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Oxytocin antagonist induced visceral pain and corticotropin-releasing hormone neuronal activation in the central nucleus of the amygdala during colorectal distention in mice

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ABSTRACT

Activation of neurons containing oxytocin and corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus, the anterior cingulate cortex (ACC), and the central nucleus of the amygdala (CeA) during colorectal distention (CRD) is likely to play a crucial role in animal models of irritable bowel syndrome (IBS). Earlier studies in rodents showed that the microbiome is involved in social behavior via oxytocin expression in the brain. However, the detailed mechanism of visceral sensation and oxytocin is largely unknown. We tested the following hypotheses: (1) that oxytocin neurons in the PVN are activated by CRD, and (2) that the activation of oxytocin neurons by CRD is related to anxiety-like behavior, visceral perception, and an activation of CRH CeA neurons or ACC neurons. Oxytocin antagonist caused visceral hypersensitivity and anxiety-like behavior. In the PVN, oxytocin neurons were activated by CRD. Noxious CRD activated the CeA, basolateral nucleus of the amygdala (BLA), and ACC. High-dose oxytocin antagonist suppressed ACC activity and activated CRH CeA neurons. These results support our hypotheses. Oxytocin likely regulates CRH CeA neurons in an inhibitory manner and the ACC in an excitatory manner. Further research into the interaction of oxytocin and CRH in visceral pain and anxiety is warranted.

1. Introduction

Irritable bowel syndrome (IBS) is a disorder of brain-gut interactions characterized by abdominal pain and bowel dysfunction (Fukudo, 2013a,b). Approximately 10–15 % of adults and adolescents have IBS symptoms (Lovell and Ford, 2012; Miwa, 2008). IBS is closely linked to psychosocial factors, including stress (Fukudo, 2013a,b), depression (Fond et al., 2014; Mudyanadzo et al., 2018), and anxiety (Fond et al., 2014). IBS can significantly impair a person's quality of life (Gralnek et al., 2000). Therefore, it is important to study the pathophysiological mechanism of IBS and its treatment options.

The exact pathogenesis of IBS is still unknown but one of its factors involves altered function of the corticotropin-releasing hormone (CRH) system in the brain (Buckley et al., 2014; Stengel and Tache, 2010; Tanaka et al., 2016). CRH is a brain–gut peptide hormone abundantly expressed in the neurons of the paraventricular nucleus (PVN) of the hypothalamus (Fukudo, 2012). CRH also serve as a neurotransmitter that targets extrahypothalamic brain circuits to mediate behavioral and autonomic activation to stress (Lowry and Moore, 2006). CRH in the PVN is released in response to physical or psychological stress and triggers hormone secretion in the hypothalamic-pituitary-adrenal (HPA) axis as well as exaggerated gastrointestinal motility (Fukudo, 2012; Lightman, 2008; Maekawa et al., 2019). Administration of exogenous CRH receptor agonist increases colonic motility, visceral hypersensitivity, and anxiety-like behavior, which are the main symptoms of IBS (Fukudo et al., 1998; Fukudo, 2012; Lightman, 2008). These symptoms are alleviated by CRH antagonists (Galbusera et al., 2017; Larauche et al., 2009; Million et al., 2013; Sagami et al., 2004). Previous studies from our laboratory reported altered distributions of gene polymorphisms of CRH receptors and CRH-binding protein in IBS patients (Komuro et al., 2016; Sasaki et al., 2016; Sato et al., 2012). CRH is

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thus likely to play a role in the stress-related pathophysiology of IBS.

Observation of gastrointestinal sensation under loading of colorectal distention (CRD) using the barostat method shows that IBS patients have a lower sensory threshold than healthy subjects, and that they are aware of stronger abdominal pain (Whitehead et al., 1990). The signal of gastrointestinal stimulation is projected onto the anterior cingulate cortex (ACC), insula, and prefrontal cortex, causing abdominal pain, abdominal discomfort, and negative emotions (Tracey and Mantyh, 2007). Furthermore, the amygdala and hypothalamus are directly innervated by the parabrachial nucleus via lamina I neurons in the spinal cord and regulate the stress response (Tillisch et al., 2011). In a meta-analysis of the brain response to CRD, IBS patients had abnormal signals in various brain regions, including the ACC and amygdala (Tillisch et al., 2011). Abnormalities of the neural network centering on the amygdala are associated with IBS symptoms (Labus et al., 2009). Abnormal activity of the ACC during CRD impairs top-down inhibitory input from the ACC to the HPA axis in IBS patients (Kano et al., 2017). However, the precise roles of various brain regions of IBS patients during CRD are still unclear.

In rodent studies, functional magnetic resonance imaging and immunostaining using c-Fos as a marker of neuronal activity have revealed that CRD activates various brain regions, including the hypothalamus, amygdala, and ACC (Lazovic et al., 2005; Mönnikes et al., 2003; Traub et al., 1996; Wang et al., 2009a,b). The rodent ACC is thought to play an important role in emotional responses associated with nociceptive signals and to also regulate visceral perception (Bliss et al., 2016; Cao et al., 2008; Gao et al., 2006; Zhuo and Kaang, 2015). The amygdala, particularly the central nucleus of the amygdala (CeA), abundantly expresses CRH (Deussing and Chen, 2018) and plays a role in gastrointestinal disorders (gastric emptying and colonic motility) and anxiety-like behaviors (Paretkar and Dimitrov, 2018; Ray et al., 1993). Central administration of CRH elicits anxiety-like behaviors while direct injection of CRH antagonists into CeA blocks these effects (Swiergiel et al., 1993). Factors that regulate the PVN, ACC, and CeA in relation to CRH neurons require further research.

In the PVN, oxytocin neurons are located next to CRH neurons (Biag et al., 2012). Oxytocin is a hormone traditionally known on peripheral for induction of labor and milk ejection (Kandel et al., 2013). Recently, the brain functions of oxytocin have been investigated using genetically modified (mainly knockout) mice. Oxytocin and oxytocin receptor knockout mice showed a reduction in ultrasonic vocalizations and exhibited anxiety-like behavior in an elevated plus maze (Winslow et al., 2000; Winslow and Insel, 2002; Wood et al., 2015). On stress response, oxytocin neurons suppress stress-induced activation of CRH neurons, regulate HPA axis activity, and exert anxiolytic effects (László et al., 2016; Windle et al., 2004). Furthermore, exogenous administration of oxytocin to IBS patients and IBS model animals alleviates visceral hypersensitivity (Louvel et al., 1996; Xu et al., 2018). Oxytocin receptors are expressed in the ACC and CeA to a moderate-to-high degree (Jurek and Neumann, 2018; Yoshida et al., 2009) and oxytocinergic neurons target these brain regions (Knobloch et al., 2012). Oxytocin is upregulated by somatic sensation (Erfanparast et al., 2018; Grinevich and Charlet, 2017; Okabe et al., 2015). Thus, oxytocin is likely to be an intracerebral regulator of visceral pain and gut-related anxiety in animals. However, it is unclear whether oxytocin is expressed by visceral sensation and regulates brain activity in the ACC and CeA in mice.

