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Water avoidance stress induces visceral hyposensitivity through peripheral corticotropin releasing factor receptor type 2 and central dopamine D2 receptor in rats

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Running title: Stress and visceral hyposensitivity
Abstract

**Background:** Water avoidance stress (WAS) is reported to induce functional changes in visceral sensory function in rodents, but the results which have been demonstrated so far are not consistent, i.e. hypersensitivity or hyposensitivity. We determined the effect of WAS on visceral sensation and evaluated the mechanisms of the action.

**Methods:** Visceral sensation was assessed by abdominal muscle contractions induced by colonic balloon distention, i.e. visceromotor response (VMR), measured electrophysiologically in conscious rats. The electromyogram electrodes were acutely implanted under anesthesia on the day of the experiment. The threshold of VMR was measured before and after WAS for 1 h. To explore the mechanisms of WAS-induced response, drugs were administered 10 min prior to the initiation of WAS.

**Key results:** WAS significantly increased the threshold of VMR, and this effect was no longer detected at 24 h after. Intraperitoneal injection of astressin2-B (200 µg/kg), a corticotropin releasing factor (CRF) receptor type 2 antagonist abolished the response by WAS. Subcutaneous (sc) injection of sulpiride (200 mg/kg), a dopamine D2 receptor antagonist blocked the response, while sc domperidone (10 mg/kg), a peripheral dopamine D2 receptor antagonist did not alter it. Naloxone (1mg/kg, sc), an opioid antagonist did not modify it either.

**Conclusions & Inferences:** WAS induced visceral hyposensitivity through peripheral CRF receptor type 2 and central dopamine D2 receptor, but not through opioid pathways. Since altered pain inhibitory system was reported to be observed in the patients with irritable bowel syndrome, CRF and dopamine signaling might contribute to the pathophysiology.

**Keywords:** corticotropin releasing factor, dopamine, hyposensitivity, visceral sensation, water avoidance stress
Key Messages

Several reports showed that water avoidance stress (WAS) alters visceral sensation in rodents, but the results which have been demonstrated so far are not consistent.

We evaluated electrophysiologically the effect of acutely submitted WAS for 1 h on the threshold of visceromotor response (VMR) to colonic distention in conscious rats.

WAS increased the threshold of VMR and this response was mediated through peripheral corticotropin releasing factor receptor type 2 and central dopamine D2 receptor.

Since disturbance of visceral pain inhibitory system is thought to possibly contribute to the pathophysiology of irritable bowel syndrome, our results might give a hint to develop a novel therapy for this disease.
INTRODUCTION

Stress alters gastrointestinal (GI) motility and visceral sensation, and central and peripheral corticotropin releasing factor (CRF) is involved in these changes. In addition to CRF, CRF-related peptides, urocortins (urocortin 1, 2 and 3) are prominently expressed in peripheral tissues where they also mediate visceral stress responses. The actions of CRF and urocortins are mediated through the activation of two receptors, CRF receptor type 1 (CRF1) and type 2 (CRF2). Activation of each CRF receptor induces distinct responses in GI tract, i.e. stimulation of colonic motility and inducing visceral hypersensitivity to colorectal distension (CRD) by CRF1 alone, and delayed gastric emptying by CRF2 exclusively. However, since stress stimulates the release of CRF and urocortins, which bind both receptors with their distinct affinity, it is reasonable to think that both CRF receptors are simultaneously activated during stress and possibly contribute to GI response to stress.

We and other researchers demonstrated that activating peripheral CRF1 enhanced colonic contractility and induced visceral sensitization, and these responses were suppressed by peripheral CRF2 signaling. These results strongly support the notion above and also suggest that the activity balance of peripheral CRF1 and CRF2 signaling may determine the functional changes in colonic motor and sensory systems. We designated this concept as the balance theory of peripheral CRF signaling, and this theory could explain well CRF and stress-induced altered colonic motor and sensory functions as described above. Moreover, we also suggested previously that peripheral CRF-induced altered gastric contractility may follow the theory.

