Impairment of gastric accommodation induced by water-avoidance stress is mediated by 5-HT$_{2B}$ receptors

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Key Points
- Psychological stress causes impaired gastric accommodation (GA), which is associated with early satiety in patients with functional dyspepsia. However, the mechanism by which stress inhibits GA remains unknown.
- The aim of this study was to investigate the influence of stress on GA, and the involvement of 5-HT$_{2B}$ receptors in the impairment of GA using an animal model.
- After subjection to water-avoidance stress, GA in conscious guinea pigs was evaluated by measuring the intrabag pressure following administration of a liquid meal.
- The present study revealed that GA was inhibited by stress, and that this inhibition was mediated by an increased responsiveness of 5-HT$_{2B}$ receptors in the gastric fundus.

Abstract
Background Psychological stress has been shown to impair gastric accommodation (GA), but its mechanism has not been elucidated. This study was conducted to clarify the role of 5-HT$_{2B}$ receptors in a guinea pig model of stress-induced impairment of GA.
Methods Gastric accommodation was evaluated by measuring the intrabag pressure in the proximal stomach after administration of a liquid meal. The guinea pigs were subjected to water-avoidance stress. The role of 5-HT$_{2B}$ receptors in impairment of GA was investigated by administering a 5-HT$_{2B}$ receptor agonist (BW723C86) or antagonist (SB215505), the traditional Japanese medicine rikkunshito (RKT), a muscarinic M$_3$ receptor antagonist (1,1-dimethyl-4-diphenylacetoxy piperidinium iodide [4-DAMP]), or a nitric oxide synthase inhibitor (N$_{\omega}$-nitro-L-arginine [L-NNA]).
Key Results In normal animals, liquid meal-induced GA was inhibited by BW723C86, but was not affected by SB215505. The inhibition of GA by BW723C86 was reversed by co-administration of 4-DAMP. Compared to normal animals, GA in stressed animals was significantly inhibited. SB215505 and RKT significantly suppressed stress-induced impairment of GA. After meal administration, the level of cyclic guanosine monophosphate in gastric fundus tissue increased by approximately twofold in normal animals, but did not change in stressed animals. The inhibition of GA by L-NNA was suppressed by SB215505 or RKT. At a dose that did not affect GA in normal animals, BW723C86 exacerbated the impairment of GA in stressed animals. Conclusions and Inferences Stress-induced impairment of GA may be mediated by an increased responsiveness of 5-HT$_{2B}$ receptors, and activation of the 5-HT$_{2B}$ receptor signaling pathway may have an inhibitory effect on nitric oxide function.

Keywords 5-HT$_{2B}$ receptor, acetylcholine, gastric accommodation, nitric oxide, stress.

Abbreviations: 4-DAMP, 1,1-dimethyl-4-diphenylacetoxy piperidinium iodide; 5-HT, serotonin; cGMP, cyclic guanosine monophosphate; cNOS, constitutive nitric oxide synthase; FD, functional dyspepsia; GA, gastric...
accommodation; HPD, hesperidin; ILG, isoliquiritigenin; L-NNA, Nω-nitro-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; RKT, rikkunshito; WAS, water-avoidance stress.

INTRODUCTION

Gastric accommodation (GA) is a physiological function that allows the stomach to accommodate large amounts of food via a reflex response in the proximal stomach that is triggered when food enters the stomach; this response leads to a decrease in gastric tone and an increase in gastric compliance. Nitricergic neurons play an important role in GA, and it has been suggested that the reflex response is induced by nitric oxide (NO) from intrinsic nerves. From investigations on the relationship between NO and GA, it has been reported that inhibition of NO synthase suppresses GA and increases satiety in healthy persons. Gastric accommodation is regulated by variations in gastric tone, which maintains tonic muscular contraction in the proximal stomach; this gastric tone is maintained by vagal cholinergic input. Previous reports have shown that blockade of the muscarinic M3 receptor increased gastric compliance. These indicate that GA is regulated by both inhibitory and excitatory motor neurons. In addition, the serotonin (5-hydroxytryptamine [5-HT])1-like receptor also modulates GA through activation of a nitricergic pathway.

Functional dyspepsia (FD) is a disorder in which symptoms, such as early satiety, bothersome postprandial fullness, epigastric pain, and epigastric burning, occur in the absence of an organic disease that can explain them. Impaired GA is seen in 40% of FD patients, and may be related to the symptoms of early satiety. However, the mechanism by which impaired GA occurs has not been fully elucidated.

