

Involvement of Neuropeptide Y Signaling in the Antidepressant-Like Effect and Hippocampal Cell Proliferation Induced by Kososan, a Kampo Medicine, in the Stress-Induced Depression-Like Model Mice

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Received May 21, 2012; accepted July 17, 2012

Neuropeptide Y (NPY) and Orexin-A (OX-A), well-known neuropeptides associated with feeding and arousal, show antidepressant-like properties via hippocampal cell proliferation. Previous studies have revealed that kososan, a Kampo (Japanese herbal) medicine, has an antidepressant-like effect in behavioral animal models of depression; the mechanisms underlying this effect may involve the orexinergic system and subsequent upregulation of hippocampal cell proliferation. However, the roles of NPY in kososan's antidepressant-like effect remain unclear. Here we investigated whether the regulation of the NPY system could play crucial roles in this effect in the stress-induced depression-like model mice. The antidepressant-like effect of kososan administered orally (1.0 g/kg) for 28 d was abolished by a continuous intracerebroventricular injection of BIBO3304, a neuropeptide Y1 receptor antagonist, for 7 d. Likewise, BIBO3304 injection blocked the kososan-induced increases in hippocampal cell proliferation and cluster formation of neural progenitor cells. On the other hand, BIBO3304 injection did not affect kososan-induced increases in hypothalamic OX-A-producing cells or in serum OX-A levels. These results suggest that the control of the NPY system in the brain plays an essential role in kososan's antidepressant-like effect and facilitates hippocampal cell proliferation, both of which could be attributed, at least in part, to the control of the NPY system subsequent to the control of the OX-A system.

Key words kososan; neuropeptide Y; antidepressant-like effect; orexin A; cell proliferation

Neuropeptide Y (NPY) is a 36 amino acid neuropeptide that is widely distributed throughout the central and peripheral nervous systems, and its physiological actions are mediated via G protein-coupled receptors known as Y1, Y2, Y4, Y5, and Y6.¹⁾ Thus, NPY plays pivotal roles in a variety of physiological processes, including food intake,^{2,3)} gastrointestinal function,⁴⁾ circadian rhythm,⁵⁾ cognition,^{6,7)} seizure,⁸⁾ and anxiety.^{9,10)} Numerous studies also indicate that NPY could be involved in the pathogenesis of depression and in the effects of antidepressant therapies. In preclinical studies, hippocampal NPY levels have been decreased in animals exposed to chronic mild stress,¹¹⁾ inescapable stress,¹²⁾ and maternal separation,^{13,14)} all of which are known as stress-based models of depression. Flinders Sensitive Line and Fawn Hooded rats, two genetic models of depression, also have significantly decreased hippocampal NPY levels compared to control rats.^{15–20)} In addition, it has been reported that the reduced hippocampal NPY levels in animal models of depression were restored by treatment with antidepressants,^{12,15,21)} electroconvulsive stimuli,^{16–19)} and running.²²⁾ Moreover, additional evidence has shown that NPY *per se* exhibits antidepressant-like activity via the Y1 receptor^{23–26)} and promotes the proliferation of hippocampal neural progenitor cells.^{27–31)} These findings suggest that the upregulation of hippocampal cell proliferation and neurogenesis, the downregulation of which is inversely associated with the pathology of depression,^{32,33)} could be required for NPY-induced antidepressant-like effects.

In clinical studies, some reports have suggested that NPY levels in the cerebrospinal fluid are decreased in patients with major depression^{34–37)} and that the NPY levels are improved by electroconvulsive therapy³⁸⁾ and treatment with an antidepressant.³⁹⁾ Improvement in the dysfunction of the NPY system might alleviate some afflictions in patients with major depression.

Similarly to NPY, several findings have indicated that orexin-A (OX-A), which is a neuropeptide produced especially in the lateral hypothalamic area (LHA), not only regulates food intake⁴⁰⁾ but could be also involved in the pathology of depression.^{41–43)} Moreover, OX-A signaling is associated with the onset of antidepressant-like activity in mice.^{44–46)} These evidences suggest that NPY and OX-A could be key mediators in the pathology of depression and in antidepressant-like effects.

Kososan, a Kampo (Japanese herbal) medicine (Xiang-Su-San in Chinese), is composed of five herbs (Cyperus Rhizoma, Perillae Herba, Aurantii Nobilis Pericarpium, Glycyrrhizae Radix, and Zingiberis Rhizoma) and is clinically applied in the treatment of depression-like symptoms associated with the initial stage of the common cold, anorexia, food-related allergic urticaria, irritable bowel syndrome, chronic fatigue syndrome, insomnia, and autonomic imbalance. It has been also clinically suggested that kososan can alleviate depression induced by interferon (IFN)- α therapy for hepatitis C.⁴⁷⁾ Our previous studies using two animal models of depression, stress- and IFN- α -induced depression-like model mice, demonstrated that the oral administration of kososan leads to an antidepressant-like effect by normalizing

The authors declare no conflict of interest.

