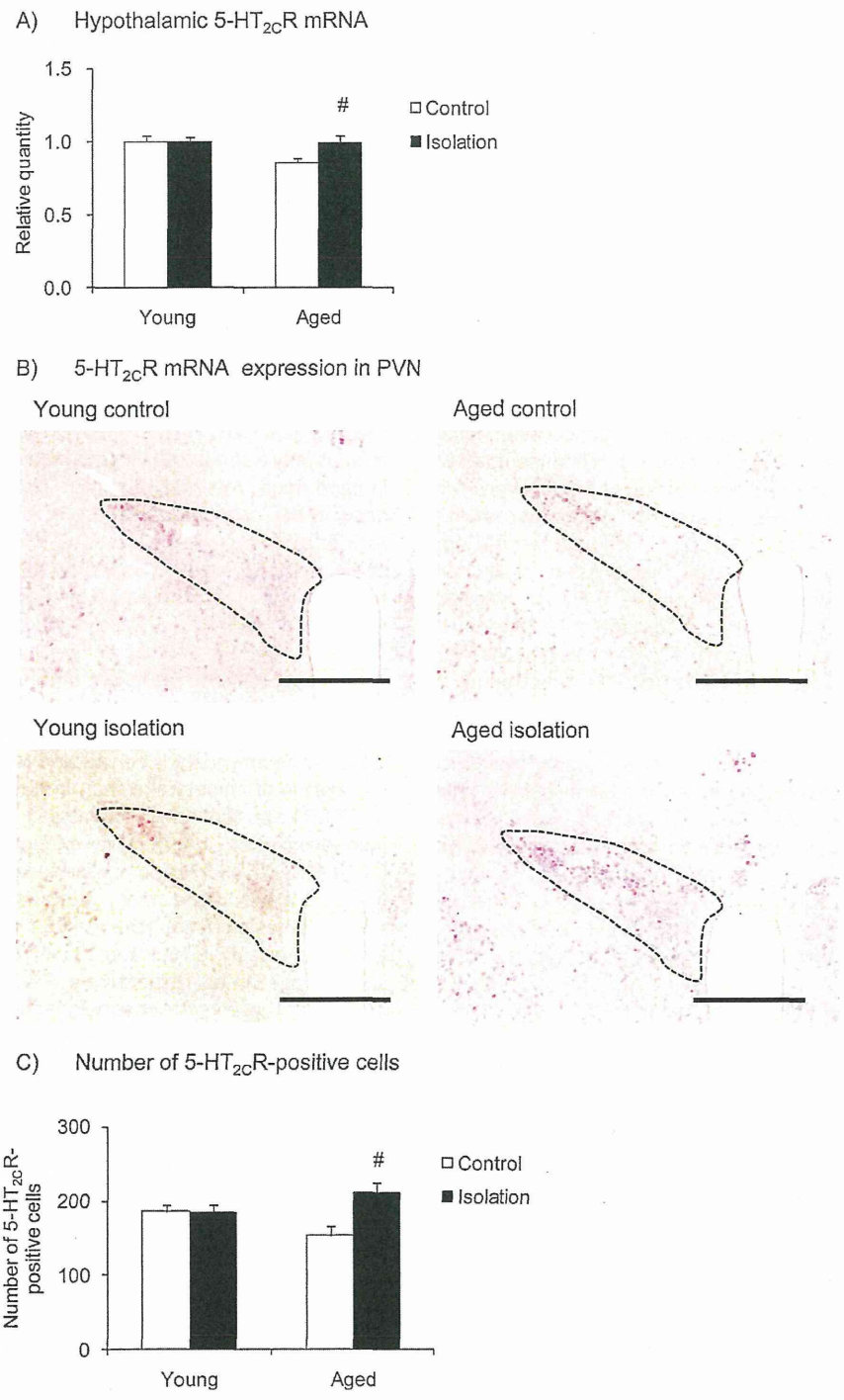

Figure 6 Hypothalamic relative expression and histological distribution of serotonin 2C receptor (5-HT_{2C}R) mRNA; Young and aged mice were exposed to the novel environment for 6 or 24 h in a freely fed condition. (A) Relative expression of hypothalamic 5-HT_{2C}R mRNA detected by reverse transcription polymerase chain reaction 24 h after isolation. (B) 5-HT_{2C}R mRNA expression in the paraventricular nucleus (PVN) detected by in situ hybridization 6 h after isolation. (C) Comparison of the number of 5-HT_{2C}R-positive cells in the PVN. Data are presented as mean ± SEM (n = 3–5). Bar: 500 μm. $^{\#}P < 0.05$ vs. aged control mice.

leads to the activation of the HPA axis observed in aged mice after novelty stress.

Our quantitative RT-PCR analysis revealed a significant increase in 5-HT_{2C}R gene expression in the hypothalamus of aged mice 24 h after exposure to a novel environment. To further confirm this observation, we performed ISH for 5-HT_{2C}R mRNA in young and aged mice with or without stress. We found that novel environmental stress caused a signifi-

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cant increase in 5-HT_{2C}R mRNA expression in PVN only in aged mice, suggesting that a novel environmental change may enhance 5-HT_{2C}R signaling in PVN in aged mice. This observation is consistent with our conclusion based on the results of a pharmacological approach. In contrast, novel environmental stress did not cause any significant change in 5-HT_{2C}R mRNA expression in other hypothalamic regions, including ARC, where appetite-regulating NPY/AGRP neurons and POMC neurons are located (data not shown).