Psychological stress can trigger the onset of FD, and decreased gastrointestinal motility is also known to be directly or indirectly involved. Studies in rodents have found that stress decreases feeding, enhances gastric contractions, stimulates colonic transit, and causes visceral hyperalgesia. These findings demonstrate that stress induces abnormalities in gastrointestinal function. However, the relationship between stress and impairment of GA has not been fully elucidated.

Peripheral release of 5-HT has been shown to be deeply involved in stress-mediated alterations of gastrointestinal function. 5-HT3B receptors are widely distributed in the gastrointestinal tract, where they mediate the contraction of smooth muscles in the gastric fundus of rats. A previous study has shown that a 5-HT3B receptor antagonist reversed stress-induced colonic hypersensitivity in a rat model of brain-gut axis dysfunction. Another study reported that 5-HT3B receptor antagonists suppressed a decrease in food intake in a rat novelty stress model. However, there have not been any studies on the relationship between 5-HT3B receptors and stress-induced impairment of GA.

In this study, we first investigated the effect of water-avoidance stress (WAS) on GA. Later, we examined the effect of an agonist and an antagonist of 5-HT3B receptors on stress-induced impairment of GA. Finally, in order to elucidate the mechanism of 5-HT3B receptor-mediated reduction in GA in stressed guinea pigs, we investigated the involvement of NO and acetylcholine.

MATERIALS AND METHODS

Animals

Male 4-week-old Hartley guinea pigs were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and used in experiments after 1 week of acclimation. During the experimental period, the animals were maintained in a 12:12-h light-dark cycle [light: 7.00 a.m. to 7.00 p.m.] at 23 ± 3 °C [room temperature] and 50% ± 20% humidity, and were provided with food and water ad libitum. All experiments were conducted between 8.00 a.m. and 5.00 p.m., and the procedures were in compliance with the guidelines established by the Animal Ethics Committee of Tsumura & Co. [12-008 and 13-042].

Animal preparation

A polyethylene bag was placed in the stomach of the animals, as reported elsewhere. Briefly, after approximately 16 h of fasting, each guinea pig was anesthetized with sodium pentobarbital [30 mg/kg bodyweight, intraperitoneally; Kyoritsu Seiyaku Corporation, Tokyo, Japan], and a 2- to 3-cm upper abdominal laparotomy was performed to expose the stomach. The polyethylene bag [maximum capacity, 14 mL; 0.01 mm in thickness] was inserted into the proximal stomach from the distal part of the stomach, and was left in place. A polyethylene tube (PE 60; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) that was connected to the polyethylene bag was threaded under the skin to exit the body from the back of the neck. A polyurethane catheter (BC-3P; Access Technologies, Skokie, IL, USA) was inserted into the right external jugular vein for systemic administration of drugs, and was left in place. To prevent blood clots, the polyurethane catheter was flushed once every 2 days with a heparin sodium solution [100 unit/mL; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan].

Water-avoidance stress

Animals were subjected to WAS by using a procedure modified from one reported elsewhere. The experiments were started...
3–5 days after the placement of the polyethylene bag. For acclimation to changes in the environment, the animals in the sham-stress and stress groups were placed on a platform (10 cm in width, 10 cm in depth, and 6 cm high) in the middle of a box (40 cm in width, 55 cm in depth, and 27 cm high) for 1 h daily for three consecutive days prior to the water avoidance task. The WAS protocol for the stress group consisted of filling the box with fresh water at room temperature up to 2 cm from the top of the platform and placing each guinea pig on the platform for 1 h daily for two consecutive days. Animals in the sham-stress group were also placed on the platform, but in a waterless box, for 1 h daily for two consecutive days. Animals in the normal group were kept in housing cages until the measurements of GA, and were not subjected to the sham stress or stress task.

The plasma corticosterone level and fecal pellet output were monitored after the sham or water avoidance task in animals without placement of the polyethylene bag. The corticosterone level was determined using an enzyme-linked immunoassay kit (ASSAYPRO, St. Charles, MO, USA).