Water-avoidance stress (WAS) is a conventional psychological stress protocol, and is well known to activate peripheral CRF signaling in addition to central one, thereby altering GI motility in rats. According to the evidence, WAS is also
thought to alter visceral sensation through peripheral CRF receptors.\textsuperscript{20} In fact, several studies demonstrated that WAS induced functional changes in visceral sensation but the results were conflict, i.e. hypersensitivity and hyposensitivity.\textsuperscript{21-25}

In the present study, we tried to clarify whether WAS induces alteration of visceral sensation through peripheral CRF receptors, and also the response by WAS is explained by the balance theory of peripheral CRF1 and CRF2 signaling. In addition, since several neural systems which may alter nociception during stress such as dopamine, opioid, etc. have been demonstrated,\textsuperscript{26, 27} the role of their systems were also determined.

MATERIALS AND METHODS

Animals

Experiments were conducted in adult male Sprague-Dawley rats (Charles River Laboratory, Atsugi, Japan) weighing about 300 g. Rats were group housed, 3−4 rats/cage, under controlled conditions of illumination (12 h light/dark cycle starting at 7 a.m.) and temperature (23−25 °C) with food (Solid rat chow, Oriental Yeast, Tokyo, Japan) and water available ad libitum.

Chemicals

Astressin\textsubscript{2}-B (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in double-distilled water. Sulpiride (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide. Domperidone and naloxone hydrochloride (Wako Pure Chemical Industries) were dissolved in saline. All drugs were prepared just before the experiment. The dose and administration route of the chemicals were determined according to the previous reports.\textsuperscript{13, 15, 17, 25, 28-30}
**Measurement of visceral sensation**

Visceral sensation was assessed by abdominal muscle contractions in response to colonic distention (visceromotor response; VMR) using electromyogram (EMG) in conscious rats, which was validated as quantitative measure of visceral nociception.\(^{15,31}\)

**Implantation of electrodes and placement of colonic distention balloon**

Under brief ether anesthesia, non-fasted rats underwent incision of skin about 5 mm in length, and four electrodes (Teflon coated stainless steel, 0.05 mm diameter, MT Giken, Tokyo, Japan) for EMG, which are positive, negative and ground ones, and the other one was for spare, were inserted approximately 2 mm into left side external oblique musculature through the incision. They were fixed to musculature by cyanoacrylate instant adhesive (Aron Alpha, TOAGOSEI, Tokyo, Japan) together with the incised skin. The electrode leads were externalized directly through this closed incision without using subcutaneous tunnel and threaded through a urethane tube. Then a distension balloon (6-Fr disposable silicon balloon-urethral catheter, JU-SB0601, Terumo Corporation, Tokyo, Japan) was inserted intra-anally with the distal end positioned 2 cm proximal to the anus. The maximal inflation volume for the balloon was 1.5 mL, and the length of the maximally inflated balloon was 1.2 cm.

**Colonic distention and monitoring abdominal muscle contractions**

After completing the surgery for the electrodes implantation and balloon placement, the rats were placed in Bollmann cages, and were allowed to recover from the anesthesia and adjusted to the experimental condition for 30 min before testing. (The animals were trained to the experimental conditions by placing them singly in Bollmann cages for 1 h before the day of experiment.) Then electrode leads
were connected to an EMG amplifier, and EMG signals were amplified, filtered (3000 Hz), digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and stored by computer software (LabChart 7, AD Instruments). Colonic distension was performed according to a previous publication with minor modification, namely, ascending method of limits phasic distension was applied in increments of 0.1 mL for 5 sec by inflating the balloon by water using a syringe manually until significant abdominal muscle contractions, i.e. VMR, were induced. The threshold of VMR was defined as the distended balloon volume (mL) inducing VMR. Tang et al. previously demonstrated using the balloon quite similar to ours that the pain threshold induced by CRD assessed by the observation of abdominal withdrawal reflex could be determined as distended balloon volume in rats and also reported that intracolonic pressure was linearly associated with intraballoon volume in the experiments. The threshold was assessed two times (2 min interval) and the mean of the threshold was calculated as the data of the animals.

**Stress protocol**

Exposure to WAS was performed as described previously. Rats were placed individually on a plastic platform (height, 8 cm; length, 6 cm; width, 6 cm) positioned in the middle of a plastic cage filled with warm water, temperature around 30 °C up to 7 cm of the height of the platform. To avoid contact with water, rats stood on a platform during the entire stress period but the tail was immersed into the water. Since cold exposure is well known to alter GI functions including visceral sensation, warm water was used in the study. Control animals were also put on the same plastic platform in a plastic cage but not filled with water (sham stress).

