Decreased plasma ghrelin contributes to anorexia following novelty stress

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Saegusa Y, Takeda H, Muto S, Nakagawa K, Ohnishi S, Sadakane C, Nahata M, Hattori T, Asaka M. Decreased plasma ghrelin contributes to anorexia following novelty stress. Am J Physiol Endocrinol Metab 301: E685–E696, 2011. First published June 28, 2011; doi:10.1152/ajpendo.00121.2011.—We hypothesized that anorexia induced by novelty stress caused by exposure to a novel environment may be due to activation of corticotropin-releasing factor (CRF) and subsequently mediated by decreasing peripheral ghrelin concentration via serotonin (5-HT) and melanocortin-4 receptors (MC4R). Each mouse was transferred from group-housed cages to individual cages to establish the novelty stress. We observed the effect of changes in feeding behavior in a novel environment using the method of transferring group-housed mice to individual cages. We investigated the effect of an intracerebroventricular injection of antagonists/agomists of CRF1/2 receptors (CRF1/2Rs), 5-HT1B/2C receptors (5-HT1B/2CR), and MC4R to clarify the role of each receptor on the decrease in food intake. Plasma ghrelin levels were also measured. The novelty stress caused a reduction in food intake that was abolished by administering a CRF1R antagonist. Three hours after the novelty stress, appetite reduction was associated with reduced levels of neuropeptide Y/agouti-related peptide mRNA, increased levels of proopiomelanocortin mRNA in the hypothalamus, and a decrease in plasma ghrelin level. Administering a CRF1R antagonist, a 5-HT1B/2C-R antagonist, an MC4R antagonist, exogenous ghrelin, and an enhancer of ghrelin secretion, rikkunshito, resolved the reduction in food intake 3 h after the novelty stress by enhancing circulating ghrelin concentrations. We showed that anorexia during a novelty stress is a process in which CRF1R is activated at the early stage of appetite loss and is subsequently activated by a 5-HT1B/2C-R and MC4R stimulus, leading to decreased peripheral ghrelin concentrations.

corticotropin-releasing factor; food intake; hypothalamus

RAPID CHANGES IN THE SOCIAL ENVIRONMENT are potential risk factors for depression and abnormal cardiovascular responses and may lead to mental/physical stress. Recent increases in such stress-related diseases have become a social problem (31, 43). Mental stress adversely affects gastrointestinal function (4), and anorexia is the most frequently observed symptom (30). Mental stress may be triggered by exposure to a novel environment; however, the underlying mechanisms remain unclear. Acute environmental changes in humans may correspond to novelty stress in animals. In this stress model, animals are removed from their home cage and placed somewhere they have never been before, i.e., a novel environment (novelty stress), and anxiety and depression are estimated (14, 35). This stress model changes internal secretion of brain neurotransmitters (15, 27).

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for stress, characterized by corticotropin-releasing factor (CRF) hypersecretion and increased blood levels of adrenocorticotropic hormone (ACTH) and corticosterone (CORT). In particular, CRF type 1 (CRF1R) and type 2 receptors (CRF2R) act in the brain to induce a reduction of food intake in rodents (10, 21, 40, 41, 45, 55). CRF1 and CRF2R-mediated anorexia appears to exhibit different time courses (45, 55). Although activating CRF1R induces short-onset anorexia and CRF2R provokes delayed-onset anorexia, much work has been carried out to clarify the mechanism that CRF inhibits feeding behavior after a novelty stress. We hypothesized that short-onset anorexia caused by CRF1 activation does not necessarily operate parallel to hyperactive HPA axis events.

Central 5-hydroxytryptamine (5-HT; serotonin) functions in fear and anxiety manifestations and is involved in appetite regulation. 5-HT1B and 5-HT2C receptor (5-HT1B/2C-R) stimulation may downregulate appetite control (13, 49). Appetite-controlling primary hypothalamic factors include the orexigenic neuropeptide Y/agouti-related peptide (NPY/AGRP) and anorexigenic proopiomelanocortin (POMC) neurons. Activation of 5-HT1B receptor expressed on NPY/AGRP neurons (17) suppresses NPY synthesis and release, and that of 5-HT2C expressed on POMC neurons promotes α-melanocyte-stimulating hormone (MSH) production (16), which contributes to appetite suppression via melanocortin-4 receptor (MC4R) activation. 5-HT2C-R is expressed on CRF neurons in the hypothalamic paraventricular nucleus (PVN) in addition to the arcuate nucleus (ARC) (18). The 5-HT1B/2C-R agonist 1-(m-chlorophenyl) piperazine (mCPP) promotes CRF secretion in the PVN, inducing activation of the HPA axis. Although the contribution of 5-HT receptor (5-HTR) to anxiety after a stress is well established, the role of 5-HTR on feeding behavior during a novelty stress remains unclear.