In recent IBS studies, microbiome functions are paid attention (Pimentel and Lembo, 2020). Gut microbiota in IBS patients are different from healthy individuals and they may cause IBS symptoms (Fukudo, 2013a,b). Depletion of microbiota reduces oxytocin expression (Desbonnet et al., 2015) and induces visceral hypersensitivity (Hoban et al., 2016). The levels of oxytocin regulated by microbiota have the potential to control the stress response or accelerate wound healing (Poutahidis et al., 2013; Varian et al., 2017).

From the above background, visceral stimulation may influence oxytocin expression in the brain. We explored the following two hypotheses in this study: (1) that oxytocin neurons in the PVN are activated by CRD, and (2) that the activation of oxytocin neurons by CRD is related to anxiety-like behavior, visceral perception, and an activation of CRH CeA neurons and ACC neurons.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice aged 10–12 weeks were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed under controlled illumination (12:12-h light:dark cycle starting at 7:00 AM) and temperature (22 ± 1 °C) with food and water ad libitum. Two or three mice were kept in one cage. Each experimental group comprised 5–8 mice. All experiments were performed between 9:00 AM and 12:00 AM. This study was approved by the Ethics Committee of Laboratory Animals, Tohoku University (Approval No. 2020MdA-122).

2.2. Colorectal distention

Mice were restrained using a restraint instrument (a 2.9 × 11.4-cm bottle) that was adjusted with tape on the outside to prevent their movement. A polyethylene barostat bag (Star Medical, Tokyo, Japan) with a maximum diameter of 2 cm was inserted into the colorectum through the anus and the bag was distended with barostat equipment (Distender Series II; G & J Electronics, Toronto, Ontario, Canada) under computer control using 8 STAR software version 6.0–19.2 (Star Medical). The CRD stimulation varied from a weak stimulus (10 mmHg) to a strong stimulus (60 mmHg). Because the 15-mmHg stimulation intensity resulted in no difference in behavior compared with the resting state in mice (Kamp et al., 2003), CRD at 10-mmHg intensity is recognized as a non-noxious stimulus. After the end of the stress exposure, the mice were sacrificed by decapitation.

2.3. Drug

The specific oxytocin antagonist L-368,899 ((2S)-2-amino-N-[(1S,2S,4R)-7,7-dimethyl-1-[[[4-(2-methylphenyl)-1-piperazinyl] sulfonyl] methyl] bicyclo[2.2.1] hept-2-yl]-4-(methylsulfonyl)) hydrochloride was purchased from MedChemExpress (Monmouth Junction, NJ). It is a blood-brain barrier-penetrating high-affinity nonpeptide (Boccia et al., 2007). L-368,899 was dissolved in saline and stored in a −80 °C freezer. Two dosages were used: 1 mg/mL and 10 mg/mL. The drug was administered intraperitoneally with the dose of 10 mL/kg mouse weight at 10 min before the mice were restrained.

2.4. Experimental protocol 1: assessment of visceral sensitivity by electromyography

This experimental protocol is summarized in Fig. 1**A**. The effects of saline and oxytocin antagonist were compared. Visceral hypersensitivity was assessed using electromyography (EMG). Mice were deeply anesthetized and an electrode (Star Medical) for EMG measurement was implanted into the left side of the abdominal muscles. The anesthetic mixture was prepared from the following drugs: 0.75 mg/kg of medetomidine hydrochloride (Domitor®, Nippon Zenyaku Kogyo, Fukushima, Japan), 4 mg/kg of midazolam

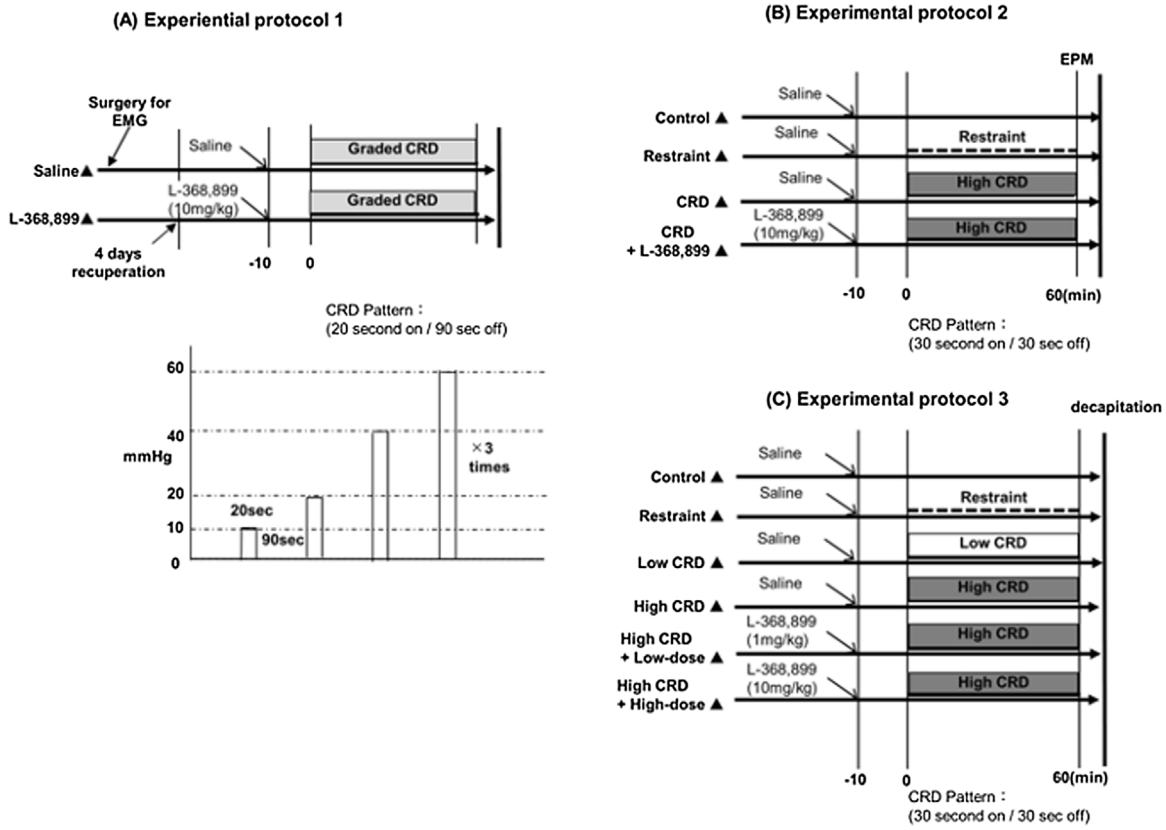

Fig. 1. Experimental protocol.
(A) Effects of the oxytocin receptor antagonist L-368,899 on a graded colorectal distention (CRD)-induced increase in the frequency of abdominal contractions. (B) Effects of L-368,899 on CRD-induced anxiety-like behavior. (C) Effects of CRD or L-368,899 on c-Fos expression and hypothalamic-pituitary-adrenal (HPA) axis activation. Free moving, no line; restraint, —; graded CRD, gray box; low CRD (10 mmHg), white box; high CRD, dark gray box.