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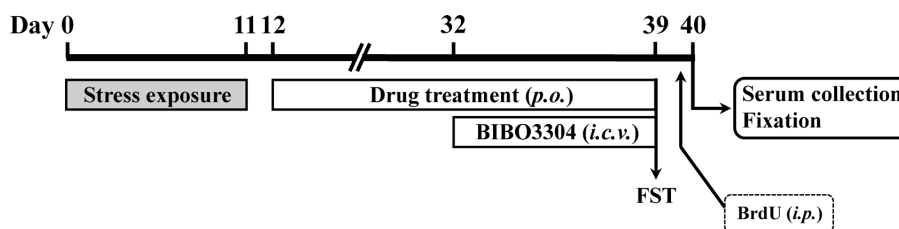


Fig. 1. Schematic Representation of Experimental Schedules

Behavioral and immunohistochemical studies on influences of BIBO3304 on kososan-elicited effects in the stress-induced depression-like model mice. Detailed explanations appear in Materials and Methods.

the dysfunction of the hypothalamic-pituitary-adrenal axis, which is strongly associated with the pathogenesis of depression.^{48,49} Furthermore, our subsequent study provided additional evidence that regulation of the orexinergic system and hippocampal cell proliferation by long-term kososan treatment plays an important role in its antidepressant-like effect in the stress-induced model mice.⁵⁰ That study also revealed that NPY-positive cells in the hippocampus are upregulated by kososan treatment.⁵⁰ However, the role of NPY upregulation in the antidepressant-like effect, hippocampal cell proliferation, and regulation of the orexinergic system by the action of kososan in the stress-induced model mice remains unclear.

To address these issues, in the present study, we performed behavioral and immunohistochemical studies to investigate the effect of the Y1 receptor (Y1R) antagonist on the antidepressant-like effect, hippocampal cell proliferation, and regulation of the orexinergic system by the action of kososan. The resulting findings suggest an additional mechanism underlying kososan's antidepressant-like effect.

MATERIALS AND METHODS

Animals We used 7-week-old male ddY mice (Japan SLC, Hamamatsu, Japan) that weighed 35–40 g at the beginning of the experiment. The mice were housed under conditions of constant temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$), with food and water available *ad libitum* (unless otherwise specified) and with a 12/12 h light–dark cycle (8:00 to 20:00) during the stress procedure. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of Kitasato University. Every effort was made to minimize the number of animals used and their suffering.

Drugs The component herbs of kososan were as follows: Cyperi Rhizoma (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.). Specimens were deposited at the Oriental Medicine Research Center, Kitasato University, Japan. The Kampo formula was decocted with 600 mL of distilled water until the volume was reduced to half. The extract was filtrated through filter paper immediately *in vacuo*. The filtrate was lyophilized and the yield of kososan extract was approximately 28% of the herbal mixture, based on its dry

weight. This extract was used in the present study. The kososan extract was dissolved in distilled water.

BIBO3304 (Tocris Bioscience, Ellisville, MO, U.S.A.), which is a selective Y1R antagonist, was dissolved in saline containing 0.167% (v/v) dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, U.S.A.). Bromodeoxyuridine (BrdU) (Roche Diagnostics, Indianapolis, IN, U.S.A.), which is a thymidine analog that labels dividing cells such as neural progenitor cells in the S-phase of the cell cycle, was dissolved in saline containing 0.007N NaOH.

Drug Treatments Kososan (1.0 g/kg, *per os* (p.o.)) was administered once daily for 28 d after stress exposure (Fig. 1). The dose was chosen based on our previous study demonstrating that oral administration of kososan leads to an antidepressant-like effect in the stress-induced depression-like model mice when administered at a dose of 1.0 g/kg.⁵⁰ Control mice were orally administered distilled water.

BIBO3304 (300 pmol/mouse/d) was injected for 7 consecutive days *via* intracerebroventricular (i.c.v.) infusion cannula joined to an ALZET micro-osmotic pump (model 1007D; DURECT, Cupertino, CA, U.S.A.), which delivers 0.5 $\mu\text{L}/\text{h}$ for 7 d (Fig. 1). Under inhaled anesthesia with Escain (isoflurane) (Mylan, Osaka, Japan), the infusion cannula was placed into the right lateral ventricle (0.3 mm posterior to bregma, 0.9 mm lateral from midline, and 2.0 mm ventral to the cortical surface) using a stereotaxic apparatus (Narishige, Tokyo, Japan). Thereafter, the osmotic pump filled with BIBO3304 solution or vehicle was implanted under the skin of the lateral back. BrdU (200 mg/kg, intraperitoneally (i.p.)) was injected once 4 h before brain fixation.