It is plausible to consider that an increase in 5-HT_{2C}R mRNA in PVN may be the primary mechanism underlying 5-HT_{2C}R hyperfunction in aged mice. Nevertheless, there may be other possibilities: an increase in synaptic 5-HT concentration, an increase in receptor numbers and/or their affinity for 5-HT, decreased receptor desensitization and/or downregulation, and changes in intracellular signal transduction systems. In aged rodents, 5-HT concentration is reportedly increased by the inhibition of 5-HT turnover in the brain due to the environmental change compared with young rodents (Miura et al., 2002). Although we did not measure 5-HT concentration in the present study, an increase in 5-HT concentration at the 5-HT_{2C}R synaptic cleft in the brain of aged mice after exposure to a novel environment may play a role in 5-HT_{2C}R activation. As with other G protein-coupled receptors (GPCRs), functioning of 5-HT_{2C}R is fine-tuned by desensitization or internalization, which can be induced by GPCR kinases and arrestins (Van Oekelen et al., 2003). Therefore, 5-HT_{2C}R signal transduction may be enhanced by changes in receptor desensitization and/or internalization in stressed aged mice. Moreover, 5-HT_{2C}R is currently the only known GPCR in which pre-mRNA can be subject to RNA editing, resulting in alteration in its function (Burns et al., 1997). Whether these mechanisms are involved in the functional upregulation of 5-HT_{2C}R in stress-loaded aged mice remains to be determined.

In our study, the administration of 5-HT_{2C}R antagonists significantly inhibited stress hormone secretion in aged and stressed mice. Therefore, it was expected that the blockade of 5-HT_{2C}R would inhibit hypothalamic CRF, pituitary POMC, and CRF1R gene expression, all of which increased continuously up to 24 h after novel environmental changes were introduced in aged mice. However, contrary to our expectations, the administration of SB242084 or RKT in aged and stressed mice increased hypothalamic CRF mRNA, albeit it significantly decreased plasma ACTH and corticosterone levels. Although the exact reason for these unexpected increases in hypothalamic CRF mRNA after the administration of SB242084 and RKT is currently unknown, this suggests that stress hormone reduction and improved food intake due to 5-HT_{2C}R antagonism cannot simply be explained by the inhibition of hypothalamic CRF and pituitary POMC synthesis. Considering that there is a time lag between stress hormone secretion and hypothalamic CRF or pituitary POMC gene expression, the timing of sample collection should be verified in more detail.

Apart from CRF neurons in PVN, 5-HT_{2C}R is also found in POMC neurons in ARC, and it is believed to induce depolarization and increased POMC gene expression (Heisler et al., 2002), leading to the release of α-melanocyte-stimulating hormone (α-MSH). α-MSH binds to MC4R in CRF neurons in PVN and orexin neurons in lateral hypothalamic area, strongly suppressing appetite (Elmquist, 2001). On the other hand, NPY/AGRP is the best characterized and probably the most important peptide involved in stimulating food intake (Halford et al., 2007; Nonogaki, 2008) via either NPY Y1 receptor activation or antagonism of α-MSH to MC4R. Thus, feeding behavior is regulated by the balance of activity among these excitatory and inhibitory appetite regulation pathways (Tecott, 2007). In the present study, neither novelty stress per se nor 5-HT_{2C}R antagonism by SB242084 or RKT caused significant changes in hypothalamic POMC gene expression level. In contrast, the administration of SB242084 and RKT enhanced NPY/AgRP mRNA expression in aged mice after isolation stress. These findings suggest that in both young and aged mice, 5-HT_{2C}R in POMC neurons in ARC peaks at the acute phase (e.g., approximately 3 h; Saegusa et al., 2011) after the introduction of stress and that it plays only a small role in sustained decrease in food intake in aged mice. Alternatively, it is also possible that in aged mice, POMC neuron dominance in ARC stimulates CRF neurons in PVN through increased α-MSH secretion (Tachibana et al., 2007). Pharmacologically, 5-HT_{2C}R antagonism may reduce the excessive release of stress hormones and counteract reduced food intake by normalizing the balance between AgRP and POMC.

Besides PVN and ARC, the limbic system (including the amygdala) is another important brain region related to stress and food intake. Among the various regions of the limbic system, the amygdala is considered to be a key region to the perception of anxiety and fear, and it is also where 5-HT_{2C}R and CRFR are abundantly expressed (Swanson et al., 1983; Pompeiano et al., 1994). Our preliminary studies showed that 5-HT_{2C}R mRNA expression tended to increase in the amygdala in stress-loaded aged mice, although the results were not statistically significant (the number of positive cells in the amygdala was 85 ± 16.1 and 135 ± 18.4 in control aged and stressed aged mice, respectively; $P = 0.087$, data not shown). This result suggests the possible involvement of the amygdala in novelty-induced hypophagia. Further studies are required to clarify the role of the limbic system in stress-related alteration in food intake.

In conclusion, exposure of aged mice to a novel environment leads to a sustained decrease in food intake and increase in stress hormone levels via 5-HT_{2C}R activation. 5-HT_{2C}R antagonists such as SB242084 and RKT showed an ameliorative effect on reduction in food intake and secretion of stress hormones. These findings indicate that excessive 5-HT_{2C}R stimulation is deeply involved in novelty-induced hypophagia in aged mice.