(Dormicum®, Astellas Pharma, Tokyo, Japan) and 5 mg/kg of butorphanol tartrate (Vetorphale®, Meiji Seika Pharma, Tokyo, Japan) and dissolved using sterilized saline (0.9 %). The anesthetic mixture was injected intraperitoneally (10 mL/kg mouse weight). The mice then recuperated for 4 days and were injected with saline or oxytocin antagonist 10 min prior to graded CRD stimulation. The catheter and EMG leads were connected to barostat equipment and a computer. To record the visceromotor response (VMR) to CRD, graded distention was performed with intra-bag pressures of 10 mmHg, 20 mmHg, 40 mmHg, and 60 mmHg and an on-stimulation time of 20 s and off-stimulation time of 90 s. VMR was used as a marker of visceral pain in animals (Kamp et al., 2003; Nakaya et al., 2016). VMR due to CRD was evaluated via calculation of the EMG amplitude.

2.5. Experimental protocol 2: elevated plus maze test for observation of anxiety-like behavior

This experimental protocol is summarized in Fig. 1**B**. Control, restraint, CRD, and CRD + oxytocin antagonist conditions were compared. After the procedures, the animals' anxiety-like behavior was measured in the elevated plus maze (EPM) for 5 min. The EPM is one of the most widely used tests to assess anxiety-like behavior in rodents (Carola et al., 2002; Walf and Frye, 2007). This test is based on the fact that mice prefer walls and avoid elevated places. The EPM device is made of white plastic and consists of one central area (5 × 5 cm) and four arms (25 cm × 5 cm) raised to a height of approximately 38 cm from the floor and placed in a dark room. The wall height of the closed arm is 17 cm. Each mouse was placed in the center of the maze, facing the closed arm. The number of crossings to each arm, the time spent, and the distance traveled in open or closed arms were recorded with a video-tracking system (LimeLight 3; Neuroscience, Inc., Tokyo, Japan) for 5 min. After each trial, the maze was washed with 70 % ethanol solution.

2.6. Experimental protocol 3: immunohistochemical examination of the brain and quantification of hormone secretion

This experimental protocol is summarized in Fig. 1**C**. Control, restraint, low-grade (10 mmHg) CRD, high-grade (60 mmHg) CRD, high-grade CRD + low-dose (1 mg/kg) oxytocin antagonist, and high-grade CRD + high-dose (10 mg/kg) oxytocin antagonist were compared. At the end of the experimental protocol, the mice were sacrificed by decapitation. Their blood was collected and brains were carefully removed.

2.7. Immunohistochemical examination of the brain

Neuronal activity in brain areas was evaluated by immunostaining using c-Fos (neural activation marker) and the activation of oxytocin and CRH neurons was evaluated by double immunostaining using c-Fos with anti-oxytocin or anti-CRH antibodies.

The brains were fixed with 4 % paraformaldehyde in Tris-buffered saline (TBS) overnight at 4 °C. The tissues were then soaked in 30 % sucrose for two nights at 4 °C. The brain was sliced at about anterior 1.10 mm and posterior 0.94 mm from bregma into 30-μm sections with a Leica CM1950 cryostat (Leica, Buffalo Grove, IL). The Paxinos and Franklin Mouse Brain Atlas was used as a reference for the correct brain placement of the sliced sections (Paxinos

et al., 2001). For PVN staining, adjacent sections were selected for staining of CRH and oxytocin.

The sections were treated according to the protocol of the M.O.M. Immunodetection Kit, Basic (FMK-2201; Vector Labs, Inc., Burlingame, CA) for double immunostaining. For the first staining, the sections were incubated with mouse monoclonal antibody against c-Fos (1:1,000, ab208942; Abcam, Cambridge, MA). Thereafter, the sliced brain sections were treated with 2 % bovine serum albumin in 0.2 % Triton X-100/TBS for 60 min at room temperature to block nonspecific protein binding and then incubated overnight at 4 °C with rabbit polyclonal antibody against CRH (1:100, ab216599; Abcam) or rabbit polyclonal antibody against oxytocin (1:1,000, AB911; Millipore, Temecula, CA). Subsequently, the sections were treated with Alexa Fluor Plus 555 goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody (1:1,000, A32732; Thermo Fisher Scientific, Rockford, IL) for 60 min at room temperature. The sections containing the ACC were immunostained with rabbit polyclonal antibody against c-Fos (1:1,000, ab190289; Abcam). The experimental process was the same as that after the use of the M.O.M. Immunodetection Kit for double staining.

Fluorescence staining was observed using an Axio plan 2 Imaging Microscope (Carl Zeiss, Jena, Germany). Boundary of the areas in sections were decided to be based on the Paxinos and Franklin Mouse Brain Atlas (Paxinos et al., 2001). For quantitative evaluation, between two and five sections were analyzed per animal, and the average value was considered to indicate neural activity. The investigator was blind to the animal number and treatment groups during the cell counting. We followed the same methods of counting cells (Núñez et al., 2010; Bardgett et al., 2014).

2.8. Quantification of hormone secretion

Immediately after decapitation, blood was collected in microtubes prefilled with 10 μL EDTA-2Na solution (0.1 mol/l). Plasma was extracted by centrifugation and stored at −80 °C until use. Plasma adrenocorticotropic hormone (ACTH) and corticosterone levels were measured using enzyme-linked immunosorbent assay kits (ACTH: EK-001−21, Phoenix Pharmaceuticals Inc., CA; corticosterone: EIACORT, Thermo Fisher Scientific).

2.9. Statistical analysis

Statistical analysis was performed with SPSS version 25 (IBM SPSS, Armonk, NY). All results were expressed as mean ± standard error. Statistical significance of VMR data was evaluated by two-way repeated-measures analysis of variance (ANOVA) and a Sidak post hoc test and the others were evaluated by one-way ANOVA and a Tukey post hoc test. A probability level of $p < 0.05$ was considered significant.

3. Results

3.1. The VMR to gradual CRD strength in CRD + oxytocin antagonist groups

High-dose oxytocin antagonist (10 mg/kg, intraperitoneally) significantly increased the amplitudes of the VMR to graded CRD at all pressures compared with saline (two-way ANOVA: group effect; $F = 18.313$, $p = 0.001$, CRD effect; $F = 91.298$, $p < 0.001$, group × CRD interaction; $F = 9.739$, $p < 0.001$, post hoc saline vs oxytocin antagonist: 10 mmHg, 128.5 ± 41.3 vs 663.0 ± 149.3, $p < 0.01$; 20 mmHg, 491.2 ± 94.5 vs 1075.7 ± 194.3, $p < 0.05$; 40 mmHg, 818.1 ± 146.0 vs 1695.1 ± 238.2, $p < 0.01$; 60 mmHg, 1150.5 ± 135.3 vs 2589.0 ± 217.0, $p < 0.01$; Fig. 2**A** and **B**). Thus, the finding suggests that oxytocin controls visceromotor response in the range from low to high strengths of CRD.

3.2. Anxiety-like behavior among control, restraint, high CRD, and high CRD + oxytocin antagonist groups

The percentages of crossings, time, and distance in open arms are shown in Fig. 3. One-way ANOVA for analysis of each categories showed significant difference (crossings: $F = 3.161$, $p = 0.040$, time: $F = 6.486$, $p = 0.002$, distance: $F = 4.747$, $p = 0.008$). There was no significant difference in the percentage of crossings into open arms (Fig. 3A). Compared with the CRD group, the CRD + oxytocin antagonist group showed a significant decrease in the percentage of time spent in open arms (CRD vs CRD + oxytocin antagonist: 40.3 % ± 7.7 % vs 15.7 % ± 7.5 %, $p < 0.05$; Fig. 3B). Similarly, the CRD + oxytocin antagonist group showed a significant decrease in the percentage of distance in open arms compared with the CRD group (CRD vs CRD + oxytocin antagonist: 43.8 % ± 7.0 % vs 17.3 % ± 6.1 %, $p < 0.05$; Fig. 3C). It became clear that the administration of oxytocin antagonist before CRD exposure caused anxiety-like behavior. In other words, oxytocin likely inhibits anxiety-like behavior due to CRD.