### Measurement of liquid meal-induced GA

Gastric accommodation was measured in conscious animals by using a protocol reported elsewhere. Briefly, to measure the baseline intrabag pressure of the polyethylene bag, the abovementioned polyethylene tube was connected to a pressure transducer [MLT0699, ADInstruments Pty. Ltd., Bella Vista, Australia], and a syringe pump (KDS-200, KD Scientific Inc., Holliston, MA, USA) was used to inject 6 mL of air into the bag at a flow rate of 2 mL/min. Five minutes after the start of air injection, the intrabag pressure was recorded for 1 min using a blood pressure amplifier (BP Amp, ADInstruments Pty. Ltd.) and a data acquisition device (PowerLab 4/26, ADInstruments Pty. Ltd.). Later, the air was withdrawn from the bag, and the animals were allowed to rest for about 10 min. Measurements of GA in animals subjected to the stress tasks were performed after the second day of the task. After the tasks, a liquid meal (4 mL, 1.7 kcal) was orally administered using a gavage needle (RZ-2, CLEA Japan, Inc., Tokyo, Japan). The meal consisted of powdered food pellets for guinea pigs [CG-7 (278.1 kcal, 18.1% (w/w) crude protein, 3.4% (w/w) crude fat, and 16.8% (w/w) crude fiber per 100 g)] and crude fiber [CLEA Japan, Inc.] suspended in distilled water at 15% (w/v) and then homogenized with a Polytron homogenizer (KINEMATICA, Luzern, Switzerland). Immediately after administration of the meal, 6 mL of air was again injected into the polyethylene bag, and the intrabag pressure was recorded for 1 min at 5, 10, 15, 20, 25, and 30 min after the start of air injection.

### Preparation and administration of pharmacological agents

The following pharmacological agents were used: the 5-HT2B receptor agonist BW732C86 (Sigma-Aldrich Co., LLC, St. Louis, MO, USA), the 5-HT2B receptor antagonist SB215505 (Sigma-Aldrich Co., LLC), the M3 cholinergic receptor antagonist 1,1-dimethyl-4-diphenylacetoxypiperidium iodide (4-DAMP, TOCRIS Bioscience, Bristol, UK), the Japanese traditional medicine rikkunshito [RKT; Tsumura and Co., Tokyo, Japan], the RKT components hesperidin (HPD; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and isoliquiritigenin (ILG; Sigma-Aldrich Co., LLC), and the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine (L-NNA, Sigma-Aldrich Co., LLC). BW732C86 (0.2–6 mg/kg) and SB215505 (1 and 6 mg/kg) were suspended in a saline solution containing 0.3% Tween 80, and orally administered by gavage 20 and 30 min before the liquid meal (premeal), respectively. 1,1-dimethyl-4-diphenylacetoxypiperidium iodide (15–135 μg/kg) was dissolved in saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and intravenously administered 15 min premeal. Rikkunshito is a powder prepared by extracting a mixture of Glycyrrhiza radix, Zingiberis rhizoma, Atractylodis lanceae rhizoma, Zizyphi fructus, Citri unshiu pericarpium, Ginseng radix, Pinelliae tuber, and Poria in hot water, and then spray drying the resulting extract. Rikkunshito [250–1000 mg/kg] was suspended in distilled water, and orally administered by gavage 30 min premeal. N-nitro-L-arginine (1–10 mg/kg) was dissolved in distilled water, and orally administered by gavage 1 h premeal.

### Measurement of cNOS activity and total NO level in fundus

Samples of gastric fundus tissue were obtained from animals in the normal and stress groups immediately after the water avoidance task on day 2; the samples were immediately frozen in liquid nitrogen. Subsequently, each sample was homogenized in phosphate-buffered saline (PBS), and the homogenate was centrifuged at 10 000 g for 20 min at 4 °C. The protein concentration of the supernatant containing 60 μg of protein was then diluted with reaction buffer (50 mM HEPES, 1 mM MgCl2, 0.2 mM CaCl2), and an Ultrasensitive Colorimetric Assay for nitric oxide synthase (NOS) (Oxford Biomedical Research, Oxford, MI, USA) was used to measure the constitutive NOS (cNOS) activity. To measure the total NO level, each supernatant sample was filtered in a Vivaspin 500 Centrifugal Concentrator (GE Healthcare, Buckinghamshire, UK), and the total NO level in 20 μL of filtrate was measured using a Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance and fluorescence intensity were measured with a SpectraMax 190 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and an Infinite 200 Multimode Reader (Tecan Group Ltd., Männedorf, Switzerland), respectively.