Role of the funding source

There was no impact from funding source(s) on any aspect of the work with the current manuscript (design, data collection, analysis, interpretation, writing, or submission).

Conflict of interest

Dr. Takeda received grant support from Tsumura & Co. Dr. Iizuka, Dr. Sadakane, Ms. Saegusa, Ms. Nahata, and Dr. Hattori are employed by Tsumura & Co. Dr. Muto., Dr. Nakagawa, Dr. Ohnishi, and Dr. Asaka have no conflict to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.03.014.

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Research Article

Rikkunshito, a Japanese Kampo Medicine, Ameliorates Decreased Feeding Behavior via Ghrelin and Serotonin 2B Receptor Signaling in a Novelty Stress Murine Model

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We investigated the effects of rikkunshito (RKT), a ghrelin signal enhancer, on the decrease in food intake after exposure to novelty stress in mice. RKT administration (500 mg/kg, *per os*) improved the decrease in 6 h cumulative food intake. In control mice, the plasma acylated ghrelin levels significantly increased by 24 h fasting. In contrast, the acylated ghrelin levels did not increase by fasting in mice exposed to the novelty stress. RKT administration to the novelty stress mice showed a significant increase in the acylated ghrelin levels compared with that in the distilled-water-treated control mice. Food intake after administering serotonin 2B (5-HT_{2B}) receptor antagonists was evaluated to clarify the role of 5-HT_{2B} receptor activation in the decrease in feeding behavior after novelty stress. SB215505 and SB204741, 5-HT_{2B} receptor antagonists, significantly improved the decrease in food intake after exposure to novelty stress. A component of RKT, isoliquiritigenin, prevented the decrease in 6 h cumulative food intake. Isoliquiritigenin showed 5-HT_{2B} receptor antagonistic activity *in vitro*. In conclusion, the results suggested that RKT improves the decrease in food intake after novelty stress probably via 5-HT_{2B} receptor antagonism of isoliquiritigenin contained in RKT.

1. Introduction

Stress is becoming a significant social problem [1, 2] and is known to influence gastrointestinal function [3, 4]. One of the psychological stressors experienced in daily life is exposure to social environmental changes, but no detailed investigation regarding the effects of stress associated with this exposure on feeding behavior has been conducted. The novelty-induced hypophagia test measures the suppression of food intake by exposure to a novel environment and is one of the few animal tests of anxiety [5, 6].

Central 5-hydroxytryptamine (5-HT; serotonin) functions by evoking fear and anxiety manifestations and is involved in appetite regulation. Acute 5-HT depletion decreases anxiety behavior that is measured by inhibition of food intake during exposure to novel stimuli [7]. 5-HT_{2C} receptor (5-HT_{2C}R) stimulation may decrease hunger [8, 9], and this type of receptor is expressed on corticotropin-releasing factor (CRF) neurons in the hypothalamic paraventricular nucleus and on proopiomelanocortin neurons in the arcuate nucleus [10]. In addition, stimulation of 5-HT_{2B}Rs, which are distributed throughout the gastrointestinal system,

negatively regulates eating behavior [11–13]. We previously confirmed that novelty stress decreases food intake by activating both CRF1R and 5-HT_{2C}R [14]. However, we have been unable to clarify the role of 5-HT_{2B}R activation in decreased food intake as a result of novelty stress.

Ghrelin is an orexigenic hormone produced in large quantities in the stomach [15]. Peripheral ghrelin binds to its specific growth hormone secretagogue receptor (ghrelin receptor) localized at the end of the vagus nerve around the stomach [16, 17]. Ghrelin secretion from the stomach is regulated by particular subtypes of some neurotransmitters. Activations of 5-HT_{2B}R and 5-HT_{2C}R lead to a reduction in the circulating ghrelin concentrations via a decrease in ghrelin secretion in the stomach [13]. In mice exposed to a novelty stress condition, plasma ghrelin levels decreased 3 h after stress application, and acylated ghrelin supplementation remedied this reaction [14].

Rikkunshito (RKT) is a Japanese Kampo medicine comprising ingredients that facilitate ghrelin signaling [13, 18, 19]. In gastrointestinal functional disorders, where 5-HT is excessively released, such as disorders following cancer chemotherapy [13] and SSRI administration [19], 5-$HT_{2B/2C}$R stimulation causes decreased peripheral and central ghrelin concentrations, and RKT restores decreased peripheral acylated ghrelin secretion to normal levels via antagonizing these receptors. We have already demonstrated that RKT administration improves decreased food intake in novel environmental stress mice [14], but the underlying mechanism remains to be sufficiently elucidated.

We hypothesized that the improvement effects of RKT on decreased feeding behavior caused by novelty stress may be mediated through 5-HT_{2B}R antagonism. To confirm this hypothesis, abnormal ghrelin dynamics in this stress model were clarified. In addition, to clarify the role of 5-HT_{2B}R in the decreased food intake in novelty stress, the administration of 5-HT_{2B}R antagonists was performed. Furthermore, we investigated the antagonistic effects of isoliquiritigenin, a component of RKT, on 5-HT_{2B}Rs and its influence on food intake.