3.3. Quantitative changes in CRH neuronal activation in the PVN

The anatomical section is shown in Fig. 4**A** while representative immunostained sections of c-Fos and CRH in the PVN are shown in Fig. 4**B**. One-way ANOVA for analysis of c-Fos and c-Fos + CRH staining showed significant difference among groups (c-Fos; $F = 56.109$, $p < 0.001$, c-Fos + CRH; $F = 39.682$, $p < 0.001$). Regarding the number of c-Fos-positive cells, the restraint group showed an increase compared with the control group (control vs restraint: 4.4 ± 1.7 vs 110.4 ± 5.1, $p < 0.01$). However, this increase was not different among all stimulated CRD groups, namely, low-grade CRD, high-grade CRD, high-grade CRD + low-dose oxytocin antagonist, and high-grade CRD + high-dose oxytocin antagonist. The expression of c-Fos in CRH-positive neurons showed the same tendency for an increase in c-Fos-positive cells. Thus, the restraint group showed an increase in c-Fos in CRH-positive neurons (control vs restraint: 1.5 ± 0.7 vs 79.6 ± 4.8) and there was no difference among the groups under restraint according to CRD or oxytocin antagonist (Fig. 4C). These results provide that the restraint caused CRH-positive PVN neuronal activation, which showed no difference between in CRD alone and CRD + oxytocin antagonist.

3.4. Measurement of plasma ACTH and corticosterone

Results of the one-way ANOVA indicated that plasma ACTH and corticosterone levels were significantly different among groups (ACTH: $F = 3.139$, $p = 0.020$, corticosterone: $F = 28.668$, $p < 0.001$). Plasma ACTH and corticosterone levels were significantly increased in restraint mice (control vs restraint: ACTH, 1.1 ± 0.1 vs 1.5 ± 0.1 ng/mL; corticosterone, 461.1 ± 63.1 vs 3306.3 ± 136.1 pg/mL; both $p < 0.01$; Fig. 5**A** and **B**). However, this increase did not differ among the groups under restraint according to CRD or oxytocin antagonist. These results indicate that the restraint stress increases plasma levels of ACTH and corticosterone. However, there was no further increase in ACTH and corticosterone by either CRD alone or CRD + oxytocin receptor antagonist.

3.5. Quantitative changes in oxytocin neuronal activation in the PVN

Representative sections immunostained with c-Fos and oxytocin in the PVN are shown in Fig. 6**A**. The statistical comparison of the positive cells among each group is shown in Fig. 6**B**. Results

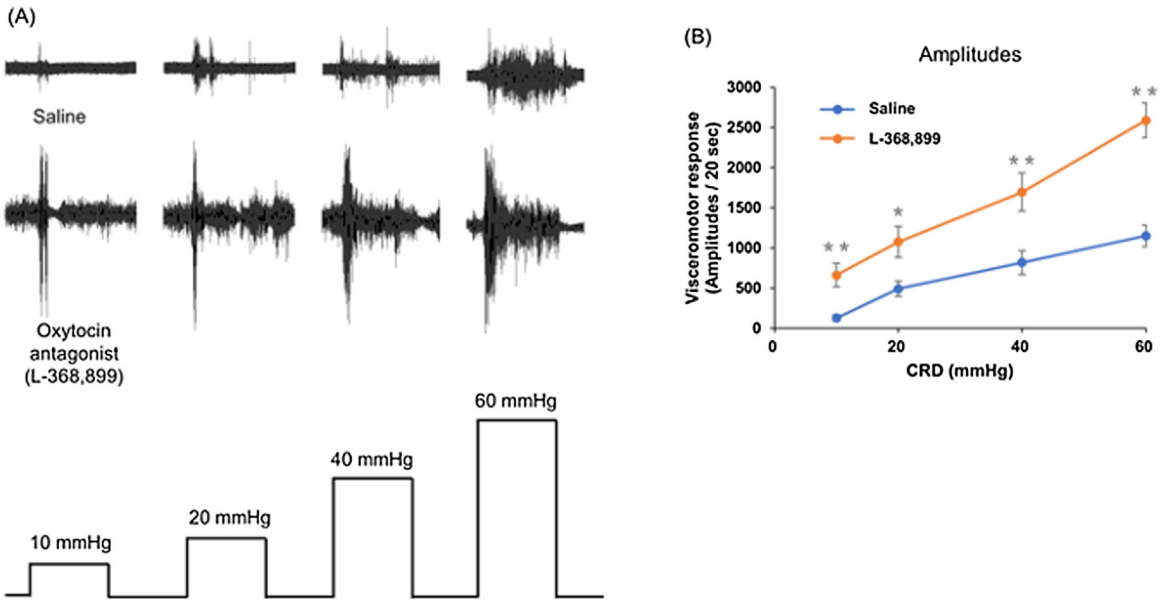

Fig. 2. Visceral sensitivity.
(A) Representative electromyographic recordings after the administration of saline or oxytocin receptor antagonist (L-368,899; 10 mg/kg) under graded (10, 20, 40, and 60 mmHg) colorectal distention (CRD) conditions. (B) Statistical analysis of the average visceromotor response to graded CRD in saline- and L-368,899-administered mice (both n = 7). Data are expressed as mean ± standard error (SE) and were assessed by two-way repeated-measures analysis of variance (ANOVA), with a Sidak post hoc test showing that the oxytocin receptor antagonist group exhibited a significant effect under graded distention compared with the saline group. *p < 0.05, **p < 0.01 vs saline.