Ghrelin is an orexigenic hormone produced in large quantities in the stomach (25). Peripheral ghrelin binds to its specific growth hormone secretagogue receptor (GHS-R) at the end of the vagus nerve around the stomach. The ghrelin signal is transmitted to the nucleus tractus solitarius (NTS) via the vagus nerve and stimulates NPY/AGRP neurons of the ARC via the noradrenaline nerve, which increases appetite (7, 37). Conflicting data are available regarding the effect of stress on ghrelin secretion. Several investigators have reported that chronic

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stress increases plasma ghrelin concentration in several stress-loaded animal models (32, 39, 54), whereas administering urocortin 1, a CRF1R and 2R stimulator, to humans and rodents reduces plasma ghrelin concentrations (8, 48). The reason for these discrepancies remains to be defined.

Ghrelin secretion from the stomach is regulated by particular subtypes of the 5-HTR system. Central 5-HT1B/2CR activation leads to a reduction in circulating ghrelin concentration (47).

Whereas appetite control factors continue to be clarified, the acute feeding control mechanisms that are successively caused by activation of the CRF1R have remained unclear. In particular, the relevance of CRFR, 5-HTR subtype, and peripheral appetite-related hormones has not been adequately clarified. Based on the above findings, we hypothesized that acute appetite suppression due to CRF1R activation after a novelty stress is caused by a chain reaction of appetite control mechanisms mediated by 5-HT1B/2CR in ARC to MC4R system in PVN, causing lowered peripheral ghrelin secretion.

We tested the possibility that subsequent lower plasma ghrelin concentration affected reduced feeding behavior after a novelty stress in mice. Furthermore, we used the novelty stress model to investigate the influence of 5-HT1B/2CR and MC4R activation on food intake and peripheral ghrelin levels to clarify the mechanism of decreased peripheral ghrelin levels that occur after CRF1R activation.

**MATERIALS AND METHODS**

Chemicals. SB-224289 (5-HT1B antagonist), mCPP (5-HT1B/2CR agonist), HS014 (MC4R antagonist), NB127914 (CRF1R antagonist), and astressin B (CRF2R antagonist) were purchased from Sigma-Aldrich (St. Louis, MO). SB-242084 (5-HT2CR antagonist), THIQ[N-(1R)-1-[(4-chlorophenethyl)ethyl]-2-(4-cyclohexyl-1-[(1H-2,4-trazol-1-ylmethyl)]-1-piperidinyl]-2-oxoethyl]-1,2,3,4-tetrahydro-3-isouquinolincarboxamide: MC4R agonist) and γ-MSH (γ-melanocyte-stimulating hormone MC3R agonist) were obtained from Tocris Bioscience (Glasgow, UK). CRF (human, rat) and ghrelin (rat) were purchased from the Peptide Institute (Osaka, Japan). [d-Lys3]GHRP-6 (GHS-R antagonist) was purchased from Bachem California (Torrance, CA). All chemicals were dissolved in sterilized physiological saline before use. Rikkunshito (RKT) was used as a powdered extract and was obtained by spray-drying the hot-water extract of a mixture of eight types of crude drugs, i.e., sojutsu (Aurantii nobilis pericarpium), hange (Pinelliae tuber), konyakun (a powdered extract and was obtained by spray-drying the hot-water extract of the Rhizoma RKT), were supplied by Tsumura & Co. (Tokyo, Japan). The components contained in RKT, glycycoumarin and isoliquiritigenin, the components contained in RKT, were supplied by Tsumura & Co. (Tokyo, Japan). Experimental animals. Male C57BL/6J mice aged 6 wk (Japan Charles River, Tokyo, Japan) were used. Before the experiment, five mice/cage were maintained in a room with controlled temperature and humidity under a 0700–0900 light cycle with free access to food and water. For the 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. Mice in each group were housed in the same room. All experiments were performed between 0900 and 1800 according to the guidelines established by the Experimental Animal Ethics Committee of Tsumura & Co., which approved the experiments.

**Intracerebroventricular infusion.** A 26-gauge stainless steel indwelling cannula was implanted 2.6 mm below the skull surface into the lateral ventricle (1.1 mm lateral to the bregma) of mice, as described previously (2, 28). Injections were performed using a 26-gauge stainless steel injector attached to PE-10 tubing fitted to a 10-μl microsyringe.