2. Materials and Methods

2.1. Chemicals. SB215505 (5-HT_{2B}R antagonist) and SB204741 (5-HT_{2B}R antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were dissolved in sterilized physiological saline before use. RKT was used as a powdered extract which was obtained by spray drying the hot water extract of a mixture of eight crude drug types: sojutsu (*Atractylodis lanceae rhizoma*), ninjin (*Ginseng radix*), hange (*Pinelliae tuber*), bukuryo (*Hoelen*), taiso (*Zizyphi fructus*), chinpi (*Aurantii nobilis pericarpium*), kanzo (*Glycyrrhizae radix*), and shokyo (*Zingiberis rhizoma*). RKT and RKT components were supplied by Tsumura & Co. (Tokyo, Japan).

2.2. Experimental Animals. Male C57BL/6J mice aged 6 weeks (Charles River Laboratories Japan, Inc., Tokyo, Japan) were used. Before the experiment, five mice per cage were maintained in a room with controlled temperature and humidity under a 07:00–19:00 light cycle with free access to food and water. For novelty stress, each mouse was transferred from group-housed cages to individual cages. Control mice were housed in individual cages for 7 days before the experiment was initiated. The mice in each group were similarly handled. All experiments were performed between 09:00 and 18:00 according to the guidelines established by the Experimental Animal Ethics Committee of Tsumura & Co.

2.3. Food Intake. All protocols were performed under a 24 h fasting condition. Time-course evaluation of the effect of the novelty stress on food intake in 24 h fasted mice was undertaken 1, 2, 3, 6, and/or 24 h after exposure to the novelty stress, and the effect was calculated as the difference between the food weights before and after the feeding period at each time interval.

To clarify the orexigenic action of RKT on food intake in stressed mice, we then investigated the effects of *per os* (PO) administration of RKT (500 mg/kg) [14] (Figure 1(a)) or RKT components (8-shogaol, nobiletin, tangeretin, glycyrrhizin, glycycoumarin, and isoliquiritigenin; 4 mg/kg, Figure 1(a)). The effect of intraperitoneal (IP) administration of SB215505 (10 mg/kg) or SB204741 (10 mg/kg) on the novelty stress-induced decrease in food intake was also investigated (Figure 1(a)). The experimental doses were chosen on the basis of a previous report [13]. RKT, RKT components, SB215505, or SB204741 was administered immediately after the onset of novelty stress.

2.4. Determining Plasma Levels of Ghrelin. To clarify the alteration of peripheral ghrelin dynamics after exposure to the novelty stress, blood was collected from mice given ether anesthesia 0.5 and 3 h after the novelty stress under the 24 h fasting and freely fed conditions. Blood collection to determine plasma ghrelin levels was performed from 10:00 to 12:00. We next investigated the effect of RKT (125, 250, or 500 mg/kg, PO) on plasma ghrelin concentration levels 3 h after exposure to the novelty stress (Figure 1(b)). RKT was orally administered 1 h before exposure to the novelty stress, and blood was collected 3 h after the exposure. The results of our evaluation of the postnovelty stress time course revealed that plasma ghrelin decreased significantly after 3 h [14]. We collected blood samples 3 h after stress to clarify the relationship between this change in plasma ghrelin levels and improved food intake.

The ghrelin levels were determined using commercial ELISA kit (Mitsubishi Chemical Medience Co., Tokyo, Japan).

2.5. Extraction of Total RNA for Reverse Transcription-Polymerase Chain Reaction (PCR). The hypothalamus and stomach in mice treated with distilled water or RKT (500 mg/kg) 3 h after exposure to novelty stress were rapidly removed and immediately frozen by placing them in a tube on dry ice. Homogenization of the isolated tissue and total RNA extraction were performed according to the protocol from the RNeasy Universal Tissue Kit (Qiagen, Valencia, CA,