### Measurement of cGMP level in fundus

Samples of gastric fundus tissue were obtained from animals in the normal and stress groups immediately after the water avoidance task on day 2 premeal, and 10 min after the liquid meal (postmeal); the samples were immediately frozen in liquid nitrogen. Subsequently, each sample was homogenized in sample diluent buffer, and the homogenate was centrifuged at 1000 g for 15 min at 4 °C. The protein concentration of the supernatant was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). The cyclic guanosine monophosphate (cGMP) level was measured using a DetectX High Sensitivity Direct Cyclic GMP Chemiluminescent Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA). Chemiluminescent intensity was measured using a FlexStation 3 Microplate Reader (Molecular Devices, LLC).

### Measurement of nNOS and 5-HT2B receptor protein expression levels in fundus

Samples of gastric fundus tissue were obtained from animals in the normal and stress groups immediately after the water

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avoidance task on day 2; the samples were immediately frozen in liquid nitrogen. Subsequently, each sample was homogenized in RIPA buffer (Thermo Fisher Scientific, LLC), and the homogenate was centrifuged at 15,000 g for 20 min at 4 °C. The protein concentration of the supernatant was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Neuronal nitric oxide synthase (nNOS) and 5-HT _{2B} receptors were detected by western blotting. To detect nNOS and 5-HT _{2B} receptors, a portion of each supernatant containing 40 and 20 μg of protein, respectively, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After blocking, immunoblotting was performed using rabbit anti-nNOS antibody (1:500, Thermo Fisher Scientific, LLC), mouse anti-5-HT _{2B} receptor IgG (1:800; BD Bioscience, San Jose, CA, USA), or goat anti-actin IgG (1:5000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), followed by incubation with a goat anti-rabbit IgG hors eradish peroxidase conjugate (1:2000, Life Technologies, Carlsbad, CA, USA) or anti-mouse IgG horseradish peroxidase-linked F(ab') fragment (1:5000, GE Healthcare). Immunoreactivity was visualized with chemiluminescence, and scanning was performed with a Typhoon 9410 Laser Module (GE Healthcare). Blots were normalized to actin for comparisons using ImageQuant software (GE Healthcare).

Detection of 5-HT-positive cells in fundus mucosa

Immediately after the water avoidance task on day 2, animals in the normal and stress groups were anesthetized with sodium pentobarbital (Kyoritsu Seiyaku Corporation), and perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Gastric fundus tissues were postfixed overnight at 4 °C in the same fixative, and dehydrated with 30% sucrose in PBS. Frozen sections cut with a cryostat at a thickness of 30 μm were immersed in PBS containing 0.3% Triton X-100 (PBST), and then blocked with Protein Block (Dako, Glostrup, Denmark). The sections were incubated in anti-5-HT goat IgG (1:2000; ImmunoStar, Inc., Hudson, WI, USA), followed by incubation with anti-goat IgG Alexa 488 (1:2000; GE Healthcare). The number of 5-HT-positive cells in the gastric fundus mucosa per 1 mm width was counted under a fluorescence microscope.

Statistical analysis

Multiple comparisons between the normal group, sham stress group, and stress group were performed using the Tukey test and Wilcoxon rank sum test. Intrabag pressure was calculated as the mean pressure over 1 min recorded using LabChart 6 (ADInstruments Pty. Ltd.). The mean change in intrabag pressure ([mean Δ intrabag pressure)] was calculated by averaging the differences between the premeal baseline and the postmeal pressures at 5-min intervals. Temporal changes in intrabag pressure (%) were analyzed with Dunnett’s test by comparing baseline values in each group with values obtained after each measurement interval. Student’s t-tests were performed to compare the normal group with the stress groups and the vehicle-administered group, and the Dunnett’s test, Williams test, or Shirley-Williams test was used to perform multiple comparisons between the vehicle-administered group and the drug-administered groups. The Tukey test or Tukey-Kramer test was used to perform all pairwise comparisons among three or four groups. All data are expressed as the mean ± SD, and were analyzed for statistical significance using StatLight (Yukms Co., Ltd., Tokyo, Japan); p < 0.05 was considered to be statistically significant.