Preparation of Stress-Induced Depression-Like Model Mice The stress-induced depression-like model mice⁴⁸ were prepared using a combination of modified forced swimming (FS)^{51,52} and chronic mild stress^{53,54} approaches (Table 1). Briefly, the mice were individually placed in separate 5-L glass beakers (height, 27 cm; diameter, 18 cm) filled with 4 L of water ($23 \pm 1^\circ\text{C}$) for 15 min on Day 0. The beakers were separated by nontransparent panels to prevent the mice from seeing each other. After 15 min in the water, the mice were removed and dried with a hot-air dryer before being returned to their home cages. After 2 d, the mice were exposed to three different stress situations: tilting of the cage 30 degrees from horizontal on Days 2 and 9, pouring of 200 mL of water into the sawdust bedding of the cage on Day 5, and shaking of the cages at 200 rpm using a Green S. Seriker II apparatus (Vision Scientific, Kyunggi, Korea) on Day 7. These stress situations were applied for 48, 24, and 24 h, respectively, with 24 h intervals. On Day 11, the mice were then placed in 5-L beakers filled with 4 L of water 1 h after the final cage tilting, and FS

Table 1. Experimental Schedule of Stress Exposure

Day	Exposure of stress	Exposure time
0	Forced swimming	15 min
2	Cage tilting (30 degrees)	48 h
5	Soiled cage (200 mL of water onto sawdust bedding)	24 h
7	Cage shaking (200 rpm)	24 h
9	Cage tilting (30 degrees)	48 h
11	Forced swimming	5 min

Detail explanation represents in Materials and Methods.

was performed for 5 min. Individual immobility times during the 5 min swim were used to assign animals to the various test groups, so that the mean immobility times were similar in all groups. This procedure was used to reduce the initial variability among groups. A mouse was considered immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water, as assessed by manual observation using a video camera.

On Day 39, the mice were again placed in 5-L beakers filled with 4L of water, and the total duration of immobility during the 5 min forced swimming test (FST) was measured. This behavioral experiment was carried out between 13:00 and 16:00.

Brain Fixation and Tissue Storage After the mice were deeply anesthetized with Escaïn, they were perfused transcardially with cold phosphate-buffered saline (PBS) and subsequently with cold 4% paraformaldehyde solution. Brains were collected and postfixed in 4% paraformaldehyde solution at 4°C overnight. Serial coronal sections (50 µm in thickness) were obtained throughout the hippocampus (bregma -1.2 mm to -2.5 mm) using a vibratome (Technical Products International, St. Louis, MO, U.S.A.) and were stored in PBS/NaN₃ at 4°C until needed for subsequent experiments.

Immunohistochemistry All stainings were conducted in 24-well plates for free-floating immunohistochemistry.

For BrdU immunohistochemistry, free-floating sections were incubated in 50% formamide/1×saline sodium citrate (SSC) for 2 h at 65°C, followed by a rinse in 2×SSC. Sections were then incubated in 2N HCl for 30 min at 37°C (to denature double-stranded DNA) and rinsed in 0.1M borate buffer (pH 8.5). After blocking for 2 h with 1% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (PBS-T), sections were incubated overnight at 4°C with mouse anti-BrdU monoclonal antibody (1:1000; Chemicon, Temecula, CA, U.S.A.). After rinsing in PBS-T, sections were incubated for 2 h at room temperature (RT) with biotinylated horse anti-mouse immunoglobulin G (IgG) (1:200; Vector Laboratories, Burlingame, CA, U.S.A.), followed by incubation with the ABC kit (Vector Laboratories) for 2 h at RT. BrdU-positive cells were visualized by incubating sections with Vector DAB (Vector Laboratories). Sections were mounted on silane-coated slides and dried, and were then counterstained with 0.05% toluidine blue (Sigma), dehydrated, and coverslipped using Permount (Fisher Scientific International, Fair Lawn, NJ, U.S.A.).

For OX-A immunohistochemistry, free-floating sections were incubated in 3% H₂O₂/80% methanol for 40 min at RT. After washing in PBS, sections were blocked for 2 h with 1% BSA in PBS-T and incubated overnight at 4°C with goat

anti-OX-A polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The sections were then rinsed in PBS-T, incubated for 2 h at RT with biotinylated donkey anti-goat IgG (1:200; Santa Cruz Biotechnology), and incubated for 2 h at RT with the ABC kit. OX-A-positive cells were visualized by incubating sections with Vector DAB. Sections were then mounted and coverslipped as described above.