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

Subsequently, to clarify the role of the CRF or 5-HT1B/2CR on food intake in control or stressed mice, we investigated the effects of the intracerebroventricular (icv) administration of the CRF1R agonist NB127914 (10 μg/mouse) (33), the CRF2R antagonist astressin B (10 μg/mouse) (36), the 5-HT2CR antagonist SB-242084 (0.5 or 1.5 μg/mouse) (1), or the 5-HT1B antagonist SB-224289 (0.5 or 1.5 μg/mouse) on the novelty stress-induced decrease in food intake during a 24-h-fasted condition (Fig. IA). We used previous reports involving rats as a reference for the experimental dosage. We converted the body weight of the mice and administered one-tenth to one-twentieth of the reported dosage. Because there was not much difference between the molecular weight of SB-224289 and that of SB-242084, we administered SB-224289 at the same dosage as SB-242084. The results of a preliminary investigation indicated that the previously reported dosage of astressin B based on body weight conversion had no effect, so we administered the same dosage given to rats in previous reports. Each drug was immediately administered after the novelty stress, and subsequently, each mouse was placed in a single housing cage with access to preweighed mouse chow from the group housing cage.

In another experiment, icv administration of CRF, a CRF1R agonist, was performed in 24-h-fasted mice (0.1, 0.5, and 1.0 μg/mouse). In addition, the 5-HT1B/2CR agonist mCPP (2, 10, and 50 μg/mouse) in 24-h-fasted mice was administered (icv) 3 h after the novelty stress (Fig. IB). This is because icv administration of the 5-HT1B/2CR agonist significantly suppressed decreased food intake for 3 h after the novelty stress was introduced in the present results. Although this is not shown in the figures or tables, 5-HT1B/2CR gene expression in the hypothalamus accelerated significantly for 3 h compared with that in control mice (5-HT1B/2CR, control: 1.00 ± 0.07; novelty stress: 1.37 ± 0.05; relative mRNA level, 5-HT2CR, control: 1.00 ± 0.05; novelty stress: 1.20 ± 0.06; relative mRNA level, P < 0.05, 0.001 vs. control). The above points led us to hypothesize that hypothalamic 5-HT1B/2CR affinity increases 3 h after a novelty stress. CRF was administered (icv) with or without mCPP to clarify the participation of CRF and 5-HT1B/2CR. CRF (0.1 μg/mouse) was administered icv to mice 15 min before the mCPP (10 μg/mouse), and food intake was evaluated 3 h after the mCPP administration.

Next, we investigated the effect of the MC4R on decreased food intake during the novelty stress. Intracerebroventricular administration of the MC4R antagonist HS014 (0.15 μg/mouse) (23) was conducted immediately in 24-h-fasted mice after the novelty stress (Fig. IA). Rat ghrelin (3.3 and 33 μg/mouse) was administered intravenously (iv) in 24-h-fasted mice, and food intake was measured to clarify the contribution of peripheral ghrelin (Fig. IA).

Next, RKT [250 or 500 mg/kg per os (po)], an enhancer of ghrelin secretion from the stomach (12, 46, 53), was administered in 24-h-fasted mice 1 h before the novelty stress in combination with the GHS-R antagonist [d-Lys3]GHRP-6 (0.12 mg/mouse) (47) or saline (Fig. 1C). Distilled water and saline, instead of RKT or [d-Lys3]GHRP-6, was administered to the control group. In another experiment, two components contained in RKT, glycyrrhizin (4 mg/kg po) and isoliquiritigenin (4 mg/kg po), which have 5-HT2CR antagonistic like-activity (47), were administered (Fig. 1A). We used a dosage at which isoliquiritigenin (a component of RKT) suppresses cisplatin-induced decreases in plasma ghrelin (47).

**Determining plasma levels of ghrelin or serum levels of CORT.** Blood was collected from mice by ether anesthesia 0.5, 1, 3, and 6 h after the novelty stress to investigate changes in plasma CORT during the novelty stress. The blood collection to determine plasma ghrelin
was performed between 10 AM and 12 PM. We first investigated the effects of NBI27914 (10 μg/mouse), astressin2B (10 μg/mouse), SB-242084 (1.5 μg/mouse), SB-224289 (1.5 μg/mouse), and HS014 (0.15 μg/mouse) on plasma ghrelin concentration in mice exposed to the novelty stress to clarify the role of 5-HT1B/2CR or MC4R on ghrelin secretion 3 h after the novelty stress. Each test drug was administered, the mice were isolated simultaneously, and blood was collected 3 h after the novelty stress (Fig. 1A). The results of our evaluation of the post-novelty stress time course revealed that plasma ghrelin decreased significantly after 3 h. We collected blood samples 3 h after administration to clarify the relationship between this change in plasma ghrelin level and improved food intake.

