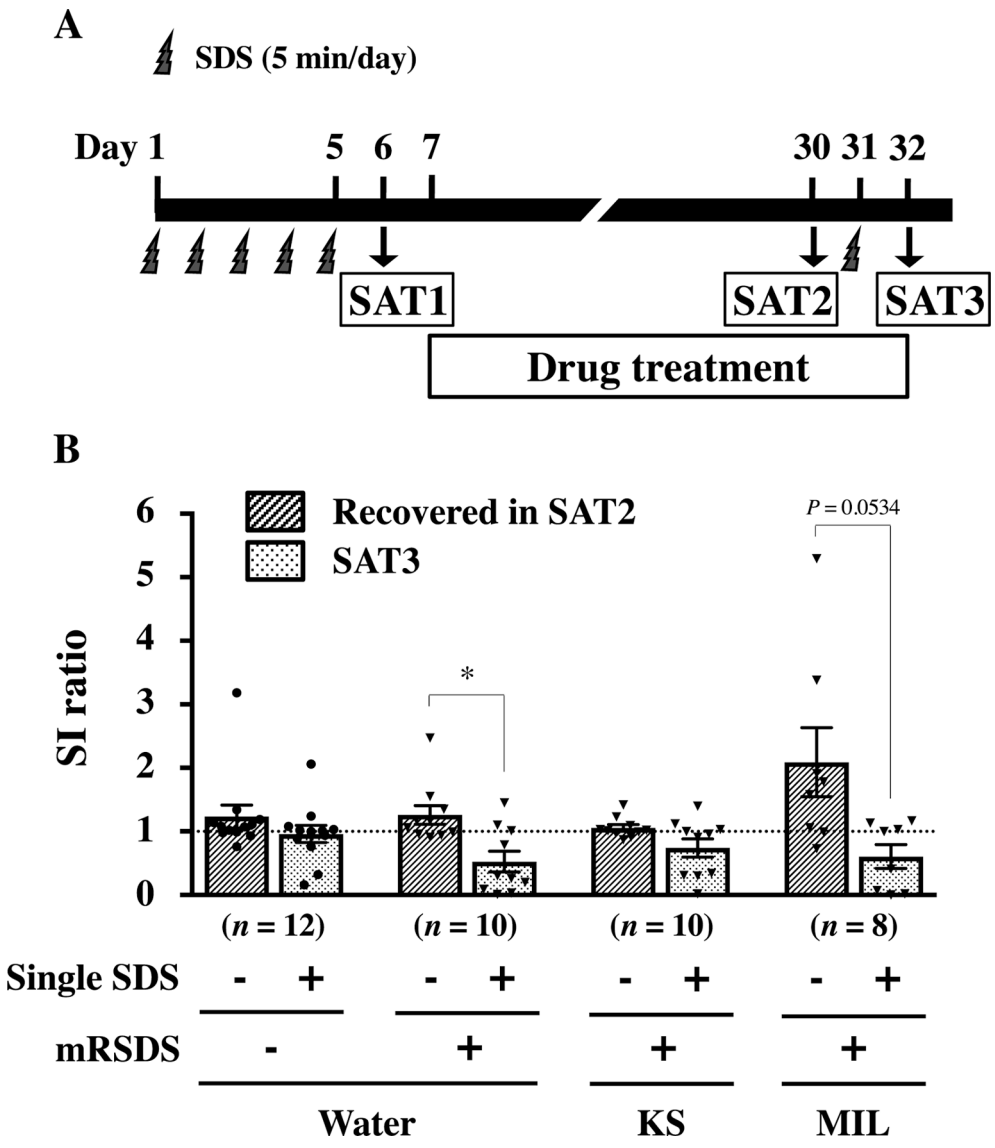


Fig. 3. Effects of drug treatment on recurrent social avoidant behaviors. (A) A schematic diagram of the experimental schedule for mRSDS and additional single SDS exposures, 1st, 2nd and 3rd SAT, and drug treatment. (B) Influences of extra single SDS exposure on SI ratio of the recovered mice in the 3rd SAT. Each column represents the mean ± SEM. Dots depict individual data of mice. $^{*}P < 0.05$ (unpaired *t*-test). KS, kososan; MIL, milnacipran; mRSDS, modified repeated social defeat stress; SAT, social avoidance test; SI, social interaction.

again for 150 s. Time spent in the SI zone during each trial was recorded by a video tracking system (EthoVision 3.0; Noldus, Wageningen, Netherlands). The SI ratio was calculated by dividing the time spent in the SI zone when the aggressor was present by the time spent in the SI zone when the aggressor was absent. Conventionally, mice with a SI ratio of <1 were regarded as susceptible mice; in contrast, mice with a SI ratio more than 1 were regarded as resistant mice (Golden et al., 2011; Krishnan et al., 2007).

2.6. Collection of blood and brain

On day 34, blood and brain were collected by decapitation. The trunk blood was centrifuged at 6000 rpm for 1 min at 4 °C, and sera were stored at −80 °C until assayed. Brain was sliced into approximately 1-mm thick coronal sections on dry ice, hippocampi were dissected from both hemispheres (bregma −1.5 to −2.5 mm), and stored −80 °C until assayed.