**Experimental procedures**
First, the effects of WAS on the threshold of VMR was tested. After measuring the basal threshold, the electrodes and distention balloon were removed. Then, either WAS or sham stress (controls) for 1 h was applied. Immediately after the cessation of stress manipulation, the animals underwent surgery for the electrodes implantation and balloon placement, and put in the Bollmann cages again. After 30 min, the threshold was determined. We also clarified the duration of the visceral sensory response induced by WAS, and the threshold was determined at basal state and 24 h after the stress.

Next, in order to explore the mechanisms of WAS-induced response, drugs were administered 10 min prior to the initiation of WAS or sham stress. Then, the visceral sensory response was compared between drug and vehicle-treated group.

**Statistical analysis**

Data were expressed as means ± S.E. Multiple comparison was performed by one-way repeated measures analysis of variance (ANOVA) or one-way ANOVA followed by Fisher's Least-Significant-Difference Test. Comparison between two groups was performed using the paired t test. SYSTAT 13 software (Systat Software, Chicago, IL, USA) was used throughout the study.

**Ethical considerations**

Approval by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#15132, approved on April 1, 2015) was obtained for all studies.

RESULTs
WAS significantly increased VMR threshold (mL) from 0.51 ± 0.019 to 0.63 ± 0.022 (Fig.1, n = 30, p < 0.05). While, this change was not detected in controls (basal 0.52 ± 0.030 vs. after 0.55 ± 0.025, n = 14, p > 0.05). There was significant interaction (group × condition, i.e. before and after stress) for the threshold (repeated measures ANOVA: F = 4.14, p < 0.05), implying two groups differed in the change of the threshold.

On the other hand, this antinociceptive effect by WAS was no longer observed at 24 h after the stress (Fig.2, basal 0.50 ± 0.018 vs. after 24 h 0.45 ± 0.024, n = 16, p > 0.05). In controls, the threshold was not changed at 24 h after sham stress either (basal 0.52 ± 0.017 vs. after 24 h 0.49 ± 0.024, n = 13, p > 0.05).

Since WAS was definitely demonstrated to increase the threshold as described above, in addition to raw data, the % change in the threshold before and after stress manipulation was also presented in the following experiments in order to assess the effect of drugs.

We tested the effect of intraperitoneal administration of astressin2-B (200 µg/kg), a CRF2 antagonist on WAS-induced response. In vehicle-treated rats, the threshold was not changed after sham stress in controls (Fig.3A, basal 0.52 ± 0.031 vs. after 0.50 ± 0.014, n = 9, p > 0.05), and it was increased after WAS (basal 0.54 ± 0.028 vs. after 0.65 ± 0.021, n = 11, p < 0.05). However, in the antagonist-treated rats, the threshold was neither changed by WAS (basal 0.54 ± 0.029 vs. after 0.52 ± 0.035, n = 13, p > 0.05) nor by sham stress (basal 0.53 ± 0.036 vs. after 0.54 ± 0.030, n = 7, p > 0.05). Figure 3B showed the % change in the threshold. The antagonist abolished the antinociceptive effect by WAS (% change, ANOVA: F = 3.0, p < 0.05, 125.1 ± 9.3 for vehicle + WAS vs. 98.9 ± 7.6 for astressin2-B + WAS, p < 0.05). Astressin2-B itself did not alter the threshold in controls (97.8 ± 4.8 for vehicle + control vs. 104.0 ± 5.3 for astressin2-B + control, p > 0.05).

Next, the effect of subcutaneous (sc) injection of sulpiride (200 mg/kg), a selective dopamine D2 receptor was tested. In vehicle-treated group, the threshold
was not changed in controls (Fig.4A basal 0.53 ± 0.026 vs. 0.54 ± 0.039, n = 10, p > 0.05) but WAS increased the threshold (basal 0.51 ± 0.035 vs. 0.61 ± 0.030, n = 8, p < 0.05). On the other hand, in sulpiride-treated group, neither WAS nor sham stress altered the threshold (sulpiride +WAS, basal 0.53 ± 0.041 vs. 0.48 ± 0.037, n = 8, p > 0.05, sulpiride + control, basal 0.54 ± 0.038 vs. 0.55 ± 0.059, n = 8, p > 0.05). As shown in Figure 4B, the antagonist abolished the effect induced by WAS (% change, ANOVA: F = 3.2, p < 0.05, 122.3 ± 7.5 for vehicle + WAS vs. 92.8 ± 7.5 for sulpiride + WAS, p < 0.05). Meanwhile, sulpiride did not modify the threshold in controls (101.9 ± 5.2 for vehicle + control vs. 102.0 ± 7.2 for sulpiride + control, p > 0.05).