Furthermore, we also investigated the effect of the 5-HT1B/2CR antagonist on plasma ghrelin levels in CRF-treated rats. CRF (0.2 μg/mouse) or mCPP (50 μg/mouse) was administered alone, CRF was coadministered with SB-242084 (1.5 μg/mouse) or SB-224289 (1.5 μg/mouse), and blood was collected 1 h later (Fig. 1D). To clarify MC4R for the decrease in food intake after the novelty stress, icv administration of THIQ (1.5 μg/mouse), an MC4R agonist, or γ-MSH (1.5 μg/mouse), an MC3R agonist, was performed, and blood was collected 1 h later (Fig. 1D). Because the results of a preliminary investigation showed that the plasma ghrelin decrease peaked 1 h after a single icv administration of CRF, mCPP, THIQ, and γ-MSH, the end point for evaluation was set at 1 h after administration.

To investigate the effects of RKT on plasma ghrelin level, RKT (500 mg/kg) was administered orally 1 h before the novelty stress, and blood was collected 1 or 3 h after the stress. We performed this 1 and 3 h after the stress (2 and 4 h after RKT administration) because it has been demonstrated that RKT restores decreased endogenous ghrelin within 2 h after it is administered (Fig. 1C) (47). The blood collection procedures to determine plasma ghrelin concentrations were as reported previously (47). To determine the serum CORT concentration
after the novelty stress, blood was collected in a laboratory dish by severing the carotid artery. Ghrelin levels were measured using the Active Ghrelin ELISA kit/Desacyl-Ghrelin ELISA kit (Mitsubishi Chemical Medience, Tokyo, Japan), and the CORT levels were measured using the Correlate-EIA Corticosterone kit (Enzo Biochem, New York, NY).

**Extraction of total RNA for RT-PCR.** The hypothalamus and stomach 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), following 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. Total RNA (1,000 ng) was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Quantitative PCR assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems), using a Prism 7900HT Sequence Detection System (Applied Biosystems). To correct the differences in the amount of total RNA added to each reaction, mRNA expression was normalized using ribosomal protein S29 (RPS29) as an endogenous control. These differences were expressed by the dCT value: dCT = ΔCt = ΔCtB − ΔCa, where A is the number of control samples and B is the number of experimental samples.

**Table 1. Changes in hypothalamic mRNA expression after novelty stress**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control mRNA Level</th>
<th>Novelty Stress mRNA Level</th>
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<tr>
<td>1</td>
<td>NPY</td>
<td>AGRP</td>
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<tr>
<td></td>
<td>1.00 ± 0.09</td>
<td>0.93 ± 0.02</td>
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<td>1.00 ± 0.08</td>
<td>0.91 ± 0.07</td>
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<td></td>
<td>1.00 ± 0.12</td>
<td>1.14 ± 0.08</td>
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<td>1.00 ± 0.05</td>
<td>0.77 ± 0.05**</td>
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<tr>
<td></td>
<td>1.00 ± 0.06</td>
<td>0.83 ± 0.06</td>
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<tr>
<td></td>
<td>1.00 ± 0.06</td>
<td>1.35 ± 0.12*</td>
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Each value indicates the mean ± SE (n = 8–10). NPY, neuropeptide Y; AGRP, agouti-related peptide; POMC, proopiomelanocortin. The relative quantity of mRNA is shown. *P < 0.05 and **P < 0.01 vs. control by Steel’s analysis, respectively.
of cycles needed to reach the threshold for the housekeeping gene (C\textsubscript{T}: threshold cycle) 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 (\textit{Rps29}: Mm02342448\_gH; \textit{Npy}: Mm00445771\_m1; \textit{Agrp}: Mm00475829\_g1; \textit{Pomc}: Mm00435874\_m1; \textit{ghrelin}: Mm00445450\_m1).

RESULTS

Effects of the novelty stress on serum CORT level and food intake. Serum CORT level significantly increased in mice 0.5 h after the novelty stress compared with control mice and recovered gradually (Fig. 2A). Food intake 1 and 3 h after the novelty stress decreased significantly compared with that in control mice (Fig. 2B).