2.7. ELISA for serum corticosterone

Corticosterone levels in sera were measured using a commercially available ELISA kit for corticosterone (AssayMax™ ELISA kit, Assaypro, St. Charles, MO, USA) in accordance with the manufacturer's instructions.

2.8. DNA microarray analysis

Total RNA was extracted from the hippocampus using Sepazol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Total RNA was quantified and quality-assessed by the OD260/280 ratio using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In this study, the RNA Integrity Numbers (RIN) was not used. The total RNA, which the A260/230 ratio was ≥ 2.0 and the A260/280 ratio was ≥ 1.8, was used for this study.

DNA microarray analysis was conducted using isolated RNAs extracted from the hippocampus as described previously (Sasaki et al., 2017). Double-stranded cDNA was synthesized from 100 ng of total RNA with the GeneAtlas 3′ IVT Express Kit (Affymetrix Inc., Santa Clara, CA, USA). Biotin-labeled amplified RNA (aRNA) was synthesized using the GeneChip 3́ IVT Express Kit (Affymetrix Inc.). Purified aRNA was fragmented using the GeneAtlas 3́ IVT Express Kit and hybridized for 16 h at 45 °C using the GeneChip Mouse Genome 430 PM microarray (Affymetrix Inc.). The chip was washed and stained in the Gene Atlas Fluidics Station 400 (Affymetrix Inc.), and the resulting image was scanned using the GeneAtlas Imaging Station (Affymetrix Inc.). Data analysis was performed using the Affymetrix expression console (http://www.affym

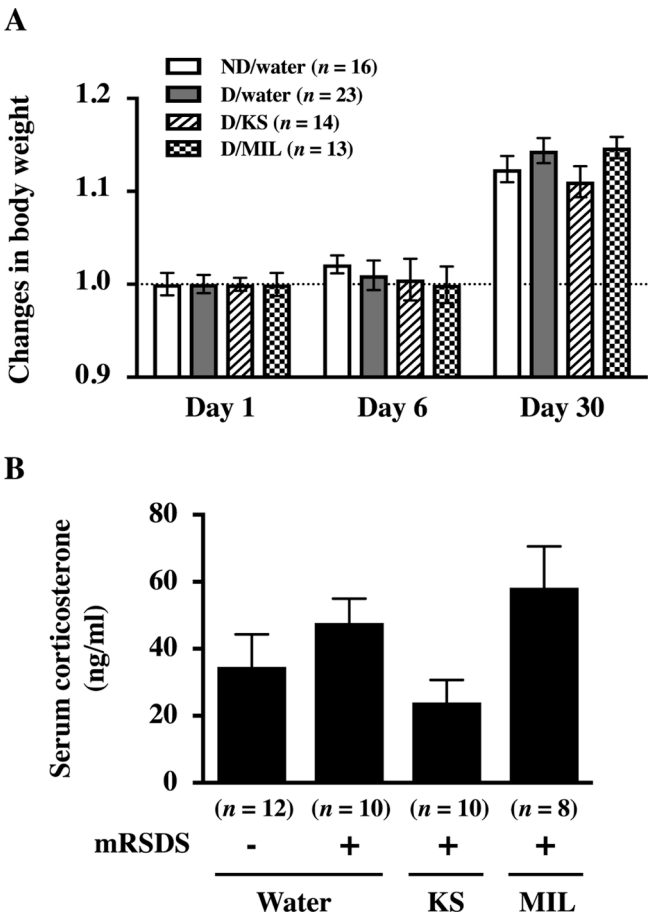


Fig. 4. Effects of drug treatment on body weight gain and serum corticosterone levels in the RSDS-exposed mice. (A) Changes in body weight on days 6 and 30 were calculated based on the deviation from body weight on day 1 (body weight each day/body weight at day 1). (B) Serum corticosterone levels on day 34. Each column represents the mean ± SEM. D, defeated; KS, kososan; MIL, milnacipran; ND, non-defeated; mRSDS, modified repeated social defeat stress.

etrix.com) and the online data tool DAVID (Database for Annotation, Visualization and Integrated Discovery, v6.8, National Institute of Allergy and Infectious Diseases, NIH, USA, https://david.ncifcrf.gov/) (Huang et al., 2007a; Huang et al., 2007b). Genes with a fold change >1.1 and P-value < 0.05 (One-way between-subject ANOVA) were considered as differentially expressed genes. Among the clustered genes, the genes that contain the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways concerning depression and inflammatory response (e.g., cytokine-cytokine receptor interaction, chemokine signaling pathway, dopaminergic synapse, glutamatergic synapse, GABAergic synapse, cholinergic synapse, and serotonergic synapse) were selected using DAVID tool and manually. Heat maps were visualized in each biological process identified by GO using Morpheus online tool (https://software.broadinstitute.org/morpheus).