Then in order to determine whether central or peripheral dopamine D2 receptor contributes to the WAS-induced visceral hyposensitivity, the effect of a peripheral D2 receptor antagonist, domperidone at a dose of 10 mg/kg, sc was tested. In vehicle-treated group, the threshold was not changed in controls (Fig.5A basal 0.53 ± 0.041 vs. 0.52 ± 0.031, n = 8, p > 0.05) but WAS increased the threshold (basal 0.51 ± 0.024 vs. 0.71 ± 0.068, n = 5, p < 0.05). The same results were also obtained in domperidone-treated group. WAS increased the threshold (basal 0.53 ± 0.033 vs. 0.75 ± 0.034, n = 6, p < 0.05) and it was not changed in controls (basal 0.54 ± 0.040 vs. 0.52 ± 0.056, n = 5, p > 0.05). As shown in figure 5B, domperidone did not modify the WAS-induced response (% change, ANOVA: F = 5.2, p < 0.05, 137.1 ± 14.7 for vehicle + WAS vs. 143.6 ± 11.4 for domperidone + WAS, p > 0.05), and it did not change the threshold in controls either (101.4 ± 7.0 for vehicle + control vs. 97.3 ± 9.7 for domperidone + control, p > 0.05).

Finally, we tested the effect of naloxone (1 mg/kg, sc) in order to determine the role of opioid system on the WAS-induced hyposensitivity. In vehicle-treated group, the threshold was not changed in controls (Fig.6A basal 0.53 ± 0.034 vs. 0.50 ± 0.016, n = 15, p > 0.05) but WAS increased the threshold (basal 0.51 ± 0.021 vs. 0.69 ± 0.049, n = 8, p < 0.05). In naloxone-treated control group, the threshold was increased a little bit after sham stress but the difference was statistically significant (basal 0.54 ± 0.018 vs. 0.59 ± 0.023, n = 8, p < 0.05). In naloxone-treated WAS group,
the threshold was increased after the stress (basal 0.52 ± 0.025 vs. 0.65 ± 0.035, n = 10, p < 0.05). Naloxone neither modified WAS-induced response (Fig.6B, % change, ANOVA: F = 6.2, p < 0.05, 135.3 ± 10.8 for vehicle + WAS vs. 127.0 ± 7.9 for naloxone + WAS, p > 0.05) nor the change in controls (97.6 ± 5.2 for vehicle + control vs. 109.6 ± 3.6 for naloxone + control, p > 0.05).

DISCUSSION

Several studies showed that WAS altered visceral sensation in rodents, but their results were not consistent. Majority of the studies demonstrated that acute and repeated WAS increased VMR to CRD, i.e. visceral hypersensitivity, which was determined by abdominal muscle contractions measured by EMG using chronically implanted electrodes.21-24 However, using a non-invasive manometry based method with acute preparation, acute WAS was shown to induce visceral hyposensitivity in rats, which was first demonstrated by Larauche et al.25 Moreover, the researchers also reported that repeated WAS (four and ten consecutive days) induced visceral hyposensitivity in rodents.22, 25, 36 The discrepancy of these results may be explained by the difference of method such as invasive vs. non-invasive, or EMG vs. manometry. Besides non-invasive acute preparation allows animals to be group housed, but single housing is needed before the experiment for several days after the surgery of EMG electrodes implantation, which seems significant stress for the animals. The surgery of electrodes implantation with single housing is an important factor to induce repeated WAS-induced visceral hypersensitivity in rodents.22

Present study clearly demonstrated that WAS induced visceral hyposensitivity. The feature of our method is acute preparation with minor surgery, which did not need single housing, which is the advantage. The disadvantage is repeated surgeries were needed. It is not known which factor such as single housing or repeated minor surgery gives a significant impact in visceral sensory response. However, as mentioned above, if the different responses induced by WAS resulted
from the difference of strength of stress related to preparation, minor stress in our method could detect the hyposensitivity response, which was similar result detected by a non-invasive method.25