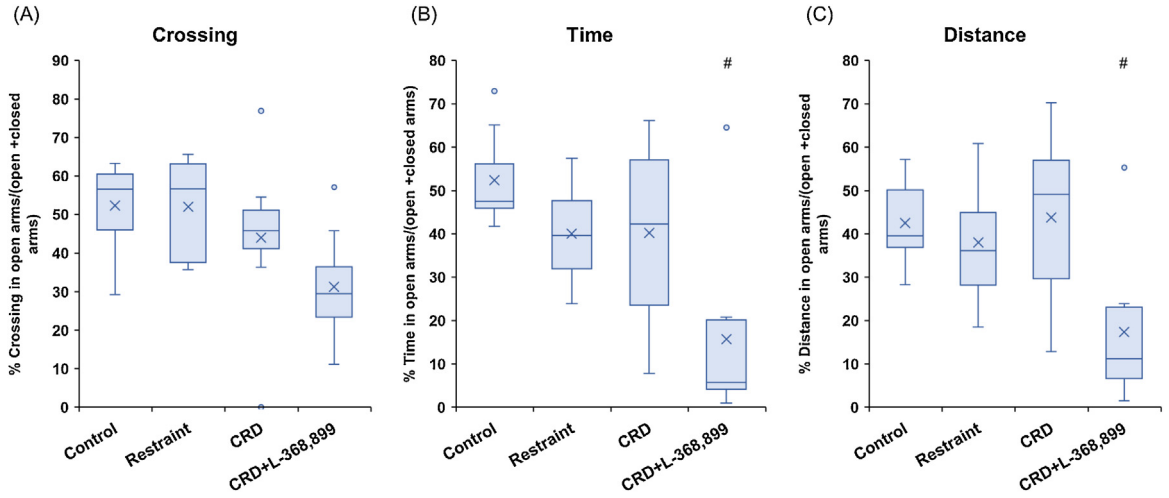

Fig. 3. Elevated plus maze (EPM) for observation of anxiety-like behavior.
Statistical analysis of the percentage of each item in the open arm. n = 8 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA, with a Tukey post hoc test performed for results showing a significant effect. (A) There was no significant difference compared with the CRD group in the percentage of crossings into open arms. On the other hand, the CRD + oxytocin receptor antagonist group showed a significant decrease in the percentage of time spent in open arms (B) and the percentage of distance traveled in open arms (C) in the EPM compared with the CRD group. #p < 0.05 vs saline.

of the one-way ANOVA indicated no significant difference among the groups in the oxytocin positive cells ($F = 1.207$, $p = 0.330$). However, significant difference in c-Fos and oxytocin positive cells among the groups was detected ($F = 22.317$, $p < 0.001$). The restraint group exhibited significantly increased expression of c-Fos in oxytocin-positive nerves compared with the control group (control vs restraint: 1.4 ± 0.2 vs 9.6 ± 1.5, $p < 0.05$). The CRD-stimulated groups (low CRD and high CRD) showed a significant increase compared with the restraint group (restraint vs low CRD and high CRD: 9.6 ± 1.5 vs 14.7 ± 1.1 [$p < 0.05$] and 16.1 ± 1.7 [$p < 0.01$], respectively). In the high CRD group, there was no significant difference versus the two groups administered oxytocin antagonist (low-dose or high-dose). Thus, these findings suggest that the CRDs activated oxytocin-positive PVN neurons.

3.6. Quantitative changes in neuronal activation in the amygdala

The amygdala is mainly composed of the basolateral nucleus of the amygdala (BLA) as the input system and the central nucleus as the output system (Fig. 7A). The CRH neurons in the CeA are involved in anxiety and visceral pain. Therefore, we measured the numbers of c-Fos- and CRH-positive cells in the CeA using double immunostaining, but not in the BLA due to its lower neuronal expression of CRH. Representative immunostained sections of c-Fos and CRH in the amygdala are shown in Fig. 7**B**.

The results of c-Fos-positive cells in the BLA are shown in Fig. 7**C**. One-way ANOVA showed significant difference among groups ($F = 13.975$, $p < 0.001$). In the BLA, the restraint group showed no difference compared with the control group. There was a significant

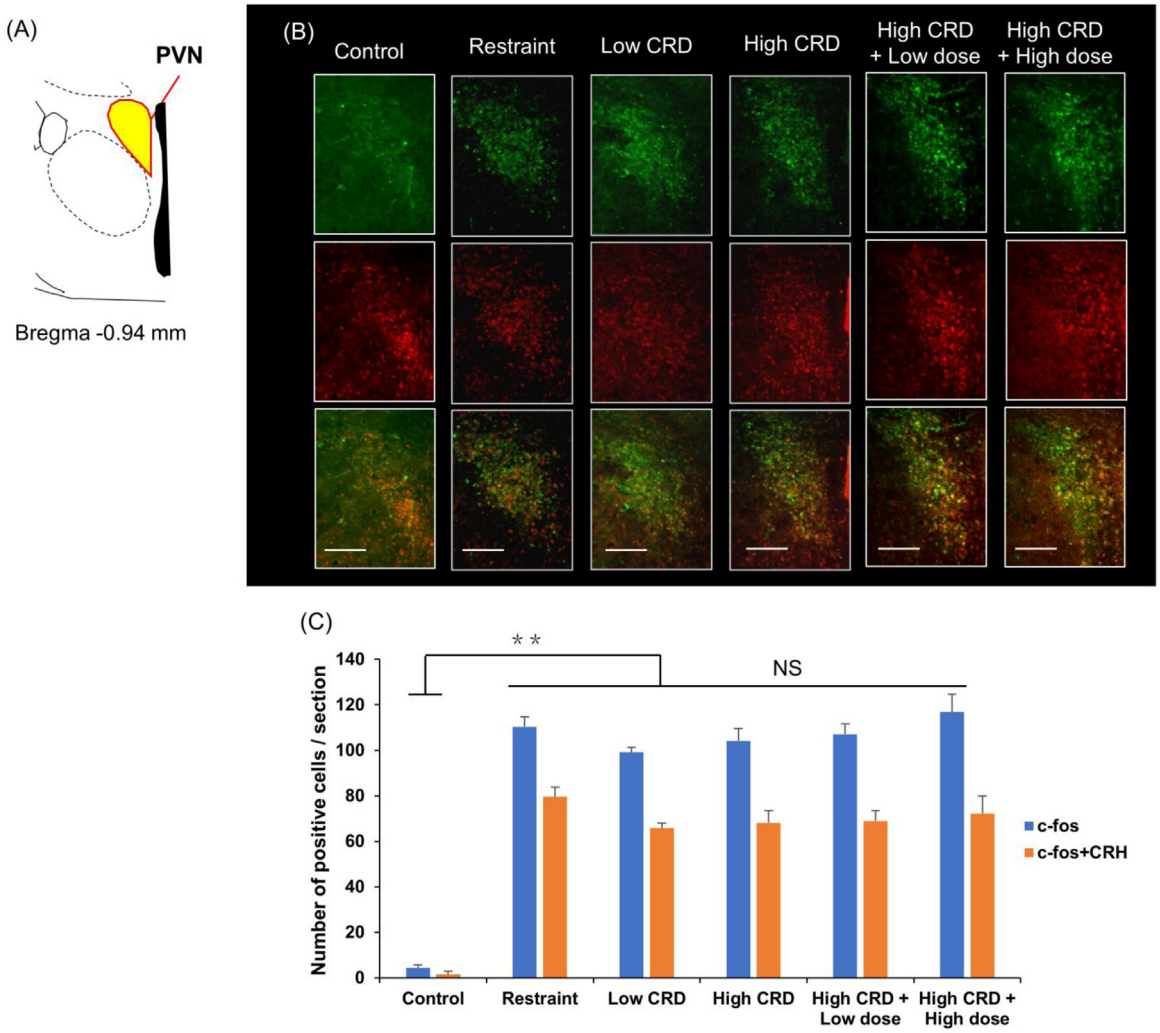

Fig. 4. Immunostaining of c-Fos and corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN).
(A) Schematic of the location where positive cells were evaluated, which represents a section level about posterior 0.94 mm from bregma. (B) Representative immunostaining using antibodies against c-Fos (green) and CRH (red) in the PVN in each group. Scale bar =100 μm. (C) The total numbers of c-Fos-positive cells and of c-Fos-positive cells colocalized with CRH-positive cells were counted. n = 5–7 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA and Tukey post hoc test. **p < 0.01 vs the restraint group. NS, no significance.

increase in c-Fos expression in the high CRD group (11.3 ± 0.9) compared with the restraint group (6.4 ± 1.0 vs, $p < 0.05$). On the other hand, c-Fos expression in the low CRD, low oxytocin antagonist, and high oxytocin antagonist groups did not differ from that in the high CRD group. These results provide the evidence that the CRD causes BLA neuronal activation but that oxytocin antagonist has no effect on this activation.