All counts of BrdU- or OX-A-positive cells and of BrdU-positive cluster-forming cells were performed on every third section throughout the hippocampus or hypothalamus (bregma -1.2 mm to -2.5 mm) at 400× and 1000× magnification using an Olympus BX-41 microscope (Olympus, Tokyo, Japan) in order to avoid counting the same cells more than once. The number of BrdU-labeled cells in the dentate gyrus of the hippocampus, or that of OX-A-positive cells in the LHA of the hypothalamus of each mouse, was defined as the total cell counts in six sections. All immunohistochemical procedures included negative controls that lacked primary antibodies. No staining was detected in the controls.

Measurement of Serum OX-A Blood samples were collected from the iliofemoral artery under deep anesthesia with Escaïn immediately before brain fixation. After the samples were collected, they were centrifuged at 6000 rpm for 1 min. The sera were stored at -80°C until use for the assay of OX-A levels.

Serum OX-A was measured using the competitive enzyme immunoassay kit (Peninsula Laboratories, San Carlos, CA, U.S.A.), according to the manufacturer's instructions. The sensitivity of the kit was 0.06–0.08 ng/mL. The intra- or inter-assay coefficient of variation was 5 or 14%, respectively.

Statistical Analyses Results are presented as means±S.E. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Correlation analysis was also performed with Pearson's correlation coefficient. The software used was Prism (GraphPad Software, San Diego, CA, U.S.A.). Differences were considered significant at $p < 0.05$.

RESULTS

Effect of BIBO3304 on Kososan-Induced Reduction of Immobility during FST in the Stress-Induced Depression-Like Model Mice The duration of immobility during the FST in the stress-exposed mice that had been injected with vehicle or BIBO3304 was significantly longer than that of the nonstressed mice injected with vehicle or BIBO3304 ($p < 0.001$, Fig. 2). BIBO3304 itself did not affect the duration of immobility in nonstressed mice. In mice injected with the vehicle, oral administration of kososan for 28 d significantly reduced the stress-induced increase in the duration of immobility ($p < 0.001$). However, BIBO3304 injection significantly attenuated this reduction ($p < 0.001$).

Effect of BIBO3304 on Kososan-Induced Increase in Hippocampal Cell Proliferation in the Stress-Induced Depression-Like Model Mice The number of BrdU-positive cells in the dentate gyrus of the hippocampus of the stress-exposed mice injected with vehicle or BIBO3304 was significantly lower than that of nonstressed mice injected with vehicle or BIBO3304 ($p < 0.001$ or $p < 0.01$, respectively; Fig. 3). BIBO3304 itself did not affect the number of BrdU-positive cells in nonstressed mice. Oral administration of kososan for

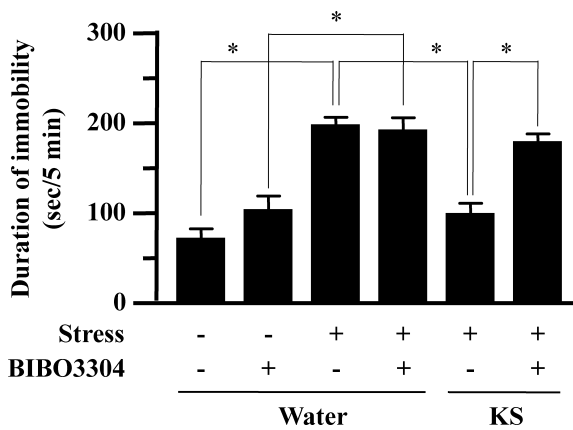


Fig. 2. Effect of BIBO3304 on Kososan-Induced Reduction in Immobility during FST in the Stress-Induced Depression-Like Model Mice

Kososan (1.0g/kg, *p.o.*) was administered to stress-induced depression-like model mice once daily for 28d according to Fig. 1. From Day 32, BIBO3304 (300pmol/mouse/d, *i.c.v.*), a selective Y1R antagonist, was injected for 7 consecutive days using an ALZET micro-osmotic pump. The duration of immobility was measured during a 5 min forced swimming test on Day 39. Data are shown as means±S.E. (*n*=13–15). **p*<0.001 with Tukey's test. KS, kososan.