2.9. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and analyzed using Prism 7 (GraphPad Software, San Diego, CA, USA). For comparison between two groups, statistical analysis was performed by unpaired or paired *t* test. For comparison between three or more groups, statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Selection of depressive-like mice and grouping

In the first SAT, SI ratio was significantly lower in socially defeated mice than in undefeated control mice ($P < 0.001$, Fig. 1C). Based on criteria, in which mice with SI ratio less than or more than 1 were regarded as resistant or susceptible mice, respectively (Golden et al., 2011; Krishnan et al., 2007), defeated mice ($n = 73$) were divided into 23 resistant and 50 susceptible mice (Fig. 1D). In this study, the susceptible mice were regarded as depressive-like mice, and assigned to 3 groups so that they had the similar SI ratio as follows: water-administered group ($n = 23$), kososan-administered group ($n = 14$), and milnacipran-administered group ($n = 13$).

3.2. Selection of mice recovered from depressive-like behaviors

In the second SAT performed on day 30 (Fig. 2A), SI ratio of water-, kososan-, and milnacipran-administered mice was significantly increased compared with that in the first SAT each group ($P < 0.05$, Fig. 2B). In this study, the minimum value of SI ratio of undefeated mice in the first SAT was set as threshold (*i.e.*, SI ratio of 0.68). In all groups, mice with SI ratio more than the threshold in the second SAT were regarded as mice recovered from depressive-like behaviors as follow (Fig. 2C): water-administered undefeated group ($n = 12$), water-administered defeated group ($n = 10$), kososan-administered defeated group ($n = 10$), and milnacipran-administered defeated group ($n = 8$). In every group, SI ratio of the recovered mice was significantly higher than that of the unrecovered mice (water-administered undefeated group, $P < 0.01$; water-administered defeated group, $P < 0.001$; kososan-administered defeated group, $P < 0.001$; milnacipran-administered defeated group, $P < 0.05$).

3.3. Effect of kososan on recurrent depressive-like behaviors

On day 31, single SDS was exposed to the recovered mice in all groups, and the third SAT was performed the following day (Fig. 3A). In the water-administered group, SI ratio was significantly reduced by single SDS exposure ($P < 0.05$, Fig. 3B). In the milnacipran-administered group, SI ratio showed a tendency toward reduction by single SDS exposure ($P = 0.0534$). In contrast, SI ratio in the kososan-administered group as well as water-administered undefeated group was not affected by single SDS exposure.

3.4. Effects of drug treatment on body weight gain and serum corticosterone levels in the RSDS-exposed mice

There was no difference in body weight gain between groups on day 30 (Fig. 4a). Likewise, no difference was found in serum corticosterone levels between groups on day 34 (Fig. 4b).

3.5. Distinct gene expression patterns in the hippocampus of mice treated with kososan or milnacipran.

Genes up-regulated and down-regulated in kososan-administered defeated mice are shown in Table 1 and Table 2. Our microarray results showed there were up-regulated 28 genes (Table 1) and down-regulated 29 genes (Table 2), which related depression and inflammatory response such as cytokine-cytokine receptor interaction, chemokine signaling pathway, dopaminergic synapse, glutamatergic synapse, GABAergic synapse, cholinergic synapse, and serotonergic synapse. In the GO analysis, the upregulated genes were associated with regulation of synaptic plasticity (GO:0048167), learning or memory (GO:0007611), modulation of chemical synaptic transmission (GO:0050804), neuron projection morphogenesis (GO:0048812), synaptic signaling (GO:0099536), trans-synaptic signaling (GO:99537),

Table 1
Higher gene expression profiles in the hippocampus of water-administered undefeated (ND/W), kososan-administered defeated (D/KS), and milnacipran-administered defeated (D/MIL) mice relative to water-administered defeated (D/W) mice.