RESULTS

Characterization of the GA in guinea pigs

Gastric accommodation was evaluated by measuring the changes in intrabag pressure (calculating the mean Δ intrabag pressure). Under the normal condition, the mean Δ intrabag pressure decreased at 30 min after liquid meal administration, indicating induction of GA. The NOS inhibitor L-NNA (10 mg/kg) significantly inhibited such GA in a dose-dependent manner (N = 5–6; Fig. 1A). The M₃ cholinergic receptor antagonist 4-DAMP [45 and 135 μg/kg] promoted liquid meal-induced GA in a dose-dependent manner (N = 5, each group) (Fig. 1B). The 5-HT _{2B} receptor agonist BW723C86 [5 mg/kg] significantly inhibited GA, and this inhibitory effect of BW723C86 was reversed by the 5-HT _{2B} receptor antagonist SB215505 [6 mg/kg; N = 5, each group; Fig. 1C]. SB215505 alone (6 and 18 mg/kg) had no effect on GA under the normal condition (N = 4–5; Fig. 1D). The inhibition of GA induced by 5-HT _{2B} receptor agonist BW723C86 [5 mg/kg] was significantly blocked by co-administration of 4-DAMP [135 μg/kg]. When 4-DAMP was administered alone, the induction of GA was significantly greater than that of the vehicle group. There was no significant difference in GA between the BW723C86+4-DAMP group and the 4-DAMP-alone group (N = 5–7; Fig. 1E).

Changes in stress-related parameters and GA induced by WAS

All experiments were performed using five animals in each group. The plasma corticosterone level and fecal pellet output, which are stress-related parameters, were measured to determine whether the water avoidance task induced a stress response in guinea pigs. The plasma corticosterone level and fecal pellet output

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Figure 1  Characterization of the GA in guinea pigs. Under normal conditions, L-NNA (10 mg/kg) inhibited liquid meal-induced GA [A]. By contrast, 4-DAMP [45 and 135 μg/kg] promoted liquid meal-induced GA [B]. BW723C86 [5 mg/kg] inhibited liquid meal-induced GA. This inhibitory effect was reversed by SB215505 [6 mg/kg; C]. SB215505 alone (6 and 18 mg/kg) had no effect on GA [D]. 4-DAMP [135 μg/kg] blocked the inhibitory effect of BW723C86 [5 mg/kg] on GA [E]. Data are expressed as the mean ± SD of four to seven animals. *p < 0.05 (Shirley-Williams test); †p < 0.05 and ‡p < 0.01 (Williams test); ††p < 0.05 (Student’s t-test); †‡p < 0.05, and NS, not significant [Student’s t-test or Dunnett’s test]; †††p < 0.01 and †‡‡p < 0.001 (Tukey-Kramer test).
were significantly higher in the stress group than in the normal group or sham stress group (Fig. 2A and B).

At 5–20 min after liquid meal administration, the intrabag pressure of the normal group significantly decreased from the basal level [decreases of 37.2–42.1%]. The same response was seen in the sham stress group. By contrast, in the stress group, the intrabag pressure showed no significant differences from the basal level after liquid meal administration [Fig. 2C]. The mean Δ intrabag pressure of the normal and sham stress groups decreased in a similar manner in the 30-min period after liquid meal administration. By contrast, the mean Δ intrabag pressure of the stress group increased, and was significantly different from those of the normal and sham stress groups, indicating inhibition of GA (Fig. 2D).

**Relationship between 5-HT₂B receptors and GA under the stress condition**

Under the stress condition, the 5-HT₂B receptor antagonist SB215505 (6 mg/kg) significantly suppressed stress-induced inhibition of GA \( (N = 5, \) each group; Fig. 3A). Furthermore, the stress-induced inhibition of GA was suppressed by administration of RKT at 500 and 1000 mg/kg \( (N = 5, \) each group; Fig. 3B). The RKT components HPD (5 mg/kg) and ILG (0.25 mg/kg) at doses equal to their estimated doses in 1000 mg/kg of RKT also significantly suppressed the stress-induced inhibition of GA \( (N = 5–7, \) Fig. 3C). In addition, the relationship between the cholinergic pathway and GA under the stress condition was also investigated; results showed that 4-DAMP at 45 and 135 \( \mu \)g/kg...
significantly suppressed the stress-induced inhibition of GA \((N = 5–6; \text{Fig. 3D})\).

**Changes in cNOS activity, total NO level, and cGMP level in fundus**

The cNOS activity, nNOS protein expression level, and total NO level in gastric fundus tissue were measured in the normal and stress groups to determine whether WAS alters the function and expression levels of NO-related molecules. For these experiments, five animals were used in each group. Compared with the normal group, none of these parameters showed a significant difference in animals immediately after WAS (Fig. 4A–C).

At pre-meal, the cGMP levels of the normal and stress groups did not differ significantly, but the level
in the stress group tended to be lower. At post-meal, the cGMP level increased by approximately twofold in the normal group, but did not increase in the stress group ($N=9$–$10$; Fig. 4D).