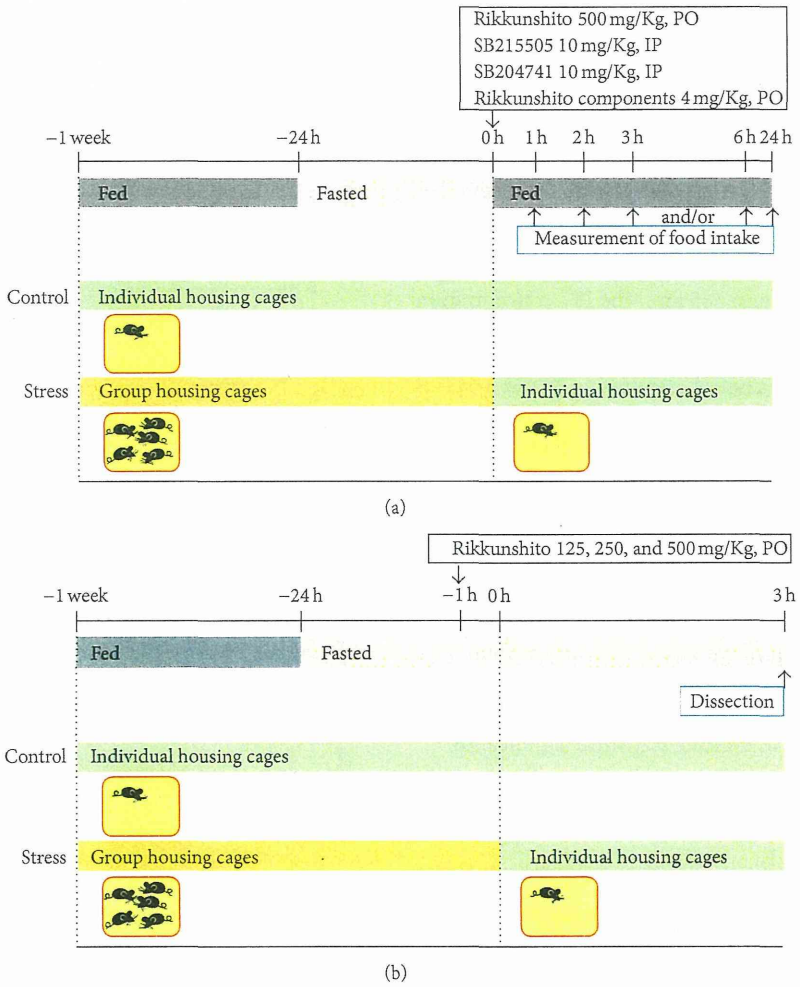

FIGURE 1: Experimental protocol. (a) Measurement of food intake. Cumulative food intake was measured at various time intervals after exposure to the novelty stress. (b) Measurement of plasma ghrelin and tissue mRNA levels. Blood, hypothalamus, and stomach were collected 3 h after exposure to the novelty stress.

USA), after which each sample was diluted to 100 ng/μL. The diluted total RNA was incubated at 70°C for 5 min and then cooled on ice. A TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's protocol to reverse transcribe the total RNA (1000 ng). A TaqMan Universal PCR Master Mix (Applied Biosystems) was used to perform quantitative PCR assays with a Prism 7900HT Sequence Detection System (Applied Biosystems). To correct the differences in the amount of total RNA added to each reaction, ribosomal protein S29 (RPS29) as an endogenous control was used to normalize mRNA expression. These differences were expressed by the ΔCt (Ct: threshold cycle) value: $\Delta Ct = 2^{(-|A-B|)}$, where A is the number of cycles that needed to reach the threshold for the housekeeping gene and B is the number of cycles needed for the target gene. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were manufactured by Applied Biosystems (RPS29: Mm02342448_gH, NPY: Mm00445771_m1, AgRP: Mm00475829_g1, preproghrelin: Mm00445450_m1, ghrelin receptor: Mm00616415_m1, orexin: Mm01964030_s1, leptin receptor: Mm00440174_m1, and CRF: Mm01293920_s1).

2.6. Binding Assay and Cell Function Assay for 5-HT_{2B}R. CHO-K1 cells stably transfected with a plasmid encoding the human 5-HT_{2B}R were used to prepare membranes in modified Tris-HCl buffer. A membrane protein was incubated with 1.2 nmol/mL [^{3}H]LSD for 60 min at 37°C. Nonspecific binding was estimated in the presence of 10 μmol/L serotonin. Membranes were filtered and washed, and then, the filters were assayed for radioactivity to determine the amount of specifically bound [^{3}H]LSD [20].

The antagonistic activities of compounds on human 5-HT_{2B}R expressed in transfected CHO-K1 cells were determined using the HTRF detection method to measure their effects on agonist-induced IP_1 production [21]. Cells were suspended in 10 mM HEPES buffer pH 7.4, plated in 96-well

microplates at a density of 4×10^4 cells/well, and preincubated for 5 min at room temperature in the presence of the buffer (basal control) or the test compound. Thereafter, the reference agonist 5-HT was added at a final concentration of 30 nM. Separate assay wells did not contain 5-HT for basal control measurements. After a 30 min incubation at 37°C, the cells were lysed, and the fluorescence acceptor (D2-labeled IP_1) and donor (anti-IP_1 antibody labeled with europium cryptate) were added. After a 60 min incubation at room temperature, the fluorescence transfer was measured at λ_{ex} = 337 nm and λ_{em} = 620 and 665 nm. The IP_1 concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm. Results were expressed as percent inhibition of the control response to 30 nM 5-HT. A concentration-response curve was generated to calculate the IC_{50} values.

2.7. Statistical Analysis. Statistical analyses of mean values of the two groups were performed using Student's *t*-test or Aspin-Welch's *t*-test after the *F*-test. Differences in multiple groups' mean values were assessed by Dunnett's analysis after Bartlett test. Cumulative food intake data were analyzed by repeated measures analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Data were expressed as the mean ± standard error of the mean (SEM) of each group, and *P* values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Changes in Food Intake in Mice Exposed to Novelty Stress. We investigated the effects of novelty stress on changes in food intake (Figure 2). Two-factor repeated measures ANOVA revealed that the effects of stress ($F(1, 42) = 47.93$, $P < 0.001$), time ($F(3, 42) = 1323$, $P < 0.001$), and stress × time ($F(3, 42) = 5.799$, $P = 0.0021$) were significant.

3.2. Effects of RKT on Food Intake in 24 h Fasted Mice. To clarify the effects of RKT on food intake, RKT (500 mg/kg, PO) was administered to 24 h fasted mice. Food intake was measured 2, 6, and 24 h after exposure to novelty stress. RKT administration restored the decreased food intake significantly ($F(1, 26) = 4.692$, $P = 0.0495$, Figure 3). Two-factor repeated measures ANOVA revealed that the effects of treatment × time ($F(2, 26) = 5.907$, $P = 0.0077$) were significant.