Effects of the CRF1R or CRF2R antagonists on the decrease in food intake in mice exposed to the novelty stress. We investigated the effects of administering either a CRF1R antagonist or a CRF2R antagonist on decreased food intake to reveal the role of the CRFR subtype in novelty stress-induced anorexia. Intracerebroventricular administration of the CRF1R...
antagonist NB127914 (10 μg/mouse) in the stress-loaded mice significantly enhanced food intake after the novelty stress compared with saline-treated mice. However, icv administration of the CRF2R antagonist astressin2B (10 μg/mouse icv) did not alter the decrease in food intake in the novelty stress-loaded mice (Fig. 2C).

Effect of CRF on food intake in 24-h-fasted mice. To clarify the effect of CRF1R activation on food intake, icv administration of CRF (0.1, 0.5, and 1.0 μg/mouse) was performed on 24-h-fasted control mice; food intake was measured 1 h after treatment (n = 6). CRF decreased food intake (control 0.68 ± 0.04 g/h; CRF 0.1 μg/mouse, 0.44 ± 0.04 g/h; CRF 0.5 μg/mouse, 0.05 ± 0.02 g/h, P < 0.001; CRF 1.0 μg/mouse, 0.03 ± 0.02 g/h, P < 0.001, by Dunnett’s analysis) (data not shown).

Hypothalamic mRNA expression in mice exposed to the novelty stress. We investigated the effects of a novelty stress on the expression of several hypothalamic appetite-related genes. Three hours after the novelty stress, the hypothalamic POMC mRNA level increased significantly compared with that in the control, whereas NPY mRNA level decreased (Table 1). The AGRP mRNA level showed a tendency to decrease compared with the control.

Effects of a 5-HT1B/2CR antagonist or agonist on the decrease in food intake in mice exposed to the novelty stress. Food intake in control mice treated with SB-224289 or SB-242084 was not significantly different from that in mice treated with saline alone (n = 7–8) (saline 0.71 ± 0.04 g/3 h; SB-224289 0.5 μg/mouse icv, 0.76 ± 0.06 g/3 h; SB-242084 1.5 μg/mouse icv, 0.73 ± 0.06 g/3 h; SB-242084 0.5 μg/mouse icv, 0.84 ± 0.04 g/3 h; SB-242084 1.5 μg/mouse icv, 0.88 ± 0.064 g/3 h). Otherwise, the icv administration of SB-224289 and SB-242084 resolved the reduction in food intake 3 h after the novelty stress (Fig. 3, A and B). mCPP (2, 10, and 50 μg/mice icv) was administered to control mice or 3-h stress-loaded mice to clarify the contribution of 5-HT1B/2CR on food intake 3 h after the stress. mCPP (10 μg/mouse) inhibited food intake in the stress-loaded mice 1 h after administration, but food intake in control mice was not affected (Fig. 3C). Additionally, 3 h after the administration of mCPP (50 μg/mouse), food intake decreased, but only in the novelty-stressed mice (data not shown).

Next, we evaluated the effect of CRF with or without mCPP on food intake to clarify their influence on food intake when central CRF1R and 5-HT1B/2CR were activated. Food intake 3 h after coadministration of CRF (0.1 μg/mouse: 0.72 ± 0.04 g/3 h; 0.2 μg/mouse: 0.70 ± 0.05 g/3 h; P < 0.05 by Dunnett’s analysis) with mCPP was reduced significantly compared with saline-treated mice (0.91 ± 0.05 g/3 h), although the CRF treatment alone failed to reduce the food intake (data not shown).

Effect on plasma ghrelin level in mice exposed to the novelty stress. We measured plasma ghrelin levels to clarify whether plasma acyl ghrelin participated in the decrease in food intake in mice exposed to a novelty stress. The plasma acyl ghrelin level at 3 h decreased compared with that of the control group and showed a tendency to recover 6 h after the novelty stress (Table 2). Administration of the CRF1 antagonist NB127914 (10 μg/mouse) inhibited the decrease in plasma acylated ghrelin level, but the CRF2 receptor antagonist astressin2B (10 μg/mouse icv) failed to inhibit it (Fig. 4A) 3 h after induction of the novelty stress.