Gene title	Gene Symbol	Fold change (vs. D/W)					
		ND/W	*p*-Value	D/KS	*p*-Value	D/MIL	*p*-Value
Connective tissue growth factor	*Ctgf*	1.81	0.512	3.01	0.145	2.29	0.02
Striatin interacting protein 2	*Strip2*	2	0.053	2.36	0.045	2.14	0.058
RAS, dexamethasone-induced 1	*Rasd1*	1.99	0.251	2.2	0.094	2.26	0.07
Troponin T1, skeletal, slow	*Tnnt1*	1.47	0.19	2.19	0.435	1.44	0.567
Myosin, light polypeptide 4	*Myl4*	2.13	0.382	2.16	0.017	2.46	0.035
Homer homolog 1 (Drosophila)	*Homer1*	1.81	0.041	2.14	0.008	1.39	0.077
Protein kinase C, delta	*Prkcd*	1.43	0.168	2.12	0.024	1.74	0.101
Transthyretin	*Ttr*	1.13	0.282	1.88	0.066	−2.09	0.017
Eph receptor A4	*Epha4*	1.7	0.065	1.81	0.023	1.64	0.008
Rad and gem related GTP binding protein 2	*Rem2*	1.27	0.166	1.79	0.068	1.6	0.18
Heat shock protein 1B	*Hspa1b*	1.84	0.06	1.78	0.02	1.45	0.13
Tescalcin	*Tesc*	1.87	0.073	1.77	0.111	1.67	0.053
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	*Sema3e*	1.7	0.054	1.75	0.031	1.8	0.012
Zinc finger protein of the cerebellum 1	*Zic1*	1.75	0.053	1.74	0.034	−1.6	0.044
Lipoprotein lipase	*Lpl*	1.59	0.168	1.73	0.024	1.72	0.101
Lymphocyte antigen 6 complex, locus E	*Ly6e*	1.33	0.015	1.73	0.002	1.17	0.074
Early growth response 1	*Egr1*	2.18	0.025	1.7	0.05	1.54	0.017
Hippocalcin	*Hpca*	1.35	0.034	1.68	0.187	1.6	0.023
Collagen, type XI, alpha 1	*Col11a1*	1.45	0.323	1.65	0.047	1.6	0.03
TRH-degrading enzyme	*Trhde*	1.57	0.035	1.64	0.017	1.59	0.115
N-myc downstream regulated gene 1	*Ndrg1*	1.11	0.697	1.58	0.006	1.27	0.101
Ectodermal-neural cortex 1	*Enc1*	−1.03	0.789	1.52	0.044	1.23	0.179
Adhesion molecule with Ig like domain 2	*Amigo2*	−1.03	0.757	1.51	0.052	1.21	0.124
Synaptopodin 2	*Synpo2*	1.37	0.012	1.5	0.048	1.3	0.03
Roundabout homolog 1 (Drosophila)	*Robo1*	1.26	0.208	1.5	0.073	1.29	0.236
Calcium channel, voltage-dependent, gamma subunit 5	*Cacng5*	1.15	0.15	1.5	0.039	1.25	0.117
Large tumor suppressor 2	*Lats2*	1.09	0.317	1.49	0.039	1.32	0.092
Ectonucleotide pyrophosphatase/phosphodiesterase 2	*Enpp2*	1.47	0.113	1.41	0.028	−1.52	0.025

Table 2
Lower gene expression profiles in the hippocampus of water-administered undefeated (ND/W), kososan-administered defeated (D/KS), and milnacipran-administered defeated (D/MIL) mice relative to water-administered defeated (D/W) mice.

Gene title	Gene Symbol	Fold change (vs. D/W)					
		ND/W	*p*-Value	D/KS	*p*-Value	D/MIL	*p*-Value
Teashirt zinc finger family member 2	*Tshz2*	−3.53	0.004	−2.48	0.038	−3.27	0.003
Neurotensin	*Nts*	−2.81	0.009	−1.98	0.015	−4.24	0.003
Coatomer protein complex, subunit gamma 2, opposite strand 2	*Copg2os2*	−2.46	0.009	−1.97	0.012	−2.15	0.013
Transcription factor 7 like 2, T cell specific, HMG box	*Tcf7l2*	−2.75	0.013	−1.97	0.013	−4.62	0.006
Protocadherin 11 X-linked	*Pcdh11x*	−1.91	0.03	−1.92	0.04	−1.71	0.086
Pre B cell leukemia homeobox 3	*Pbx3*	−2.01	0.017	−1.87	0.021	−2.45	0.018
Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	*Slc17a6*	−2.44	0.009	−1.85	0.036	−2.47	0.034
5-hydroxytryptamine (serotonin) receptor 2C	*Htr2c*	−1.73	0.016	−1.78	0.041	−3.52	0.008
Ethanolamine phosphate phospholyase	*Etnppl*	−1.33	0.477	−1.75	0.003	−1.5	0.072
LIM domain binding 2	*Ldb2*	−2.28	0.04	−1.74	0.067	−2.63	0.025
Hemoglobin alpha, adult chain 1 /// hemoglobin alpha, adult chain 2	*Hba-a1 /// Hba-a2*	−3.34	0.002	−1.7	0.004	−2.98	0.002
Amnionless	*Amn*	−1.88	0.012	−1.68	0.03	−1.94	0.006
Single-stranded DNA binding protein 2	*Ssbp2*	−1.05	0.535	−1.66	0.031	−1.47	0.006
Solute carrier family 6 (neurotransmitter transporter), member 20A	*Slc6a20a*	−1.9	0.023	−1.64	0.053	−3.58	0.011
Inter-alpha trypsin inhibitor, heavy chain 3	*Itih3*	−1.8	0.035	−1.62	0.029	−1.76	0.021
Nephroblastoma overexpressed gene	*Nov*	−1.68	0.017	−1.61	0.011	−1.98	0.001
Fucosyltransferase 9	*Fut9*	−1.65	0.034	−1.61	0.036	−1.34	0.078
Small nuclear RNA activating complex, polypeptide 3	*Snapc3*	−1.3	0.032	−1.44	0.031	−1.38	0.023
Calbindin 2	*Calb2*	−1.22	0.648	−1.42	0.0002	−2.03	0.008
Microtubule-associated protein 2	*Map2*	−1.23	0.303	−1.39	0.023	−1.1	0.085
EGF-like and EMI domain containing 1	*Egfem1*	−1.15	0.037	−1.38	0.008	−1.12	0.126
Suppressor of cancer cell invasion	*Scai*	−1.22	0.275	−1.38	0.047	1.03	0.675
RNA binding motif protein 5	*Rbm5*	−1.22	0.021	−1.36	0.045	−1.1	0.402
DENN/MADD domain containing 6B	*Dennd6b*	−1.25	0.06	−1.34	0.055	−1.24	0.143
CDK5 and Abl enzyme substrate 1	*Cables1*	−1.13	0.194	−1.34	0.003	−1.13	0.008