3.3. Changes in Plasma Ghrelin Levels and Effects of RKT in Mice Exposed to Novelty Stress. We measured plasma ghrelin levels to clarify whether plasma acylated ghrelin played a role in decreasing food intake in the mice exposed to novelty stress. Plasma acylated ghrelin and des-acyl ghrelin levels at 0.5 and 3 h under ad libitum feeding were not significantly different between the control and novelty stress groups. Under the 24 h fasting condition, plasma acylated ghrelin levels in the stress group were not significantly increased compared with those under ad libitum feeding; however, the des-acyl ghrelin level significantly increased, while still being lower than that in the control group (0.5 h; $P < 0.001$, 3 h; $P < 0.001$, Figure 4).

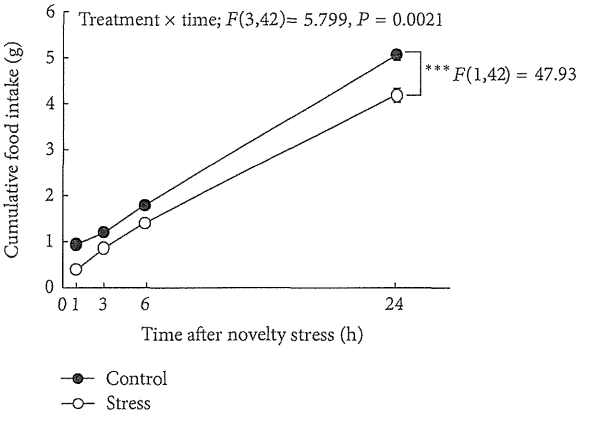

FIGURE 2: Changes in cumulative food intake after exposure to a novelty stress condition. Data are expressed as the mean ± SEM of 8 mice. $^{***}P < 0.001$ when analyzed by two-factor repeated measures ANOVA.

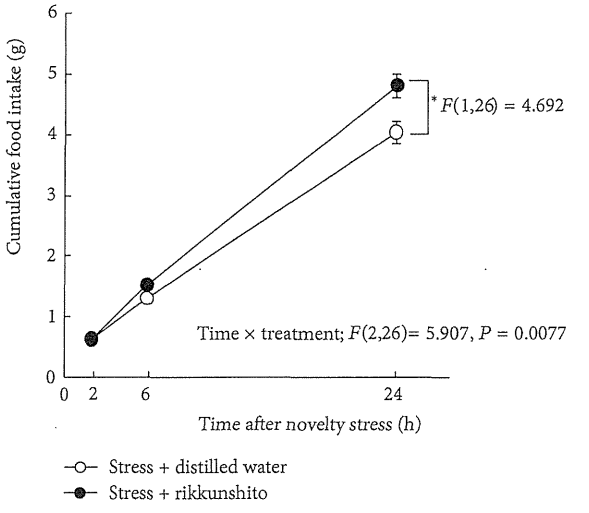

FIGURE 3: Effect of rikkunshito on food intake under a novelty stress condition. Data are expressed as the mean ± SEM of 7-8 mice. $^{*}P < 0.05$ versus distilled-water-treated mice exposed to novelty stress conditions by two-factor repeated measures ANOVA.

Next, we detected the effects of RKT administration on plasma ghrelin levels under the 24 h fasted condition. RKT administration prevented a decrease in plasma acylated ghrelin levels compared with distilled water 3 h after exposure to novelty stress ($P = 0.0074$, Figure 5). The RKT-treated mice showed a trend toward increased plasma des-acyl ghrelin levels compared with the distilled water-treated stress mice, but the difference was not significant.

3.4. Effects of RKT on mRNA Expression of Orexigenic Factors in Mice Exposed to Novelty Stress. To clarify the effects of RKT on gene expression of orexigenic factors after exposure

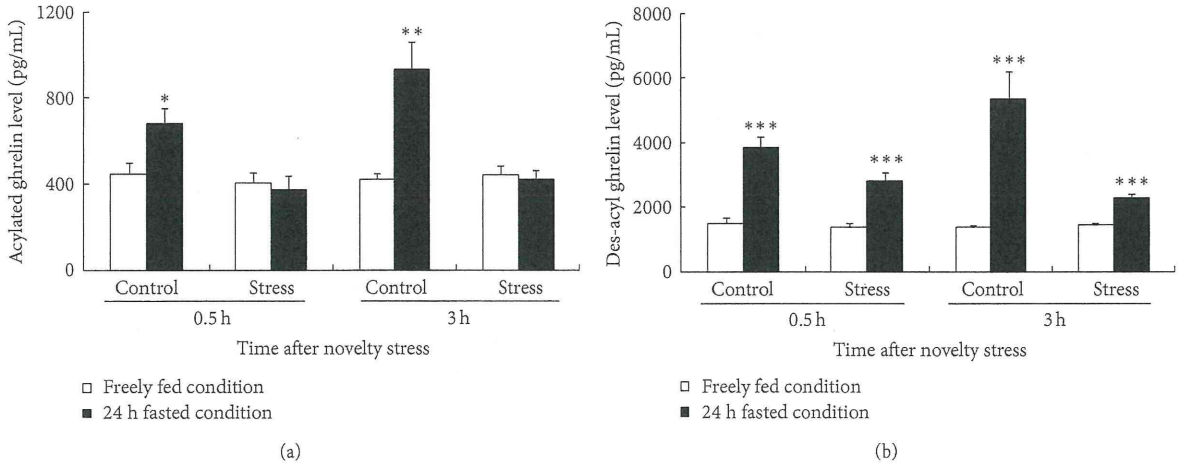

FIGURE 4: Changes in plasma ghrelin levels under the freely fed or 24 h fasted condition. (a) The plasma acylated ghrelin level. (b) The plasma des-acyl ghrelin level. Data are expressed as the mean ± SEM of 7-8 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus mice fed freely by Student's t-test or Aspin-Welch's t-test.