CRF system is known to contribute to stress-induced altered visceral sensation. We and other researchers reported that WAS or CRD activated central and peripheral CRF1, thereby inducing visceral hypersensitivity in rats.15, 37, 38 Meanwhile, peripheral CRF2 stimulation attenuates CRD-induced visceral sensitization,12, 13, 15 which raises the possibility of contribution of peripheral CRF2 to the antinociceptive response induced by WAS. Then, we tested the role of peripheral CRF2 and demonstrated that astressin2-B blocked the WAS-induced hyposensitivity. It has been thought that endogenous CRF system mediates exclusively stress-induced visceral hypersensitivity but our result demonstrated for the first time, it may also be involved in visceral antinociceptive response induced by stress. In other words, both CRF receptor subtypes are activated by stress, and those may be simultaneously engaged in stress-induced altered visceral sensation, which may further support the validity of our proposed balance theory as described before.15, 16

The basic concept of the balance theory is as follows. Colonic motor and sensation may be determined by the state of the intensity of CRF1 signaling. CRF2 signaling may be involved in the CRF1-triggered enhanced colonic contractility and visceral hypersensitivity by modulation of CRF1 activity. The activity balance of peripheral CRF1 and CRF2 signaling possibly determines the functional colonic changes. Several reports also supported this concept in not only colonic motility14 but also duodenal sensation39 and the excitability of amygdala neurons.40 Acute stress induces integrated responses to maintain homeostasis and warrant survival of organisms. In the absence of adequate counter regulation, the stress response runs in an overdrive state that can become fatal.41 In this context, CRF2 signaling seems to be counter regulation against CRF1-induced stress response. Other researchers also suggested that CRF1 is responsible for initiating a stress response
and CRF2 plays an important role in maintaining and terminating this response.\textsuperscript{42} The signaling balance might be changed over time during stress, which may induced by altering CRF receptor expression profile during stress. Expression of CRF receptor subtypes is known to be altered by acute stress.\textsuperscript{43}

According to the theory, what should be remembered is that stimulation of CRF2 itself does not alter colonic function in the basal state because of a lack of activation of CRF1 signaling, i.e. existence of activated CRF1 signaling is needed for exhibiting effect of CRF2.\textsuperscript{15} Thus our result suggested that WAS activates not only CRF2 but also CRF1. Since WAS was reported to activate peripheral CRF1 to modify GI motility,\textsuperscript{18, 19} this notion is consistent with the evidence. Therefore, blocking CRF2 is thought to disinhibit CRF1 signaling, thereby emerging pure CRF1 action, namely, reducing the pain threshold. In addition, regarding this result, an important point to emphasize is blocking CRF2 just normalized the threshold but did not induce hypersensitivity. Activated CRF1 signaling induced by WAS without interfering by CRF2 would induce to reduce the threshold below the basal level. These results suggest that there may be still other systems to cancel out the CRF1-evoked action.

Incidentally, we also presented the raw data for each individual animal, which demonstrated the threshold response of each rat. According to these data, in control with vehicle group (Fig.3A), 1 rat became hyposensitive (11 %), 2 rats developed hypersensitivity (22 %) and 6 rats demonstrated no change of the threshold (67 %). On the other hand, 3 rats were hyposensitive (23 %), 6 rats were hypersensitive (46 %) and 4 rats demonstrated no change (31 %) in WAS with astressin\textsubscript{2}-B group. These results may indicate that the response profile may be different between these two groups, even though % change was not different, suggesting astressin\textsubscript{2}-B did not really bring the visceral sensation to the basal state. This notion may indicate that other systems besides the CRF2 signaling contribute to the WAS-induced visceral sensory response, and existence of relatively large percentage of rats developing hypersensitivity might result from the disinhibited
CRF1 signaling by astressin2·B in these rats, which may further support the notion as discussed above.

The definite action sites of peripheral CRF in modulating visceral sensation has not been determined. Since CRF receptors are proved to be expressed in dorsal root ganglia,⁰¹ CRF may act spinal afferents directly and modulate visceral sensation. Enterochromaffin cells and mast cells have CRF receptors and release various chemical mediators such as serotonin, prostaglandins and cytokines through activating the receptors.⁴⁴⁻⁴⁶ Since these mediators are thought to contribute to visceral hypersensitivity through activating spinal afferents or dorsal root ganglia neurons,⁴⁷⁻⁴⁹ these cells may also be target of CRF modulating visceral sensation. According to these lines of evidence, the interaction of CRF1 and CRF2 may occur in these proposed action sites. Gourcerol et al.¹⁴ speculated that CRF2 activation may share intracellular signaling targets of CRF1, leading to inhibition of CRF1 signaling.