neuron development (GO:0048666), neuron differentiation (GO:0030182), neurogenesis (GO:0022008), and nervous system development (GO:0007399) (Fig. 5A), while downregulated genes were related to glial cell differentiation (GO:0010001), aging (GO:0007568), negative regulation of nervous system development (GO:0051961), MAPK cascade (GO:0000165), negative regulation of signal transduction (GO:0009968), negative regulation of cell communication (GO:0010648), regulation of cell death (GO:0010941), apoptotic process (GO:0006915), cell death (GO:0008219), and cell population proliferation (GO:0008283) (Fig. 5C). Along with the GO process, the upregulated genes were enriched in the Cholinergic synapse (mmu04725), Wnt signaling pathway (mmu04310), Neurotrophin

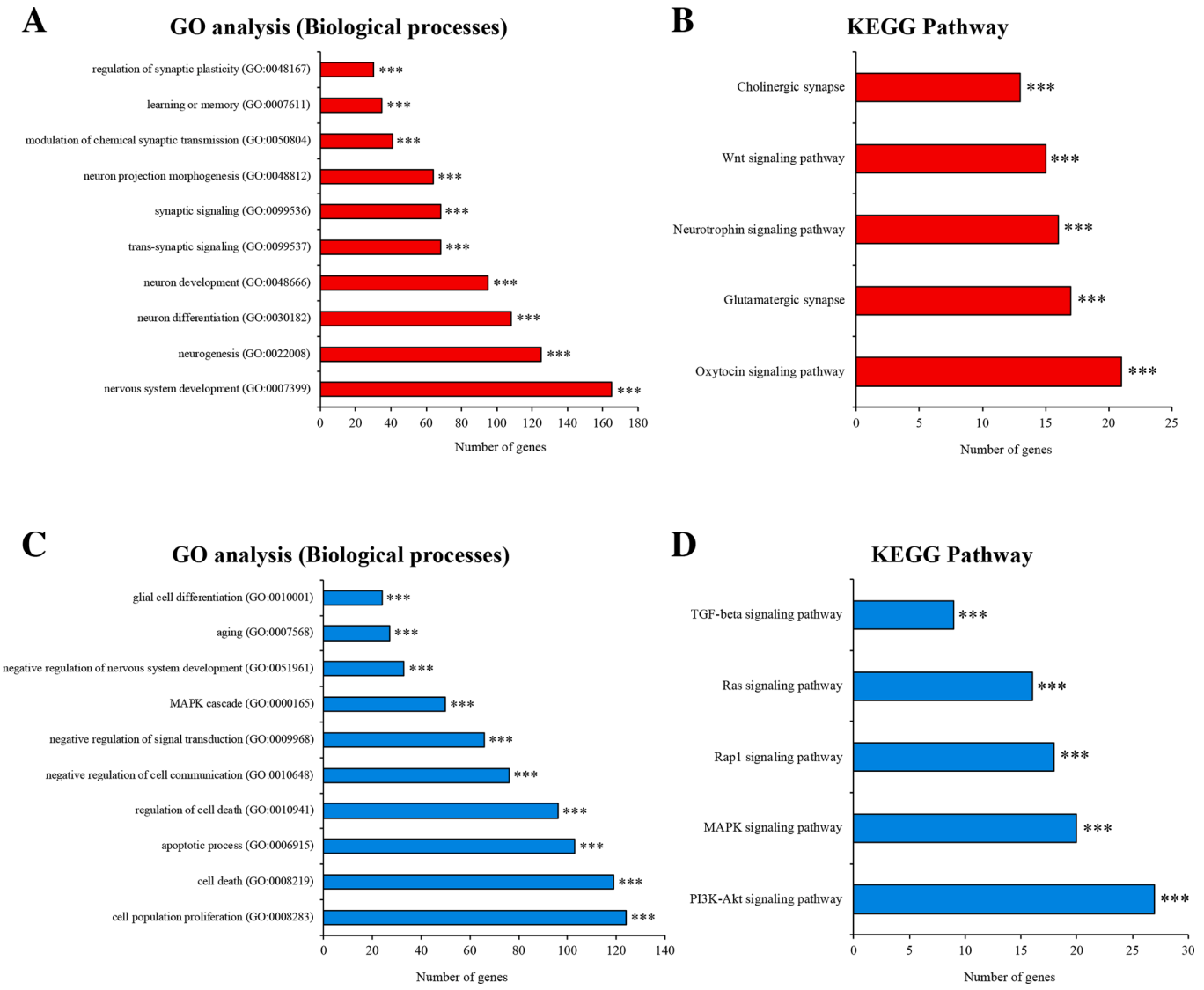


Fig. 5. Hippocampal gene expression profile in kososan- and milnacipran-administered defeated group compared to water-administered defeated group. Gene ontology (GO) process analysis (enriched biological process) and KEGG pathway for the up-regulated genes (A and B) and down-regulated genes (C and D) from microarray results with *p*-value as $^{***}P < 0.001$.