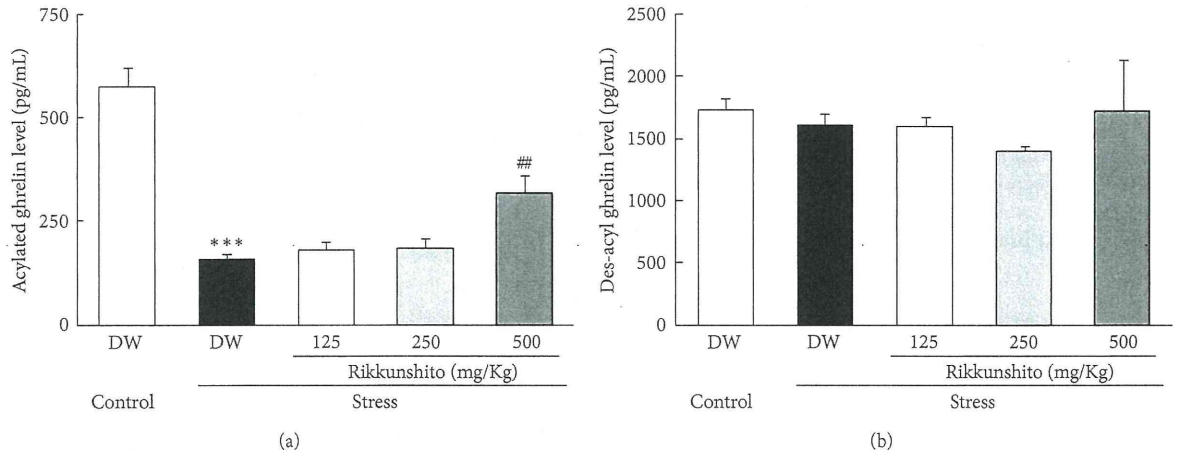

FIGURE 5: Effect of rikkunshito on plasma ghrelin levels in mice exposed to novelty stress. Plasma ghrelin levels were determined 3 h after onset of novelty stress. (a) Plasma acylated ghrelin level. (b) Plasma des-acyl ghrelin level. Data are expressed as the mean ± SEM of 8 mice. $^{***}P < 0.001$ versus control group by Aspin-Welch's t-test and $^{\#\#}P < 0.01$ versus the distilled-water-treated mice exposed to stress by Dunnett's analysis. DW: distilled water. The partial data of acylated ghrelin level indicated in this figure are derived from [14].

to novelty stress, we evaluated this effect on hypothalamic and gastric mRNA expression 3 h after exposure. In the stress group, hypothalamic NPY and AgRP mRNA showed a trend toward decreased mRNA expression. RKT administration (500 mg/kg, PO) showed a tendency to increase NPY and AgRP mRNA expression compared with stress, but the difference was not statistically significant (Figure 6). Preproghrelin gene expression was significantly increased by RKT treatment ($P = 0.036$), although this remained unchanged 3 h after novelty stress. Levels of ghrelin receptor mRNA in the RKT-treated mice showed an increasing trend, but the difference was not statistically significant. Orexin mRNA expression in the RKT-treated mice was significantly different from that in the distilled-water-treated stress mice ($P = 0.023$). There were no significant changes in leptin receptor and CRF mRNA expression among all groups. In addition, there were no significant changes in gastric preproghrelin mRNA expression among all groups (control group, 1.0 ± 0.03; novelty stress group, 1.1 ± 0.02; stress + RKT group, 1.1 ± 0.03 relative quantity of mRNA; data are not shown in figures and tables).

3.5. Effects of 5-HT_{2B}R Antagonists on Food Intake. Cumulative food intake was decreased in the group exposed to novelty stress compared to control mice in the first 6 h after exposure to the stress ($F(1, 80) = 7.647$, $P = 0.0086$, Figure 7). Two-factor repeated measures ANOVA revealed that the effects of stress × time were not significant ($F(2, 80) = 1.789$, $P = 0.17$). Administration of a 5-HT_{2B}R antagonist

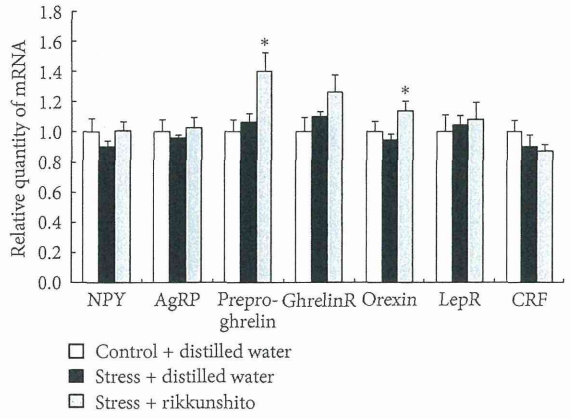

FIGURE 6: Effects of rikkunshito on hypothalamic appetite-related factor gene expression in mice exposed to a novelty stress condition. The hypothalami were collected after a 3 h exposure to novelty stress condition (4 h after rikkunshito 500 mg/kg, PO). The data are expressed as the mean ± SEM of 8 mice. $^{*}P < 0.05$ versus distilled-water-treated mice exposed to a novelty stress condition by Student's t-test or Aspin-Welch's t-test.