Next, to clarify the role of 5-HT1B/2CR on plasma ghrelin secretion under a novelty stress condition, we first detected the effect of administering 5-HT1B/2CR antagonists on plasma ghrelin levels. The icv administration of SB-242084 or SB-224289 prevented the decrease in plasma acyl ghrelin level compared with saline-treated mice 3 h after the induction of the novelty stress (Fig. 4B). The plasma acyl ghrelin level in mCPP- and CRF-treated mice was reduced significantly compared with saline-treated mice. Intracerebroventricular administration of SB-224289 or CRF-treated mice also prevented the decrease in plasma acyl ghrelin level compared with saline-treated mice (Fig. 4C).

Effects of an MC4R on the decrease in food intake and plasma ghrelin level in mice exposed to the novelty stress. To clarify the effect of MC4R, which is downstream of 5-HT1B/2CR, on the novelty stress, we evaluated the effect of an MC4R antagonist on food intake and plasma ghrelin levels 3 h after the novelty stress. Intracerebroventricular administration of the MC4R antagonist HS014 (0.15 μg/mouse) recovered the decrease in food intake 3 h after the novelty stress (Fig. 4D). As observed in Fig. 4E, an icv injection of HS014 also inhibited the decrease in plasma acyl ghrelin in animals exposed to the novelty stress. An icv injection of THIQ (an MC4R agonist), but not γ-MSH (an MC3R agonist), decreased acyl ghrelin in control mice (Fig. 4F).

Effects of exogenous ghrelin on food intake and effects of RKT on plasma acyl ghrelin level and food intake. Exogenous ghrelin significantly prevented the decrease in food intake (Fig. 5A). Although the plasma acyl ghrelin level at 1 h in the stressed group showed a tendency to decrease compared with that in the control group, the oral administration of RKT (500 mg/kg po), which enhances ghrelin secretion from the stomach (46, 53), increased plasma acyl ghrelin concentrations 1 h after the novelty stress (Fig. 5B). RKT (500 mg/kg po) significantly inhibited the decrease in acyl ghrelin level 3 h after the novelty stress. RKT (500 mg/kg po) inhibited the decrease in food intake 1 and 3 h after the novelty stress, and the effect of RKT was abolished by coadministering the GHS-R antagonist [D-Lys3]GHRP-6 (0.12 mg/mouse iv) (Fig. 5C).

To clarify whether the actions of RKT on inhibiting anorexia and enhancing acyl ghrelin are due to the 5-HT2CR antagonism by the RKT ingredients, we selected two ingredients that display 5-HT2CR antagonism in the in vivo assay (46). Administering glycycoumarin and isoliquiritigenin (4 mg/kg po) inhibited the decrease in food intake 3 h after the novelty stress (Fig. 5D).
DISCUSSION
The results of this study may offer an explanation for appetite loss during mental stress. We demonstrated that an acute environmental change created by transferring mice from group to individual breeding cages caused a decrease in food intake within 1–3 h. We also determined that the decrease in food intake during the early stage was dependent on central CRF1R activation rather than CRF2R activation.

Fig. 4. Effects of a CRF1R antagonist, a 5-HT1B/2CR agonist/agonist, or a MC4R antagonist on plasma ghrelin levels in mice exposed to a novelty stress or in CRF-administered mice. A: effect of CRF1R antagonist (10 μg/mouse icv) or CRF2R antagonist (10 μg/mouse icv) on plasma acyl ghrelin in mice exposed to a novelty stress (refer to Fig. 1A for the experimental protocol). Effect of the 5-HT1B antagonist (1.5 μg/mouse icv) or the 5-HT2CR antagonist (1.5 μg/mouse icv) on plasma acyl ghrelin in mice exposed to the stress (B: refer to Fig. 1A for the experimental protocol) and in CRF-administered mice (C: refer to Fig. 1D for the experimental protocol). Effect of the MC4R antagonist HS014 (0.15 μg/mouse icv) on the decreases in food intake (D: refer to Fig. 1A for the experimental protocol) or plasma acyl ghrelin (E: refer to Fig. 1A for the experimental protocol) 3 h after the novelty stress. Effect of MC4R agonist THIQ (1.5 μg/mouse icv) or MC3R agonist γ-MSH (1.5 μg/mouse icv) on plasma acyl ghrelin in control mice (F: refer to Fig. 1D for the experimental protocol). Data are means ± SE. **P < 0.01, ***P < 0.001 vs. control mice by Student’s t-test. ###P < 0.001 vs. saline-treated novelty stressed mice. †††P < 0.001 vs. CRF-treated mice; n = 5–8.
and that subsequent signal enhancement of 5-HT<sub>1B/2C</sub>R contributes to the loss of appetite at a later stage (3 h after the stress). Furthermore, we found that plasma acyl ghrelin decreased significantly in the novelty-stressed mice and that CRF1R, 5-HT<sub>1B/2C</sub>R, and MC4R antagonism abolished the decrease. Moreover, 5-HT<sub>1B/2C</sub>R antagonism inhibited the CRF-treated plasma acyl ghrelin reduction. These results suggest that suppressed feeding behavior accompanying a novelty stress may be induced by interactions between central CRF1R, 5-HT<sub>1B/2C</sub>R, MC4R, and peripheral ghrelin.
Recent studies have demonstrated that central 5-HT1B/2CR activity occurred after the novelty stress and induced a reduction in feeding behavior.