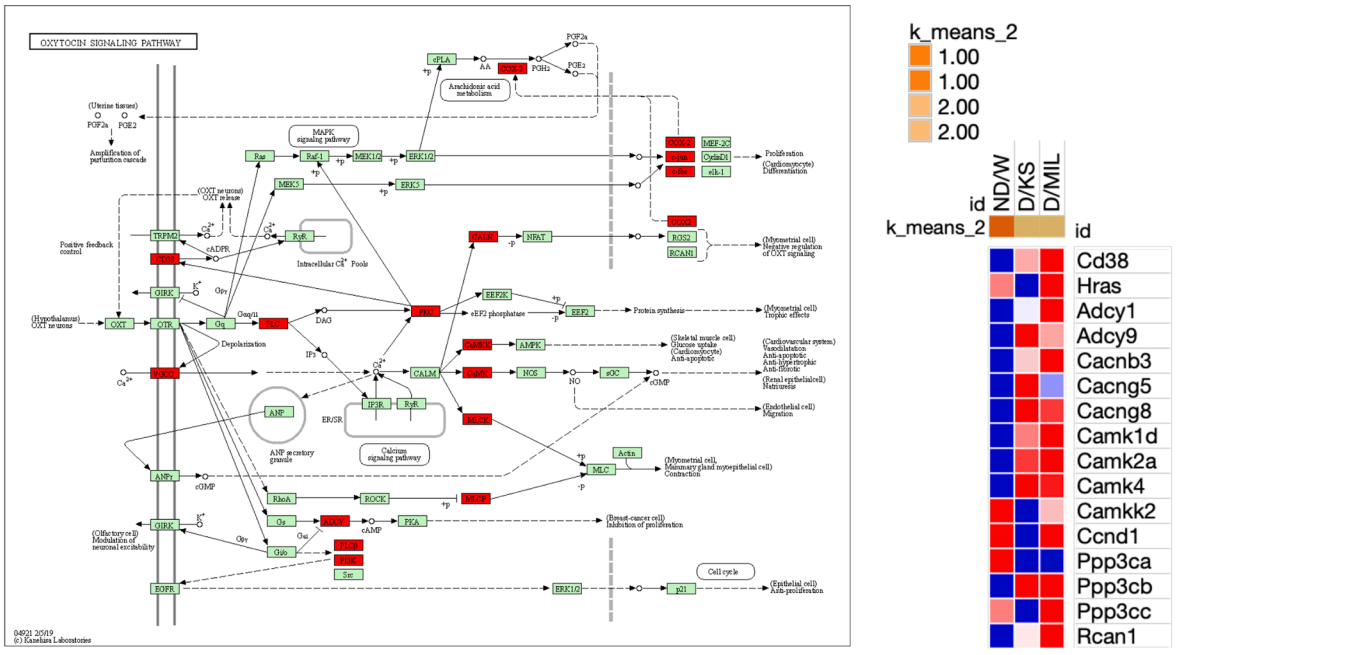
signaling pathway (mmu04722), Glutamatergic synapse (mmu04724), and Oxytocin signaling pathway (mmu04921) in KEGG analysis (Fig. 5B). Moreover, TGF-beta signaling pathway (mmu04350), Ras signaling pathway (mmu04014), Rap1 signaling pathway (mmu04015), MAPK signaling pathway (mmu04010), and PI3K-Akt signaling pathway (mmu04151) pathways enriched by downregulated genes were in KEGG pathway (Fig. 5D).

In addition, among these genes, especially, higher hippocampal mRNA expression of *transthyretin* (*Ttr*), *Zinc finger protein of the cerebellum 1* (*Zic1*), *and Ectonucleotide pyrophosphatase/phosphodiesterase 2* (*Enpp2*) was found in kososan-administered defeated mice than water-administered defeated mice (Table 1), whereas these genes in milnacipran-administered defeated mice were lower than those in water-administered defeated mice. Difference in mRNA expression of *Troponin T1, skeletal, slow* (*Tnnt1*) and *Homer homolog 1* (*Homer1*) in the hippocampus between kososan-administered defeated mice and water-administered defeated mice was also larger than that in between milnacipran-administered defeated mice and water-administered defeated mice. Lower hippocampal mRNA expression of *Suppressor of cancer cell invasion* (*Scai*) was found in kososan-administered defeated mice than water-administered defeated mice (Table 2), whereas this gene expression in milnacipran-administered defeated mice was comparable to that in water-administered defeated mice. Numerous genes, which are downstream of the oxytocin signaling pathway, were upregulated in kososan- and milnacipran-administered defeated mice relative to water-administered defeated mice (Fig. 6).

4. Discussion

Recurring depression has been recognized as a pathological condition that is difficult to control with medication. In this study, we provided the first evidence for suppressing a recurrence of depression by kososan treatment in the mRSDS mouse model. In the hippocampus, we also found that some genes may be related to a possible mechanism underlying kososan's effect on recurrent depression. Therefore, our results would be valuable in terms of potential therapeutic strategies for overcoming the recurrent depression.