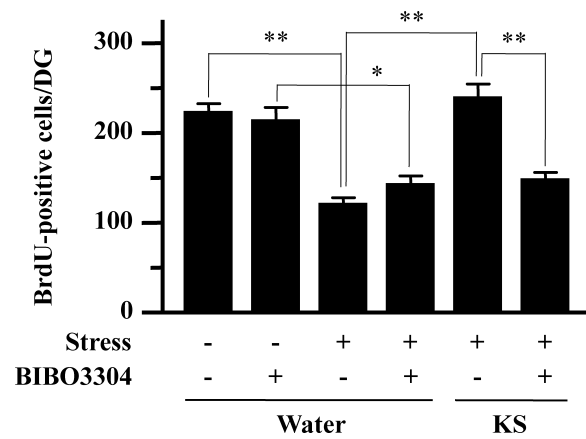


Fig. 3. Effect of BIBO3304 on Kososan-Induced Increase in Hippocampal Cell Proliferation in the Stress-Induced Depression-Like Model Mice

BrdU (200mg/kg, *i.p.*) was injected once at 4h before brain fixation on Day 40. BrdU immunohistochemistry was performed on brain slices prepared from animals that were sacrificed one day after the FST. The BrdU-positive cells in the dentate gyrus were counted. Data are shown as means±S.E. (*n*=7–9). **p*<0.01 and ***p*<0.001 with Tukey's test. BrdU, bromodeoxyuridine; DG, dentate gyrus; KS, kososan.

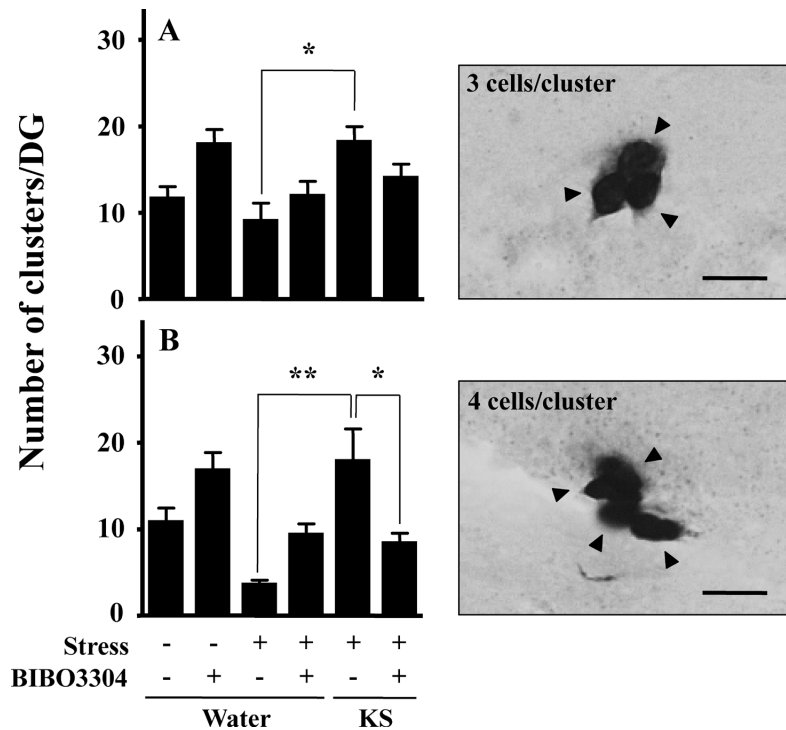


Fig. 4. Effect of Kososan with or without BIBO3304 on the Number of BrdU-Positive Clusters in the Dentate Gyrus of the Hippocampus in the Stress-Induced Depression-Like Model Mice

BrdU (200mg/kg, *i.p.*) was injected once at 4h before brain fixation on Day 40. BrdU immunohistochemistry was performed on brain slices prepared from animals that were sacrificed one day after the FST. The BrdU-positive clusters (A, three BrdU-positive cells/cluster; B, four or more BrdU-positive cells/cluster) in the dentate gyrus were counted. Representative photomicrographs show clusters formed by three or four BrdU-positive cells (arrowheads) in the subgranular zone of the dentate gyrus. Scale bar=10µm. Data are shown as means±S.E. (*n*=7–9). **p*<0.05 and ***p*<0.001 with Tukey's test. KS, kososan; DG, dentate gyrus.

28d without BIBO3304 injection significantly reversed the stress-induced decrease in the number of BrdU-positive cells (*p*<0.001). However, BIBO3304 injection markedly attenuated the increase in the number of BrdU-positive cells induced by kososan treatment (*p*<0.001).

Effect of Kososan with or without BIBO3304 on the Number of BrdU-Positive Clusters in the Dentate Gyrus of the Hippocampus in the Stress-Induced Depression-Like

Model Mice The number of clusters of either three or more than four BrdU-positive cells in the dentate gyrus of the hippocampus was significantly greater in mice administered kososan than in water-administered control mice in the stress-induced depression-like model mice (*p*<0.05 and *p*<0.001, respectively; Fig. 4). However, BIBO3304 injection significantly inhibited the kososan-induced increase in the number of clusters consisting of more than four BrdU-positive cells (*p*<0.05,

Fig. 4B). In contrast, the number of clusters consisting of two BrdU-positive cells was not affected by either stress exposure or kososan treatment (data not shown).