**Relationship between 5-HT$_{2B}$ receptors and NOS inhibitor-induced impairment of GA**

These experiments were performed using five animals in each group. The inhibition by L-NNA was significantly suppressed by the 5-HT$_{2B}$ receptor antagonist SB215505 at 1 and 6 mg/kg [Fig. 5A], RKT at 250, 500, and 1000 mg/kg [Fig. 5B], and ILG at 0.8 mg/kg [Fig. 5C].

**Alteration of 5-HT$_{2B}$ receptor responsiveness to an agonist under the stress condition**

Under the normal conditions, the 5-HT$_{2B}$ receptor agonist BW723C86 at 0.2 and 0.6 mg/kg had little influence on liquid meal-induced GA. Under the stress condition, however, BW723C86 at 0.6 mg/kg exacerbated the inhibition of GA ($N=5$–$7$; Fig. 6A).
There was no significant difference in the 5-HT$_{2B}$ receptor protein expression level in the gastric fundus between the normal group and the stress group immediately after WAS ($N = 5$, each group; Fig. 6B). In the fundus of animals in the normal and stress groups, there was positive staining for 5-HT in the lower mucosal layer (Fig. 6C). There was no significant difference in the number of 5-HT-positive cells per unit length of mucosa between the two groups ($N = 5$, each group; Fig. 6D).

**DISCUSSION**

In this study, we found that: GA was impaired by stress, and administration of a 5-HT$_{2B}$ receptor antagonist reversed that impairment, administration of a
5-HT$_{2B}$ receptor agonist to stressed animals exacerbated the inhibition of GA, and the inhibition of the NO pathway was involved in 5-HT$_{2B}$ receptor-mediated impairment of GA.

We first examined the effects of reference drugs to clarify the characterization of GA in guinea pigs. Gastric accommodation is controlled by the balance between the activation of inhibitory motor neurons and the activation of excitatory motor neurons, such as that from acetylcholine. In humans, GA increases as a result of the activation of nitrergic neurons, and decreases as a result of the activation of cholinergic neurons. In this study, the NOS inhibitor L-NNA inhibited liquid meal-induced GA, while the muscarinic M$_3$ receptor antagonist 4-DAMP promoted liquid meal-induced GA; these results are in agreement with those of previous reports. Next, in the normal condition, we examined the role of 5-HT$_{2B}$ receptors on GA, and the relationship between 5-HT$_{2B}$ receptors and acetylcholine; we found that the 5-HT$_{2B}$ receptor agonist BW723C86 inhibited GA in normal animals, but interestingly, the 5-HT$_{2B}$ receptor antagonist SB215505 did not inhibit GA in normal animals. These results suggest that 5-HT$_{2B}$ receptors are not actively involved in the regulation of GA that occurs under the physiological conditions of normal animals. Furthermore, the inhibition of GA induced by BW723C86 was blocked by co-administration of 4-DAMP. This result indicated that the activation of 5-HT$_{2B}$ receptors can cause the release of acetylcholine. However, in strips of guinea pig gastric fundus, stimulation of 5-HT$_2$ receptors induced tetrodotoxin (TTX)-insensitive contractions. In addition, in strips of circular muscle from rat gastric fundus, the 5-HT-induced smooth muscle

Figure 6: Alteration of 5-HT$_{2B}$ receptor responsiveness to an agonist under the stress condition. BW723C86 at a dose of 0.6 mg/kg that did not affect GA in the normal group exacerbated the stress-induced inhibition of GA (A). The expression level of 5-HT$_{2B}$ receptor protein in gastric fundus (B) and the number of 5-HT-positive cells in fundus mucosa (C and D) did not differ significantly between the normal and stress groups. Data are expressed as the mean ± SD of five to seven animals. *p < 0.05 (Dunnett’s test); NS, not significant (Student’s t-test). Scale bar, 100 μm.
contraction response was blocked by a 5-HT$_{2B}$ receptor antagonist; this blocking was not affected by the presence of atropine or TTX. Furthermore, in the presence of atropine, contraction induced by 5-methoxytryptamine, a 5-HT$_{3B}$ and 5-HT$_{4}$ receptor agonist, was inhibited in strips of rat fundus. These findings suggest two possibilities: that the 5-HT$_{2B}$ receptor-mediated smooth muscle contraction effect results from direct action on the receptors of smooth muscle cells and/or on TTX-insensitive and non-cholinergic neurons. In other words, there may be two mechanisms for the effects of 5-HT$_{2B}$ receptors in gastric fundus relaxation: direct action on fundus smooth muscle, and indirect action by the release of acetylcholine. Further studies are necessary to clarify the precise mechanisms.