The results of c-Fos- and c-Fos + CRH-positive cells in the CeA are shown in Fig. 7**D**. Results of the one-way ANOVA indicated that c-Fos and c-Fos + CRH were significantly different among groups (c-Fos: $F = 33.907$, $p < 0.001$, c-Fos + CRH: $F = 31.192$, $p < 0.001$). In the CeA, the restraint group showed no significant difference in c-Fos expression compared with the control and low CRD groups. The high CRD group exhibited a significant increase in c-Fos expression (11.1 ± 1.8) compared with the restraint (3.3 ± 0.7, $p < 0.01$) and low CRD (5.6 ± 0.6, $p < 0.05$) groups. Furthermore, c-Fos expression was significantly higher in the low oxytocin antagonist group (17.3 ± 1.6, $p < 0.05$) and high oxytocin antagonist group (19.2 ± 1.5, $p < 0.01$) than in the high CRD group. Regarding the expression of c-Fos + CRH, there was a similar trend for c-Fos-only expression. The restraint group showed no significant difference compared with the control and low CRD groups. The high CRD group (8.8 ± 1.3) demonstrated a significant increase compared with the restraint group (2.3 ± 0.5, $p < 0.01$) but not the low CRD group. The high oxytocin antagonist group showed significantly increased expression of c-Fos + CRH (14.8 ± 1.6) compared with the high CRD group (8.8 ± 1.3, $p < 0.01$). Thus, these results show that the high CRD causes activation of CRH-positive CeA neurons. Moreover, high CRD with the high dose oxytocin antagonist induces further activation of CRH-positive CeA neurons.

3.7. Quantitative changes in neuronal activation in the ACC

The stereotaxic location of the ACC examined is shown in Fig. 8**A**. Similar to the BLA, there were fewer CRH neurons in the ACC than in the CeA. Only c-Fos-positive cells were counted in the ACC (Fig. 8B). One-way ANOVA showed significant difference among groups ($F = 18.951$, $p < 0.001$). The restraint group showed a significant increase in c-Fos-positive cells (33.8 ± 2.9) compared with the control group (13.3 ± 1.1, $p < 0.01$). The high CRD group showed a further increase in c-Fos-positive cells (50.1 ± 3.9) compared with the restraint group (33.8 ± 2.9, $p < 0.01$) but not compared with the low CRD group (41.3 ± 3.9). Although the low-dose oxytocin antagonist group had a similar number of c-Fos-positive cells versus the

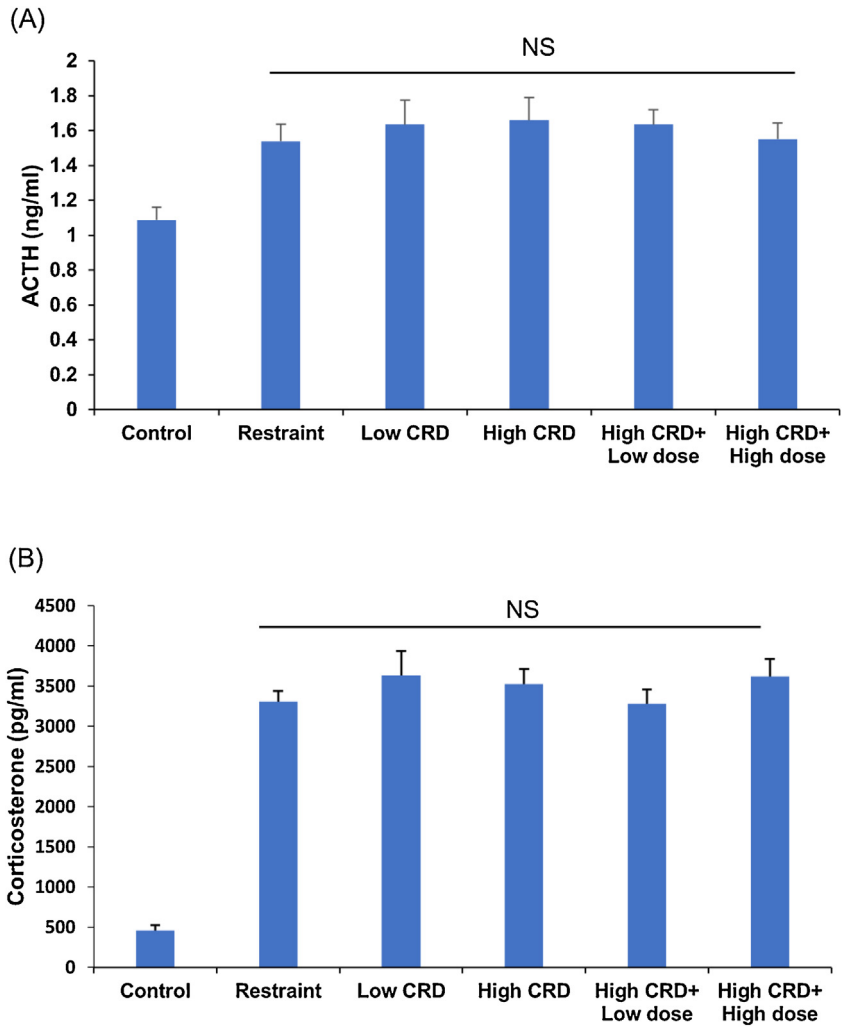

Fig. 5. HPA axis responsivity.
Integrated adrenocorticotropic hormone (A) and corticosterone (B) values in control, restraint, low CRD, high CRD, high CRD + low-dose oxytocin antagonist, and high CRD + high-dose oxytocin antagonist groups. n = 5–7 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA and Tukey post hoc test. NS, no significance.

high CRD group, the high oxytocin antagonist group showed significantly decreased expression of c-Fos-positive cells (36.5 ± 1.6) compared with the high CRD group ($p < 0.05$; Fig. 8C). Thus, the results provide that the high CRD causes ACC neuronal activation, which is suppressed by the high dose oxytocin antagonist.

4. Discussion

This is the first study to show that CRD activates oxytocin neurons in the PVN of mice. We also showed that administration of oxytocin antagonist causes visceral hypersensitivity in mice. In rats, intracerebroventricular administration of an oxytocin antagonist sensitizes visceral nociception to noxious CRD stimuli (Larauche et al., 2019). Earlier studies reported that oxytocin alleviates visceral hypersensitivity in IBS patients (Louvel et al., 1996) and in a stress-induced animal model of IBS (Xu et al., 2018). The results of this study together with these earlier reports which oxytocin controls visceral sensitivity, suggest that endogenous oxytocinergic tones modulate visceral pain. Oxytocin receptors expression has been demonstrated in not only brain but also colon (Monstein et al., 2004; Welch et al., 2009, 2014). The activation of oxytocin in peripheral nervous system is suggested to be involved in modulation of pain nociception (Boll et al., 2018). In this study, oxytocin receptor antagonist was intraperitoneally administered. Thus, it is possible that oxytocin antagonist has an effect on peripheral regions and might be involved in pain processing. We also demonstrated that administration of oxytocin antagonist under CRD stimulation causes anxiety-like behavior in mice, since they spent more time in the closed arms of the EPM. This indicates that oxytocin receptors are involved in anxiolytic properties in the EPM. Our results regarding the effect of oxytocin antagonist agree with those of other reports involving behavioral experiments using oxytocin antagonists (Grinevich and Charlet, 2017; Ring et al., 2006). Thus, the first hypothesis and the behavioral aspects of the second hypothesis were supported.