Effects of BIBO3304 on Kososan-Induced Increase in Hypothalamic OX-A-Positive Cells and Serum OX-A Levels There were significantly fewer OX-A-positive cells in the LHA of the hypothalamus in water-administered stressed mice than in that of water-administered nonstressed mice ($p < 0.001$, Fig. 5A). Kososan treatment for 28 d significantly ameliorated the stress-induced decrease in OX-A-positive cells in the LHA ($p < 0.001$, Fig. 5A). However, BIBO3304 injection failed to inhibit the kososan-induced increase in the number of OX-A-positive cells in the LHA of stress-induced depression-like model mice. Likewise, serum OX-A levels in the water-administered stressed mice were significantly lower than those of water-administered nonstressed mice ($p < 0.001$, Fig. 5B). Kososan treatment, with or without BIBO3304 injection, significantly enhanced the stress-induced decrease in serum OX-A levels. There was a significant positive correlation between the number of OX-A-positive cells in the LHA and serum OX-A levels ($r = 0.48$, $p < 0.001$; Fig. 5C).

DISCUSSION

In this study, we found for the first time that kososan exerts an antidepressant-like effect and induces hippocampal cell proliferation through the control of the NPY system accompanied by that of the OX-A system. This finding supports our previous study about a possible mechanism underlying kososan's antidepressant-like effect on the OX-A/NPY system.

Our previous study has shown that oral administration of kososan for 4 weeks reversed the stress-induced decrease in the number of hippocampal NPY-positive cells, but that the decreased number of NPY-positive cells was not affected by an antidepressant milnacipran treatment.⁵⁰ Thus, we focused on further understanding the role of NPY system in mechanism underlying the antidepressant-like effect of kososan in this study. Throughout all of the experiments, treatment with BIBO3304, a selective Y1 receptor antagonist, was administered by i.c.v. injection using the ALZET micro-osmotic pump to investigate whether BIBO3304 would affect kososan-induced effects. NPY-producing cells are distributed not only in the brain but also in the peripheral organs, especially in the adrenal gland and small intestine.^{55,56} In our previous study, we also observed that long-term kososan treatment reversed the decrease in the number of hippocampal NPY-positive cells in the stress-induced depression-like model mice.⁵⁰ Therefore, to sustainably suppress NPY's action in the brain, we administered BIBO3304 by i.c.v. injection using the osmotic pump. In the stress-induced depression-like model mice, the antagonism of the Y1 receptor with BIBO3304 blocked the kososan-induced reduction of immobility (Fig. 2). This suggests that NPY signaling in the brain is, at least, necessary for kososan's antidepressant-like effect to occur. Continuous i.c.v. injection of BIBO3304 alone also did not affect the duration of immobility (Fig. 2) or spontaneous locomotor activity (data not shown) in the nonstressed mice. These results imply that BIBO3304's inhibition of the kososan-induced reduction of immobility does not result from the pharmacological action of BIBO itself on physiological behaviors and functions.

In the present study, BIBO3304 treatment significantly

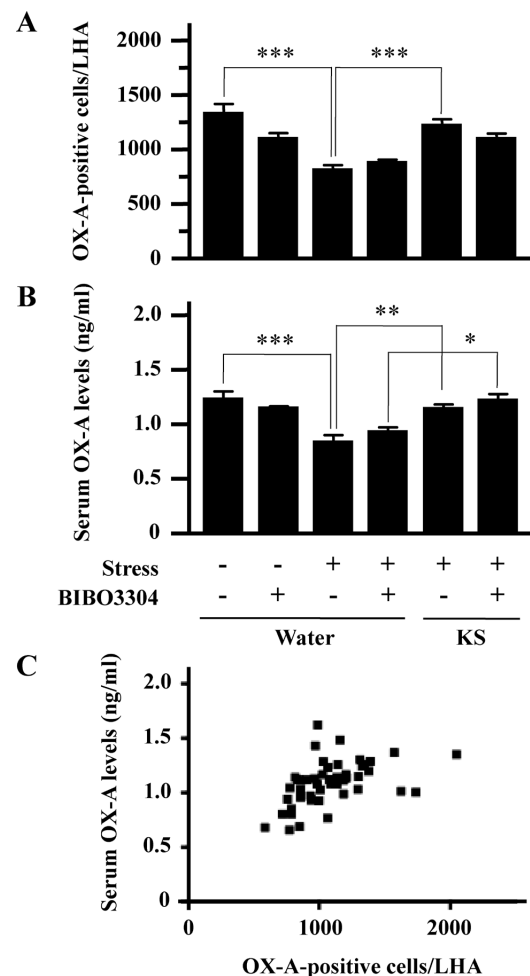


Fig. 5. Effect of BIBO3304 on Kososan-Induced Increase in Hypothalamic OX-A-Positive Cells and Serum OX-A Levels