Basic and clinical evidence suggested that central dopamine D2 receptor has direct antinociceptive effect for somatic pain.⁵⁰⁻⁵³ In addition, we have very recently demonstrated that dopamine system also mediates antinociceptive effect in visceral pain.³⁰ Brain dopamine system is activated by stress, such as restraint,⁵⁴ and Zhang et al.⁵⁵ showed that chronic unpredictable stress increased dopamine D2 receptor mRNA in the striatum. These lines of evidence suggest that central dopamine D2 receptor may contribute to the WAS-induced visceral hyposensitivity, and it actually happened in our study that sulphiride abolished but domperidone did not modify the response by WAS. These results also suggest that central dopamine might be one of the pathways to cancel out the CRF1-evoked visceral sensitization, which was predicted by the data regarding astressin2·B as discussed before.

Dopamine receptors have been found at spinal cord and brain.⁵⁶⁻⁵⁸ These includes ventral tegmental area, ventro-lateral orbital cortex/prefrontal cortex or insular cortex, as well as the striatum.⁵⁷ We would suggest that release of dopamine during WAS may act these receptors to modulate visceral pain perception.
Endogenous opioid system has been known to mediate stress-induced somatic hypoalgesia.\textsuperscript{27, 59} Larauche et al.\textsuperscript{25} showed that acute or repeated WAS exhibited visceral analgesia in both female and male rats, that was mainly naloxone-dependent in females, but naloxone-independent in males. In our result, although naloxone itself slightly increased the threshold, it did not altered the WAS-induced response in male rats, which is consistent with the above findings. Further studies are needed to explore the effect of naloxone in basal state, but the present finding suggests that opioid system may not mediate the WAS-induced antinociception in our experimental settings.

Our results suggest that several systems to mediate visceral sensation are activated, and the response induced by WAS may result from the summation of the effect of each system such as CRF1, CRF2, dopamine, etc. Moreover, dominant activated signaling might be changed with the lapse of time from the stress load. In fact, Larauche et al.\textsuperscript{25} showed that female rats exposed to WAS for four consecutive days displayed visceral hypoalgesia in immediately after the last WAS session but visceral hyperalgesia at 24 h after it. CRF signaling balance might be also influenced by the time course as described before, and difference of gender of tested animals and strength of stress related to measuring visceral pain may be also involved, leading to the conflict results regarding the WAS-induced altered visceral sensation.

Our results had several limitations. We did not test the effect of CRF1 antagonist because all now available selective CRF1 antagonists have been designed to cross the blood-brain barrier.\textsuperscript{60} Therefore, the role of peripheral CRF1 cannot be clarified directly at present. Additionally, the contribution of central CRF system was not determined in our study. Although the balance theory may explain the visceral response by WAS through peripheral CRF signaling, it is not known whether the theory is also effective in central CRF signaling. Further studies are needed.
It is now widely accepted that an altered visceral sensation plays an important role in the pathogenesis of IBS.\textsuperscript{61-63} Previous studies indicate that majority of IBS patients display increased visceral sensitivity to rectal balloon distention.\textsuperscript{64, 65} Visceral hypersensitivity may result from not only facilitating pain sensation pathways but also disturbances in inhibitory pathways in response to stress as demonstrated in somatic pain studies.\textsuperscript{66} Moreover, altered descending inhibitory pathways have been described in IBS patients,\textsuperscript{67, 68} and acute mental stress increased visceral sensory threshold in control subjects but it did not alter it in IBS patients.\textsuperscript{69} Thus exploring the mechanisms of stress-induced visceral hyposensitivity is essential to understand the pathophysiology of IBS, and our results indicated that dopamine system might play a role in addition to CRF system.\textsuperscript{16}

In summary, we demonstrated WAS induced visceral hyposensitivity, and this response was peripheral CRF2 and central dopamine D2 receptor-dependent, but not mediated by opioid system. These new findings may contribute to further understanding the mechanisms of stress-related alterations of visceral sensation and the pathophysiology of IBS.

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DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTION

TN designed and performed the experiment, analyzed the data and wrote the paper. SM and RN performed the experiment. KT contributed to establishing the experimental system monitoring visceral sensation. TO was involved in the study concept, study supervision and critical revision of the manuscript.
REFERENCES


difference in opioid involvement. *Neurogastroenterol Motil* 2012; **24**: 1031-e1547.