Water-avoidance stress is widely used as a model of physical and psychological stress. In the colon, WAS stimulates motility mediated by the central corticotropin-releasing factor (CRF) pathway and causes colonic hyperalgesia. In the stomach, WAS delays gastric emptying and enhances gastric contractions mediated by peripheral CRF1 receptors. Suspecting that WAS might also impair GA, we used the WAS technique in this study. Administration of a liquid meal decreased the pressure of the intrabag implanted in the proximal stomach of guinea pigs, but WAS suppressed that decrease. Similar to a previous report, the plasma corticosterone level and fecal pellet output were higher in the stress group than in the normal (unstressed) group or the sham stress group in our study. The above results showed that WAS induced impairment of GA under a state of stress.

We inferred that the change in environment when the animals were transferred from the housing cages to the cages used for the water avoidance task caused stress; so, we included a sham stress group. There was no difference in GA between the normal and the sham stress group. However, the plasma corticosterone level was increased in the sham stress group when compared to the normal group. We conducted the normal group as a control of the stress group to evaluation of GA.

This study showed that a 5-HT$_{2B}$ receptor agonist inhibited GA in normal animals, and that a 5-HT$_{2B}$ receptor antagonist reversed the decrease in GA in stressed animals while having no effect in normal animals. These results suggest that 5-HT$_{2B}$ receptors control GA by exerting suppressive effects under pathophysiological conditions such as stress. Rikkunshito is known to enhance GA in humans, dogs, and guinea pigs. A WAS-induced decrease in GA was suppressed by RKT and its components HPD and ILG, which have also been reported to have 5-HT$_{2B}$ receptor-antagonist activity. These results provide additional evidence that stress-induced impairment of GA is mediated by the activation of 5-HT$_{2B}$ receptors.

Administration of the 5-HT$_{2B}$ receptor agonist to stressed animals at doses that did not affect GA in normal animals exacerbated the impairment of GA in the stressed animals, indicating increased responsiveness of 5-HT$_{2B}$ receptors. Factors that can affect the responsiveness of a receptor include changes in the receptor expression, activation of the receptor’s signal transduction system, and changes in the affinity of the receptor for ligands. In this study, there was no difference in the 5-HT$_{2B}$ receptor protein level in the gastric fundus wall between the normal and stress groups. Some animal studies have demonstrated that CRF released from the central nervous system by stress promotes peripheral release of 5-HT. In addition, CRF1/2 ligands can stimulate the release of 5-HT from endocrine-like cells. Based on that indirect evidence, one could hypothesize that WAS inhibits GA as a consequence of causing contraction of fundus smooth muscle by stimulating the local release of 5-HT in the stomach and thereby activating 5-HT$_{2B}$ receptors. Because cisplatin-treated dogs showed increased numbers of 5-HT-immunoreactive cells in ileal mucosa, we counted the 5-HT-positive cells in the epithelial layer of the gastric fundus mucosa of our guinea pigs. However, the number of these cells per unit length of mucosa did not differ between the normal group and the stress group. In a clinical study, it was reported that patients with FD had lower postprandial plasma 5-HT levels when compared to asymptomatic healthy subjects. In another study, plasma 5-HT levels were higher only in patients with epigastric pain syndrome, and not in those with postprandial distress syndrome. These reports suggested that plasma 5-HT levels are not increased in FD patients, even those with postprandial distress syndrome, and the significance of plasma 5-HT in the pathogenesis of FD should be further investigated. Regarding this study, it is likely that stress-induced 5-HT$_{2B}$ receptor activation may be caused by the reactivity of the receptors rather than by increased 5-HT$_{2B}$ receptor expression or 5-HT in the gastrointestinal tract.

Nitric oxide from intrinsic nerves is also involved in the regulation of GA, and NOS inhibitors have been shown to impair GA in rodents and healthy humans. Nitric oxide of neuronal origin is synthesized from L-arginine by nNOS, and it acts on the cells of smooth muscle, which is an effector, inducing a relaxation response. Because they are constitutively expressed in tissues, both nNOS and endothelial NOS
are referred to as cNOS. In this study, there was no difference between the normal and stress groups in cNOS activity, nNOS protein expression level, or total NO level in gastric fundus tissue. By contrast, the level of cGMP, which is a second messenger for NO in gastric fundus, increased by twofold in the normal group after induction of GA by administration of a liquid meal; this increase was not seen in the stress group. Although it is technically difficult to measure nNOS activity and NO of nNOS origin selectively, the cGMP level results suggest that stress may cause a decrease in the level of NO produced by nNOS and/or an acceleration of cGMP hydrolysis.