To investigate visceral sensitivity, the CRD intensity ranged between 10 mmHg and 60 mmHg. As defined by an earlier mouse study, 10-mmHg intensity is considered a non-noxious stimulus while 60 mmHg is considered noxious (Kamp et al., 2003). Regarding somatic sensations, oxytocin has been reported to be induced by both noxious and non-noxious stimuli (Erfanparast et al., 2018; Grinevich and Charlet, 2017; Okabe et al., 2015). In this study, high CRD as a noxious stimulus activated mouse oxytocin neurons. This result is consistent with the study of rats exposed to noxious CRD (Wang et al., 2009a,b). We also showed that non-noxious CRD stimulates oxytocin PVN neurons more than restraint stimuli alone, suggesting that non-noxious visceral sensations also stimulate oxytocinergic neurons in the PVN.

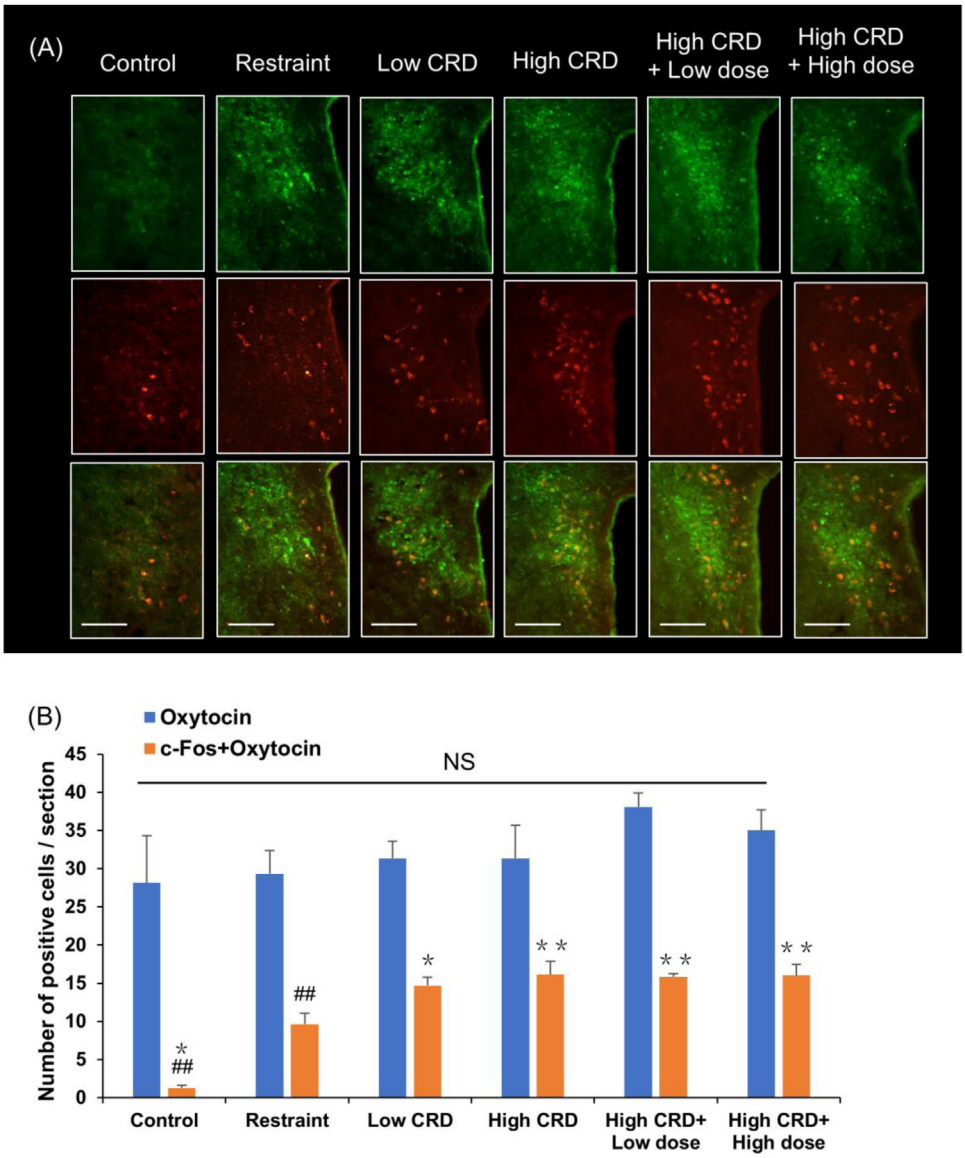

Fig. 6. Immunostaining of c-Fos and oxytocin in the PVN.
The location used to evaluate positive cells was the serial sections in Fig. 4A. (A) Representative immunostaining using antibodies against c-Fos (green) and oxytocin (red) in the PVN in each group. Scale bar =100 μm. (B) The total numbers of oxytocin positive cells and of c-Fos positive cells colocalized with oxytocin positive cells were counted. n = 5–7 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA and with Tukey post hoc test if significant. *p < 0.05, **p < 0.01 vs the restraint group; ##p < 0.01 vs the High CRD group. NS: not significant.

Visceral afferent signals due to the CRD are conducted via not only the vagus nerve but also the primary afferent neurons in the pelvic plexus (Brierley et al., 2018; Fukudo, 2013a,b). Oxytocin neurons have been proven to be activated by afferent electrical stimulation of the vagal nerves and sciatic nerves whose dorsal root gaglia of the L4-S3 are shared with the lumbar and sacral afferent fibers from the colorectum (Stock and Uvnas-Moberg, 1988; Ueta et al., 2000). The activation of oxytocin PVN neurons by CRD seen in this study may presumably be caused by visceral afferent neurons. Interestingly, the signal pathway may be same as the regulation of oxytocin levels by microbiome (especialy *Lactobacillus*). The chronic stress significantly changes the bacterial community structure, reduced species richness and diversity, and causes visceral hypersensitivity (Xu et al., 2014). These changes are rescued by administration nonabsorbed oral antibiotic (rifaximin), which caused increase of the relative abundance of *Lactobacillus* (Xu et al., 2014). The *Lactobacillus reuteri* upregulates oxytocin level in the brain, by a vagus nerve-mediated pathway (Poutahidis et al., 2013; Varian et al., 2017). Considering the results of this study, some microbiome may change the ascending visceral sensory signal tone and regulate oxytocin expression in the brain.