(A) OX-A immunohistochemistry was performed on brain slices prepared from animals that were sacrificed one day after FST (Day 40). The OX-A-positive cells in the LHA were counted. (B) Serum OX-A levels were measured by the competitive enzyme immunoassay kit. Data are shown as means \pm S.E. (A, $n = 13-15$; B, $n = 7-8$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ with Tukey's test. (C) Correlation between the number of OX-A-positive cells in the LHA and serum OX-A levels. Each plot represents the individual data acquired from each mouse. Pearson's correlation coefficient test was performed ($r = 0.48$, $n = 46$, $p < 0.001$). OX-A, orexin-A; LHA, lateral hypothalamic area; KS, kososan.

attenuated the kososan-induced increase in the number of BrdU-positive cells in the dentate gyrus of the hippocampus (Fig. 3). This suggests that NPY signaling in the brain participates in kososan-induced hippocampal cell proliferation. The positive role of NPY signaling in hippocampal cell proliferation is supported by several *in vitro* and *in vivo* studies.²⁷⁻³¹ Other remarkable findings of the present study are that kososan treatment significantly increased the numbers of clusters consisting of either three or more than four BrdU-positive cells compared with water-treated model mice, and that BIBO3304 treatment significantly blocked the kososan-induced increases in the number of clusters, especially of clusters containing more than four BrdU-positive cells (Fig. 4). It has been reported that the antidepressant fluoxetine, a selective serotonin-reuptake inhibitor, increased the number of clusters of BrdU-positive cells as well as the number of BrdU-positive cells in the hippocampus.⁵⁷ This indicates that the increased number of clusters could reflect increased

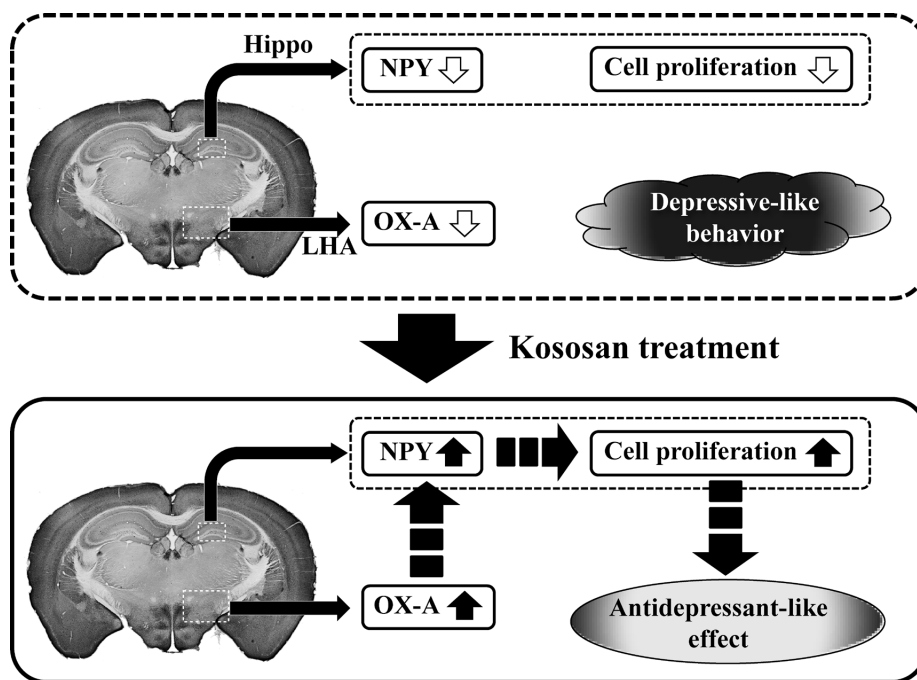


Fig. 6. A Putative Mechanism Underlying the Antidepressant-Like Effect of Kososan through OX-A/NPY System in the Stress-Induced Depression-Like Model Mice

The diagram represents a putative mechanism of kososan's antidepressant-like effect deduced from the present findings, our previous studies⁵⁰⁾ and several reports.^{23,25,27,29)} Hippo, hippocampus; LHA, lateral hypothalamic area.

hippocampal cell proliferation, which in turn denotes that the number of clusters of BrdU-positive cells can be an indicator of hippocampal cell proliferation. Therefore, our results regarding the clusters support the involvement of NPY signaling in kososan-induced hippocampal cell proliferation. However, BIBO3304 treatment *per se* appeared to be a slight increase in the number of clusters under nonstress and stress conditions (Fig. 4). Although several findings on hippocampal cell proliferation of NPY *via* the Y1 receptor have also been reported,²⁷⁻³¹⁾ the molecular mechanism by which hippocampal cell proliferation itself exerts an antidepressant-like effect as a beneficial behavior remains unclear. Thus, efforts are currently under way to clarify these issues.