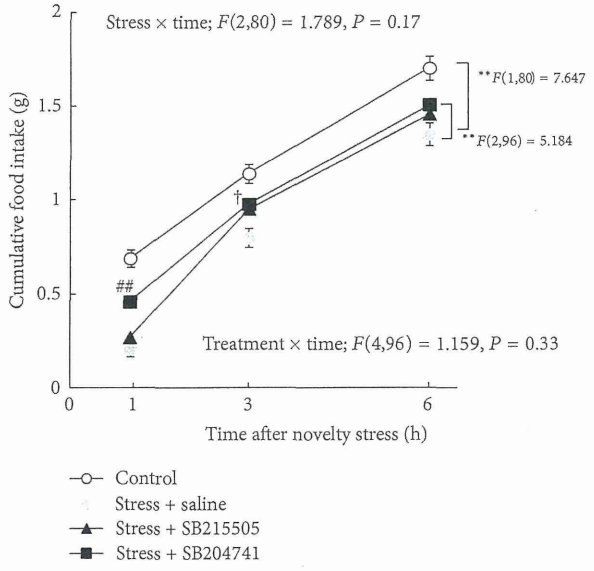

FIGURE 7: Effects of SB215505 or SB204741, 5-HT_{2B} receptor antagonists, on cumulative food intake in mice exposed to a novelty stress condition. Data are expressed as the mean ± SEM of 10–21 mice. $^{**}P < 0.01$ when analyzed by two-factor repeated measures ANOVA. $^{\#\#}P < 0.01$ SB204741 treatment versus saline-treated mice exposed to novelty stress by Dunnett's *post hoc* analysis. $^{\dagger}P < 0.05$ SB215505 treatment versus saline-treated mice exposed to novelty stress by Dunnett's *post hoc* analysis.

(SB215505; 10 mg/kg, IP or SB204741; 10 mg/kg, IP), significantly ameliorated the decrease in food intake ($F(2, 96) = 5.184$, $P = 0.0092$). Administration of SB215505 significantly prevented the decrease in food intake 3 h after exposure to novelty stress ($P = 0.044$), whereas SB204741 prevented the decrease in food intake for 1 h after exposure to stress ($P = 0.0015$).

3.6. Effects of Herbal RKT Components on Food Intake. We investigated the effects of six components of RKT on decreased food intake after exposure to novelty stress. The 6 h cumulative food intake was significantly decreased in the novelty stress-exposed mice compared with that in the control mice ($P = 0.0012$, Figure 8). Isoliquiritigenin administration (4 mg/kg, PO) prevented a decrease in cumulative food intake ($P = 0.045$). Glycycoumarin (4 mg/kg, PO) administration showed a tendency to alleviate decreased food intake in stressed mice, although the effect was not statistically significant. The other RKT components investigated exerted no effects on decreased food intake.

3.7. IC_{50} Values for 5-HT_{2B}R. Table 1 shows the 5-HT_{2B}R-binding inhibitory and cell function activities of isoliquiritigenin contained in RKT. Isoliquiritigenin showed an IC_{50} for 5-HT_{2B}R binding of 6.3 ± 0.0 μmol/L and an inhibitory cell function activity of 2.1 ± 0.2 μmol/L.

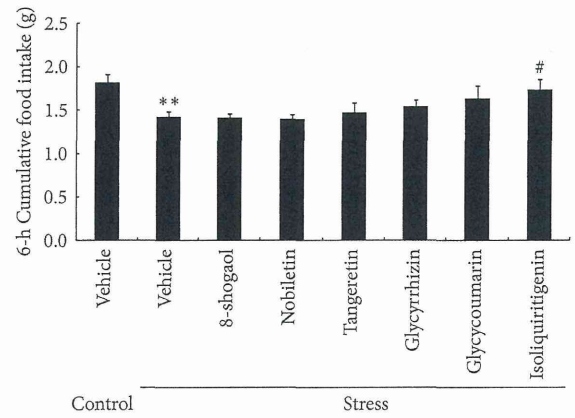

FIGURE 8: Effects of rikkunshito components on 6 h food intake in mice exposed to a novelty stress condition. Data are expressed as the mean ± SEM of 8–16 mice. $^{**}P < 0.01$ versus control mice by Student's t-test and $^{\#}P < 0.05$ versus vehicle- (0.5% carboxymethylcellulose-) treated mice exposed to a novelty stress condition by Dunnett's analysis.

4. Discussion

In this study, we demonstrated that the novelty stress decreased food intake and suppressed a physiological increase in plasma acylated ghrelin levels after fasting in mice. Oral RKT administration significantly suppressed this novelty-induced hypophagia and decrease in acylated ghrelin levels during fasting. Furthermore, decreased food intake caused by the novelty stress was significantly suppressed by 5-HT_{2B}R antagonists and isoliquiritigenin, an ingredient of RKT which has 5-HT_{2B}R antagonistic activity *in vitro* that exhibits the same effect.