A RSDS (also called chronic SDS) paradigm is widely used as a validated animal model of psychosocial stress-induced depression, and utilized to evaluate compounds of which repeated, but not single, treatment is expected to exhibit antidepressant-like effects. However, little has been reported on its usage as a mouse model of recurrent



C

Gene Title	Gene Symbol	Fold change (vs. D/W)					
		ND/W	*p*-Value	D/KS	*p*-Value	D/MIL	*p*-Value
CD38 antigen	*Cd38*	1.17	0.066	1.25	0.011	1.29	0.041
Harvey rat sarcoma virus oncogene	*Hras*	1.15	0.100	1.06	0.567	1.18	0.007
adenylate cyclase 1	*Adcy1*	1.02	0.953	1.17	0.038	1.34	0.042
adenylate cyclase 9	*Adcy9*	1.30	0.009	1.61	0.023	1.51	0.046
calcium channel, voltage-dependent, beta 3 subunit	*Cacnb3*	1.17	0.172	1.20	0.153	1.22	0.107
calcium channel, voltage-dependent, gamma subunit 5	*Cacng5*	1.15	0.150	1.50	0.039	1.25	0.117
calcium channel, voltage-dependent, gamma subunit 8	*Cacng8*	1.00	0.996	1.27	0.020	1.24	0.065
calcium/calmodulin-dependent protein kinase ID	*Camk1d*	1.21	0.326	1.33	0.189	1.37	0.224
calcium/calmodulin-dependent protein kinase II alpha	*Camk2a*	- 1.05	0.899	1.09	0.645	1.36	0.304
calcium/calmodulin-dependent protein kinase IV	*Camk4*	1.28	0.527	1.49	0.015	1.48	0.059
calcium/calmodulin-dependent protein kinase kinase 2, beta	*Camkk2*	1.31	0.091	1.23	0.059	1.28	0.040
cyclin D1	*Ccnd1*	1.32	0.295	1.09	0.577	1.32	0.059
protein phosphatase 3, catalytic subunit, alpha isoform	*Ppp3ca*	1.34	0.165	1.19	0.322	1.20	0.288
protein phosphatase 3, catalytic subunit, beta isoform	*Ppp3cb*	1.08	0.292	1.24	0.048	1.24	0.047
protein phosphatase 3, catalytic subunit, gamma isoform	*Ppp3cc*	1.17	0.169	1.08	0.123	1.20	0.052
regulator of calcineurin 1	*Rcan1*	1.06	0.483	1.19	0.060	1.30	0.029

Fig. 6. Hippocampal upregulation of oxytocin signaling-related genes in kososan- and milnacipran-administered defeated mice. (A) Increased gene expression in oxytocin signaling pathway (KEGG mmu04921) is highlighted in red compared with water-administered defeated mice using DNA microarray analysis. (B) Heat map showing genes involved in oxytocin signaling pathway between kososan- and milnacipran-administered defeated group compared to water-administered defeated group. (C) Upregulation of oxytocin signaling-related genes are shown in the Table. D, defeated; KS, kososan; MIL, milnacipran; ND, non-defeated; W, water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

depression so far. In our study, we provided a unique animal model for recurrent depression using mRSDS paradigm previously reported (Ito et al., 2020). Notably, mice recovered from the social avoidance in the second SAT retriggered the social avoidant behaviors when re-exposed to single SDS by which water-administered undefeated group was not affected in the third SAT. The recurrent social avoidant behaviors were not found in the kososan-administered mice. These results raise the possibility that kososan alleviates the recurrence of depression-like behaviors in mice. Our previous findings have shown that kososan exhibits therapeutic and preventive effects on depression-like behaviors and their possible mechanisms of action in several animal models (Hori et al., 2015; Ito et al., 2017; Ito et al., 2012; Ito et al., 2006; Ito et al., 2009; Nagai et al., 2015; Nagai et al., 2008). Given these findings, the medication with kososan is likely to show the preventive role against recurrent depression as well as the antidepressant-like effect.

Corticosterone in rodents is a glucocorticoid hormone that is well-known as a reliable biological marker of stress (Möstl and Palme, 2002; Tanaka, 1999). Numerous preclinical studies have demonstrated that stress exposure is elevated in serum corticosterone levels (Biggio et al., 2014; Dominguez et al., 2019; Iñiguez et al., 2014; Mouri et al., 2018; Niraula et al., 2018), which coordinates the onset of depression-like behaviors and that its long-lasting elevation induces neural damage and affects brain functions. Since the exogenous repeated administration of corticosterone also causes behavioral deficits such as depression-like behaviors (Ali et al., 2015; Lebedeva et al., 2017; van Donkelaar et al., 2014; Zhao et al., 2008), repeated treatment of corticosterone in rodents has been widely available as a depression-like animal model. More recently, it has been reported that cyclical injection of corticosterone, presumably mimicking recurrent depression, exacerbates depression-like behaviors via downregulation of hippocampal reelin (Lebedeva et al., 2020). In our study, serum corticosterone levels in the kososan-administered defeated mice appeared to be reduced, but not significantly, when compared with the water- or milnacipran-administered defeated mice. This result raises the possibility that kososan blocks recurrent depression, partially through regulation of corticosterone levels. However, we collected serum samples the next day after single SDS exposure in this study. Since corticosterone is generally the highest level immediately after stress exposure, significant differences in serum corticosterone levels between groups might have been found if serum sampling had been conducted just after single SDS exposure, warranting further exploration.