In this study, serum CORT increased 0.5–1 h after the novelty stress and returned to a control level. Therefore, the HPA axis was activated immediately after the stress and was restored to the control level by negative feedback. The decrease in food intake was maintained for 3 h after the novelty stress. These results suggest that activation of the HPA axis and changes in food intake do not necessarily occur in parallel. Intracerebroventricular administration of NBI27914, a CRF1R antagonist, initially suppressed the decrease in food intake induced by this stress. Our data are in agreement with previous studies in that a CRF1R blockade inhibited a decrease in food intake after the induction of a stress (21, 41). Although it is well known that CRF2R contributes to the decrease of appetite in stressed animals (10, 40), icv administration of a CRF2R antagonist did not improve the decrease in food intake, although the CRF2R antagonist concentrations were sufficient to block functions through activation of CRF2R (10, 40). Thus, it appears that CRF1R regulates feeding behavior immediately after a novelty stress. However, the effect on the decreased food intake because of administering the CRF1R antagonist was observed only 1 h after the novelty stress, implying that CRF1R participates in feeding behavior only during the initial stage of the novelty stress.

We found that hypothalamic NPY mRNA decreased significantly 3 h after the novelty stress, whereas POMC mRNA increased. Intracerebroventricular administration of the 5-HT1B/2CR antagonist resulted in inhibiting appetite suppression 3 h after the novelty stress. Furthermore, the decrease in food intake level 3 h after the novelty stress was significantly higher than that of the control mice in the mCPP experiment. Recent studies have demonstrated that central 5-HT1B/2CR participates in controlling appetite (18, 29, 38). Inhibiting NPY/AGRP synthesis and secretion occurs because of the activation of 5-HT1B in NPY/AGRP neurons of the hypothalamic ARC (17). Moreover, activating 5-HT2CR in POMC neurons increases α-MSH release (16), thereby inducing a decrease in food intake. Our results suggest that an increase in 5-HT1B/2CR activity occurred after the novelty stress and induced a reduction in feeding behavior.

Coadministering CRF and mCPP extended the reduction in food intake by 3 h after the administration compared with CRF alone. Intracerebroventricular administration of CRF regulates 5-HT and 5-hydroxyindoleacetic acid (a 5-HT metabolite) levels in the RN (24, 44) and hypothalamus (9). Circulating CORT increases hippocampal 5-HT2CR mRNA expression (19, 20), and social isolation also enhances mCPP-responsiveness (11). On the basis of these findings, it is probable that activating CRF1R during the early stages after the novelty stress may stimulate an affinity of 5-HT1B/2CR with regard to the decrease. However, some studies have shown that CRF mRNA expression and ACTH secretion are enhanced by 5-HT2CR activation when 5-HT is administered to the PVN (18, 22). These reports suggest that 5-HT2R activation affects activation of the HPA axis. It is probable that the CRF1R and 5-HT2R signals mutually influence one another. We did not show directly that CRF1R activated 5-HT1B/2CR; hence, it will be necessary to conduct a further detailed investigation.

In this study, plasma ghrelin decreased in mice exposed to the novelty stress compared with that in the control group, and the decrease was restricted by treatment of CRF1R antagonist. Furthermore, an icv injection of CRF or mCPP also decreased plasma ghrelin levels 1 h after injection, and the decreased acyl ghrelin level following the novelty stress or CRF injection was completely abolished by a 5-HT1B/2CR receptor antagonist. Reduced peripheral ghrelin has been demonstrated in fed mice overexpressing CRF (42). We established previously that an intraperitoneal injection of mCPP inhibits ghrelin secretion from the stomach to the circulation (47). These results suggest that a central increase in CRF release and/or CRFR1 activation may be involved in the decreased ghrelin secretion via 5-HT1B/2CR activation, leading to a sustained decrease in feeding behavior.