34. Martínez V, Wu SV, Taché Y. Intracisternal antisense oligodeoxynucleotides to the thyrotropin-releasing hormone receptor blocked vagal-dependent stimulation of gastric emptying induced by acute cold in rats. *Endocrinology* 1998; **139**: 3730-3735.


**Figure legends**

Figure 1.

The effect of water avoidance stress (WAS) on visceromotor response (VMR) threshold. WAS significantly increased the threshold but control rats did not display any significant change. NS represents no significant difference. \(*p < 0.05.\) Horizontal lines show mean values.

Figure 2.

Water avoidance stress (WAS)-induced visceral hyposensitivity was no longer detected at 24 h after the stress. The threshold of visceromotor response (VMR) was not changed between basal and after sham stress (control) or WAS. NS represents no significant difference. Horizontal lines show mean values.

Figure 3.

The effect of astressin\(_2\)-B (200 µg/kg) on water avoidance stress (WAS)-induced visceral hyposensitivity. A, The threshold of visceromotor response (VMR) was not changed after sham stress in vehicle or astressin\(_2\)-B-treated rats. WAS increased it in vehicle injected rats but it was not changed in astressin\(_2\)-B-treated WAS group. NS represents no significant difference. \(*p < 0.05.\) Horizontal lines show mean values. B, % change in the threshold between basal and after stress. Astressin\(_2\)-B abolished the response by WAS. The antagonist itself did not influence the threshold of VMR. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. \(*p < 0.05\) vs. vehicle + control group. \(\#p < 0.05\) vs. vehicle + WAS group.
Figure 4.
The effect of sulpiride (200 mg/kg) on the antinociceptive response by water avoidance stress (WAS). A, In controls, the threshold of visceromotor response (VMR) was not changed by sham stress in vehicle or sulpiride-treated group. On the other hand, it was increased by WAS in vehicle-treated rats but was not changed in sulpiride-treated group. NS represents no significant difference. *p < 0.05. Horizontal lines show mean values. B, Sulpiride itself did not modify the threshold change in controls but abolished the response by WAS. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle + control group. #p < 0.05 vs. vehicle + WAS group.

Figure 5.
The effect of domperidone (10 mg/kg) on water avoidance stress (WAS)-induced visceral hyposensitivity. A, The threshold of visceromotor response (VMR) was not changed between before and after sham stress (control) with or without domperidone. WAS increased the threshold in both vehicle and domperidone-treated rats. NS represents no significant difference. *p < 0.05. Horizontal lines show mean values. B, Domperidone did not alter the threshold change in controls, and it did not block the response by WAS. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle + control group.

Figure 6.
The effect of naloxone (1 mg/kg) on water avoidance stress (WAS)-induced increased threshold of visceromotor response (VMR). A, In controls, the threshold was not changed in vehicle group, but it was slightly but significantly increased after sham stress in naloxone-treated rats. In both vehicle and naloxone-treated rats, WAS
increased the threshold. NS represents no significant difference. *p < 0.05.
Horizontal lines show mean values. Naloxone did not modify the response by
WAS. In controls, the % change in the threshold was not different between vehicle
and naloxone-treated groups. Each column represents the mean ± S.E. Number of
rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle + control group.
A

VMR threshold (ml)

Basal | After
Vehicle + Control | NS
Basal | After
Astressin_{2-B} + Control | NS
Basal | After
Vehicle + WAS | *
Basal | After
Astressin_{2-B} + WAS | NS

B

% Change in VMR threshold

Vehicle + Control | (9)
Astressin_{2-B} + Control | (7)
Vehicle + WAS | *(11)
Astressin_{2-B} + WAS | #(13)
A

![Graph showing VMR threshold (ml) for different conditions](image)

- Basal Vehicle + Control
- After Vehicle + Control
- Basal Sulpiride + Control
- After Sulpiride + Control
- Basal Vehicle + WAS
- After Vehicle + WAS
- Basal Sulpiride + WAS
- After Sulpiride + WAS

B

![Bar chart showing % Change in VMR threshold](image)

- Vehicle + Control
- Sulpiride + Control
- Vehicle + WAS
- Sulpiride + WAS

*(10), *(8), *(8)*
A

![Graph A](image)

B

![Graph B](image)