We also examined the relationship between 5-HT2B receptors and the reduction in GA caused by L-NNA-induced inhibition of NO function. The reduction in GA induced by L-NNA was suppressed by the 5-HT2B receptor antagonist SB215505, RKT, and ILG. It has been reported that aortic contraction response to 5-HT and 5-HT2B receptor agonists is greater in type 2 diabetic mice than in normal mice, and that activation of the RhoA/Rho kinase pathway in vessels is involved in that excessive response.38,39 These findings indicated that RhoA/Rho kinase is involved in the 5-HT2B receptor signaling pathway, and that activation by some kind of factor causes the increased responsiveness of 5-HT2B receptors. Contractile agonists decrease cGMP levels by activating cGMP-specific phosphodiesterase type 5 (PDE5) via the RhoA pathway in gastric muscle cells.40 By contrast, cGMP/cGMP-dependent protein kinase inactivates RhoA signaling by phosphorylating RhoA,41 suggesting that the cGMP and RhoA/Rho kinase pathways influence each other. Combining these findings, it is likely that L-NNA caused the decrease in cGMP and the activation of the RhoA/Rho kinase pathway in guinea pigs fundus, suggesting increased responsiveness of 5-HT2B receptors to 5-HT. Thus, the 5-HT2B receptor antagonist recovered the impaired GA induced by L-NNA. In addition, in this study, the stress-induced increase in responsiveness of 5-HT2B receptors also may be mediated by the activation of the RhoA/Rho kinase pathway in fundus, because the postprandial cGMP level in fundus was not increased in the stress group when compared to the normal group.

Another possibility is that 5-HT2B receptors regulated the plasma 5-HT levels, because it has been reported that stimulation of 5-HT2B receptors triggers a transient increase in plasma 5-HT.42 As the circular muscle in rat fundus presents the relaxant 5-HT2A and 5-HT4 receptors,19 it is possible that the ameliorating effects of the 5-HT2B receptor antagonist on the inhibition of GA are mediated by the plasma 5-HT levels and/or by stimulating other types of relaxant 5-HT receptors, such as 5-HT2A and 5-HT4.

Impairment of distention-induced GA in FD patients can be improved by cholinergic blockade,43 and Wistar Kyoto rats have impaired GA due to increased gastric vagal cholinergic tone.44 We also examined the relationship between impairment of GA induced by stress and the cholinergic pathway. Administration of the

![Figure 7 Schema of putative pathogenic mechanisms of the impairment of gastric accommodation induced by water-avoidance stress.](image-url)
muscarnic M₃ receptor antagonist 4-DAMP reversed the impairment of GA induced by stress, suggesting that WAS may cause activation of the cholinergic pathway. Contractile agonists, such as acetylcholine, attenuate cGMP levels by stimulating phosphorylation of cGMP-specific PDE5 via activation of the RhoA pathway. Activation of excitatory pathways and hypofunction of inhibitory pathways may be caused by stress in fundus.

From the above results, we hypothesize that collapse of the balance between excitatory motor neurons that have acetylcholine as a neurotransmitter and inhibitory motor neurons that have NO as a neurotransmitter may cause an increase in the contractile response mediated by 5-HT₂₅ receptors (Fig. 7).

In this study, we were unable to clarify the relationship between 5-HT₂₅ receptors and acetylcholine. In other words, it remains unknown why the responsiveness of 5-HT₂₅ receptors is increased by stress, and how 5-HT₂₅ receptors and NO function are related. Studies focusing on intracranial pathways, and those using fundus strips or genetic ablation would be necessary to clarify such mechanisms.

In conclusion, WAS caused impairment of GA, which may have been mediated by the activation of 5-HT₂₅ receptors. In addition, inhibition of NO function may have been involved in the increase in responsiveness of 5-HT₂₅ receptors.

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AUTHOR CONTRIBUTION
HM designed the research study and contributed to interpretation of data and wrote the manuscript; JK contributed to collection, analysis, and interpretation of data; TO and HT were a supervisor in this study and revised the paper critically for important intellectual content; YK and JW designed the research study and contributed to interpretation of data; TK, HF, TT, and YO contributed to technical support. All authors approved the final version of the manuscript.

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