In the hippocampal gene expression analysis, several differences were found between kososan and milnacipran-administered defeated mice. Especially, upregulation of *Tnnt1, Homer1, Ttr, Zic1 and Enpp2* expression, and downregulation of *Scai* expression were greater in the kososan-administered defeated mice than milnacipran-administered defeated mice. It would be plausible to assume that these genes are linked to the preventive effect of kososan on recurrent depression. Lowe and Wyrobek (2012) have reported that *Tnnt1*, a gene involved in calcium homeostasis, may be a molecular stress biomarker of central nervus system for early diagnosis and treatment of neuropsychiatric disorders. Numerous animal studies have shown that *Homer 1*, a family of scaffolding protein that is located in the postsynaptic density proteins including metabotropic glutamate receptors and has some isoforms (*Homer1a/b/c/d*), mediates synaptic plasticity and learning (Clifton et al., 2017; Gerstein et al., 2012), stress vulnerability (Li et al., 2019; Wagner et al., 2015), cognitive function (Wagner et al., 2013), motivational behavior (Wagner et al., 2014), depression-like behavior (Sun et al., 2021), the preventive effect of electroconvulsive therapy (Kaastrup Müller et al., 2015), and neuroprotection against traumatic brain injury (Luo et al., 2014). Transthyretin has been reported to be neuroprotective in Alzheimer's model mice (Buxbaum et al., 2008), and its decrease is probably involved in stress-induced anxiety- and depression-like behaviors in mice (Joo et al., 2009). Some findings have reported that *Zic1* plays roles in maintaining neural precursor cells (Inoue et al., 2007) and cognitive function (Hong et al., 2021). It has been reported that *Enpp2*, a gene associated with myelin formation, mediates oligodendrocyte function in patients with depression (Aston et al., 2005) and cognitive function in senescence-accelerated mouse prone 8 mice (Wang et al., 2017). A recent study has implicated that splice variants of *Scai*, a gene involved in the suppressor of cancer invasion (Brandt et al., 2009) and cisplatin resistance (Zhao et al., 2019), affect neuronal morphology in the brain (Mizukoshi et al., 2020). Given these findings, our results imply that hippocampal altered expression of these genes and their interactive regulation may be involved in preventive effect of kososan on recurrent depression.

It is also noteworthy that either of kososan and milnacipran is likely to drive oxytocin signaling, although the enhancement of oxytocin signaling was inconsistent with behavioral recovery of kososan. Results regarding GO and KEGG provide the possibility that enhanced oxytocin signaling is involved in brain homeostasis, which may be supported by many studies demonstrating that oxytocin plays a modulatory role in brain function containing neural circuit, emotion, social recognition, and immune response. For example, oxytocin transiently coordinates excitatory/inhibitory neuronal balance for brain homeostasis (Ripamonti et al., 2017) and enhances neurogenesis (Lin et al., 2017). Anti-inflammatory effect of oxytocin (Mairesse et al., 2019; Yuan et al., 2016) is also linked to an antidepressant-like effect (Amini-Khoei et al., 2017; Loyens et al., 2013). More interestingly, hippocampal oxytocin signaling is necessary for discriminating social stimuli (Raam et al., 2017), the impairment of which causes psychiatric disorders (Kennedy and Adolphs, 2012). Therefore, it may be assumed that the upregulation of oxytocin signaling observed in this study is attributed to antidepressant-like activities of kososan and milnacipran (Ito et al., 2017; Ito et al., 2006; Ito et al., 2009; Maj et al., 2000), and only its upregulation is unlikely to be sufficient to improve the recurrent depression. Further investigation on the association of oxytocin signaling with genes altered in this study may delineate the prevention of recurrent depression by kososan.

In conclusion, this study demonstrates that kososan holds the promise of improving recurrent depression induced by re-exposure of stress. Moreover, we found several genes which could be implicated in the preventive effect of kososan. Further studies on clarifying role of genes identified in this study would provide a new avenue for therapeutic strategies of recurrent depression.

CRediT authorship contribution statement

Naoki Ito: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Funding acquisition, Writing – original draft. **Kazunori Sasaki:** Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Eiji Hirose:** Investigation, Methodology. **Takayuki Nagai:** Methodology, Writing – review & editing. **Hiroko Isoda:** Conceptualization, Writing – review & editing. **Hiroshi Odaguchi:** Conceptualization, supervision, Writing – review & editing.

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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Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files. Microarray data are deposited in the Gene Expression Omnibus (GEO) under

Accession Number: GSE148776 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179968, accessed on 13 July 2021).

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