POMC, a precursor to the endogenous MC4R agonist α-MSH, is produced in only two locations in the brain, the ARC of the hypothalamus and the commissural NTS. It is believed that POMC neurons in the ARC project to the PVN.
and stimulate MC4R in the PVN via release of α-MSH (16, 18). Surprisingly, we observed that the icv administration of an MC4R antagonist also inhibited the decrease in plasma ghrelin level in mice exposed to the novelty stress. It has not been reported that activating MC4R, following POMC neuron activation, leads to a decrease in peripheral ghrelin concentrations. These results indicate that activating 5-HT1B/2C-R and MC4R may contribute to the decrease in ghrelin secretion after the stress. In contrast, MC4R is highly expressed in the dorsomedial nucleus (DMN), which is the point of origin of the vagal efferent nerves as well as the PVN (50). c-Fos expression in the DMN is facilitated in CRF-overexpressing mice (42). Moreover, POMC neurons in the ARC project to the NTS of the dorsal vagal complex (3), which projects to the DMN (6). These observations support the possibility that activating MC4Rs in the NTS by activating 5-HT1B/2C receptors on POMC neurons in the ARC may suppress ghrelin secretion from the stomach via efferent vagal nerves.

Our results showed that circulating acylated ghrelin levels in control mice gradually increased from 1 h to 3 h. Circadian rhythms in blood ghrelin levels have also been reported in rodents (5) under no influence of fasting condition (26). It is likely that the changes in ghrelin levels in the control mice may be due to the diurnal rhythms.

To clarify whether a decrease in peripheral ghrelin levels participated in the appetite loss caused by the novelty stress, we used exogenous ghrelin and RKT, a drug that enhances ghrelin secretion in rodents (12, 47, 51) and humans (34). Intravenous administration of ghrelin inhibited the reduction in food intake caused by the novelty stress, and administering the GHS-R antagonist reversed the decrease in food intake compared with mice exposed to the novelty stress. Administration of exogenous ghrelin appeared to suppress stress-induced decreases in food intake by temporarily raising blood ghrelin above the physiological concentration.

Oral administration of RKT inhibited the reduction of food intake at 1 and 3 h, and coadministration of the ghrelin receptor antagonist [d-Lys3]GHRP-6 with RKT abolished this effect in mice exposed to the novelty stress. Additionally, RKT also increased plasma acyl ghrelin concentrations at 1 and 3 h after the novelty stress, suggesting that blocking the decrease in endogenous peripheral ghrelin in mice exposed to the novelty stress also acts to sustain feeding behavior. Bolus administration of exogenous ghrelin and GHS-R agonists is often used when investigating the medicinal action of ghrelin. However, pharmacological maintenance of endogenous circulatory hormones such as ghrelin is extremely difficult in mice and other rodents. RKT has also been studied using facilitated ghrelin secretion to clarify the role of endogenous ghrelin (3, 52). Although RKT is crude, a single dose of RKT stabilizes endogenous ghrelin concentration and maintains the level (for ~6 h) (unpublished data). Administration of RKT may be suited to examine the role of endogenous ghrelin.

We found that the oral administration of glycyccoumarin and isoliquiritigenin inhibited the reduction in food intake as well as RKT in mice exposed to the novelty stress. Glycycoumarin and isoliquiritigenin potently inhibit 5-HT2C receptor ligand binding (47). We have reported that orally administering RKT abolishes the decrease in food intake in mCPP-treated rats (51). These findings support the possibility that RKT improved the decreases in food intake and plasma ghrelin levels via 5-HT2C-R antagonism-like action in mice exposed to the novelty stress. In addition, glycyccoumarin and isoliquiritigenin also have agonistic-like action for the other subtypes of 5-HTR, such as 5-HT2AR (47). Because we did not investigate the possibility that the activation of the other 5-HTR subtypes may be associated with the decrease in peripheral ghrelin level, it will be necessary to conduct a further detailed investigation.

On the basis of these results, we demonstrated for the first time that a decrease in plasma ghrelin may participate in the decrease in food intake caused by an environmental change via 5-HT1B/2C-R and MC4R activation.

In conclusion, factors contributing to appetite loss may be altered during each stage of the novelty stress. We conclude that activating CRF1R results in appetite loss during the early stage in mice exposed to an environmental change. Subsequently, central 5-HT1B/2C-R and MC4R is activated, facilitating appetite suppression in later stages. By decreasing peripheral ghrelin via central 5-HT1B/2C-R and MC4R activation, a continuous decrease in food intake may further facilitate the maintenance of appetite suppression.

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