Our previous study revealed that kososan treatment significantly reversed the stress-triggered decreases in the numbers of OX-A- and NPY-positive cells in the LHA and the hippocampus, respectively.⁵⁰⁾ In addition, treatment with SB-334867, an orexin receptor 1 (OXR1) antagonist, blocked the kososan-induced increase in the number of NPY-positive cells in the hippocampus, as well as the antidepressant-like effect and the increase in hippocampal cell proliferation. These results suggest that orexin signaling participates not only in kososan's antidepressant-like effect and hippocampal cell proliferation but also in the regulation of the hippocampal NPY system. However, it remains unclear whether NPY's regulatory effect could be involved in kososan's antidepressant-like effect and its upregulation of the orexin system in the LHA. In the present study, antagonism of Y1 signaling with BIBO3304 barely downregulated the kososan-induced increase in OX-A-positive cells in the LHA (Fig. 5A). Likewise, the alteration of serum OX-A levels by stress and drug treatments was detected in the same manner as that of OX-A-positive cells in the LHA, with a positive correlation (Figs. 5B, C). Since it has been reported

that OX-A could pass through the blood-brain-barrier,⁵⁸⁾ the alteration of serum OX-A levels may reflect the alteration of OX-A-positive cells in the LHA. These results raised the possibility that hippocampal NPY signaling appears not to influence kososan-induced upregulation of either OX-A-positive cells in the LHA or serum OX-A levels, although NPY signaling is involved in kososan's antidepressant-like effect and hippocampal cell proliferation. However, these results do not mean that the mediation of NPY signaling is independent of that of OX-A signaling for the kososan-induced antidepressant-like effect, because we previously demonstrated that OX-A signaling is necessary for hippocampal upregulation of NPY levels.^{46,50)} It has also been reported that NPY neurons in the arcuate nucleus of the hypothalamus are directly activated by OX-A *via* OXR1.⁵⁹⁾ These results, together with those of our previous study, suggest that the hippocampal NPY system does not regulate the OX-A system in the LHA, but that the NPY system could be modulated by OX-A signaling through OXR1 in the hypothalamic-hippocampal neural network. These results also suggest that the kososan-induced antidepressant-like effect and hippocampal cell proliferation could be regulated, at least in part, by the control of the OX-A system in the LHA, followed by that of the hippocampal NPY system.

In the brain, NPY-expressing neurons are localized not only in the hippocampus but also in the arcuate nucleus of the hypothalamus.⁶⁰⁾ The NPY neurons in the arcuate nucleus project axons to orexin neurons in the LHA,^{61,62)} and the projections are involved in the regulation of feeding behavior^{63,64)} and arousal.⁶⁵⁾ Although it remains unclear whether the projections of NPY neurons to the hypothalamus from the arcuate nucleus are related to the onset of an antidepressant-like effect, our previous study demonstrated that i.c.v. injection of OX-A

produced an antidepressant-like property with an increase in the hippocampal, but not hypothalamic, NPY level.⁴⁶ So far, it has been assumed that the mediation of hypothalamic NPY appears not to be required for the onset of an antidepressant-like effect. The characteristic difference in the distribution of NPY between the hippocampus and the hypothalamus also may be based on region-specific actions of NPY.

In conclusion, this study provides additional proof that the control of hippocampal NPY signaling plays an essential role in the antidepressant-like effect and increase in hippocampal cell proliferation brought about by long-term treatment with kososan, as assessed in the stress-induced depression-like model mice. More interestingly, we propose a hypothesis that the antidepressant-like effect and hippocampal cell proliferation by kososan treatment could be due to the control of the hippocampal NPY system subsequent to the control of the hypothalamic OX-A system (Fig. 6). These findings also provide valuable information with which to obtain a better understanding of the mechanism underlying the antidepressant-like effect of kososan, and they underscore the potential differences between kososan and antidepressants in the mechanisms by which they exert antidepressant-like effect.⁵⁰ Further studies of the molecular mechanisms underlying the production of NPY and OX-A by kososan treatment or stress exposure will contribute not only to the development of novel therapeutic drugs targeting orexinergic and NPYergic systems, but also to the further elucidation of the pathophysiological mechanisms of depression. In addition, it is expected that the orexinergic and NPYergic system-modulating drugs may be effective in anorexia and insomnia concomitant with depression, and further studies are needed to clarify the therapeutic potential.

Acknowledgments We are grateful to Ms. R. Oishi for her technical help. This work was supported by a JSPS Grant-in-Aid for Young Scientists (B) (21790631) and by a Kitasato University Research Grant for Young Researchers (2008–2010).

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