Oxytocin has been reported to suppress CRH neuronal activation (Jamieson et al., 2017) and the HPA axis (László et al., 2016). Moreover, direct administration of oxytocin antagonists into the PVN enhances the HPA axis response to stress (Neumann et al., 2000). However, our results showed no difference in c-Fos + CRH neuronal activity of PVN among restraint, low CRD, high CRD, low oxytocin antagonist, and high oxytocin antagonist groups. Similarly, there was no difference in plasma ACTH and corticosterone levels among the restraint loading groups. These results suggest that oxytocin neurons activated by CRD do not regulate CRH neurons in the PVN and that HPA axis activation exhibits a ceiling effect under restraint stress.

Our study indicated that high CRD activates BLA and CeA neurons. Earlier work showed that CRD activates amygdala neurons (Bliss et al., 2016; Lazovic et al., 2005; Mönnikes et al., 2003;

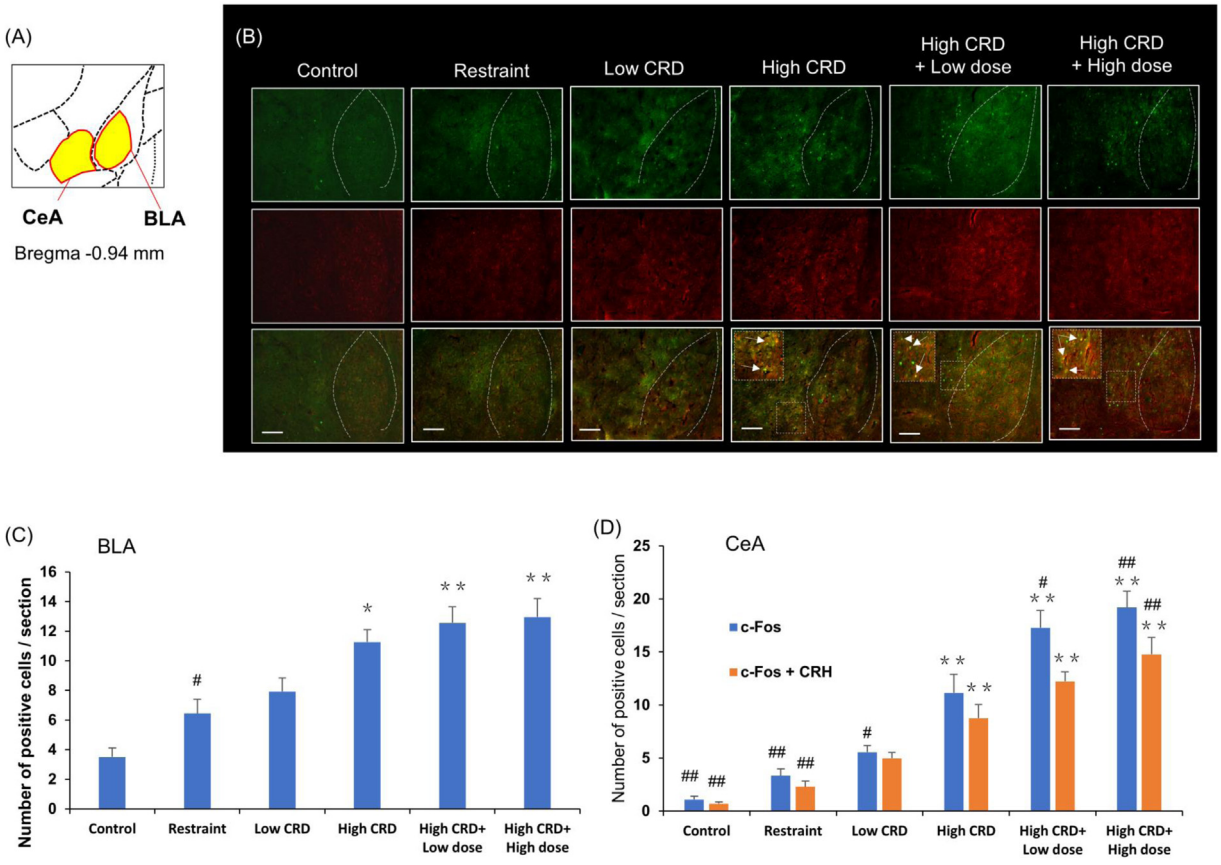

Fig. 7. Immunostaining of c-Fos and CRH in the amygdala.
Schematic of the location where positive cells were evaluated. (A) The section shown is about posterior 0.94 mm from bregma, the same location as in the PVN. (B) Representative immunostaining using an antibody against c-Fos (green) and CRH (red) in the amygdala in each group. The arrowheads indicate positive cells in the BLA and double-positive cells in the CeA. Scale bar =100 μm. (C) Only c-Fos-positive cells were counted in the BLA. (D) The total numbers of c-Fos-positive cells and of c-Fos positive cells colocalized with CRH positive cells within the CeA were counted. n = 5–7 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA and by Tukey post hoc test if significant. *p < 0.05, **p < 0.01 vs the restraint group; #p < 0.05, ##p < 0.01 vs the 60-mmHg group.

Wang et al., 2009a,b). The administration of high oxytocin antagonist before high CRD increases neuronal activation in the CeA but not in the BLA compared with the high CRD alone procedure. The amygdala plays an important role in emotional or fear memory processing and pain regulation (Neugebauer, 2015; Pare and Duvarci, 2012; Phelps and LeDoux, 2005). In particular, CeA is an important brain site for IBS symptoms due to its high content of nociceptive neurons (Neugebauer and Li, 2002) and CRH CeA causes visceral hypersensitivity (Su et al., 2015) and anxiety behavior (Pomrenze et al., 2019; Swiergiel et al., 1993). Indeed, in this study, the results of CeA neuronal activation paralleled the results of double immunostaining using c-Fos and CRH, which indicated that the neurons further activated by high-dose oxytocin antagonist were CRH neurons. Indeed, the CeA shows higher expression of oxytocin receptor and nerve fiber than the BLA (Ferretti et al., 2019; Jurek and Neumann, 2018; Yoshida et al., 2009). These findings suggest that oxytocin neurons specifically affect the CeA and negatively regulate CRH neurons in CeA.

Stress stimuli via the thalamus and cortex project to the BLA and are transmitted to the CeA (Jankord and Herman, 2008). CRH-R1 receptors are abundantly expressed in the BLA (Ray et al., 1993). Oxytocin neurons projected into the CeA may control visceral perception, since oxytocin signals downstream of CRH receptors in the brain have been suggested to regulate stress-induced visceral perception (Larauche et al., 2019). The oxytocin receptors within the CeA are expressed in gamma-aminobutyric acid (GABA) neurons as inhibitory neurons (Neugebauer et al., 2020), whose receptor binding is thought to mediate GABAergic transmission (Huber et al., 2005; Rosenfeld et al., 2011). Inhibition of CRH neurons in CeA and inhibition of anxiety-like behavior by oxytocin may be mediated by GABA (Han et al., 2018). A sheep study showed that GABA and CRH levels are increased in the amygdala in response to stress (Cook, 2004). On the other hand, microinjection of oxytocin into the CeA has anxiolytic effects (László et al., 2016) and oxytocin projecting from the PVN to CeA decreases fear responses (Ebner et al., 2005; Huber et al., 2005; Knobloch et al., 2012). Lesion studies have shown that the anxiety-like behavior induced by the EPM is dependent on the CeA and not on the BLA and ACC (Bissiere et al., 2006; Möller et al., 1997). The mechanism underlying the ability of oxytocin to reduce visceral pain and anxiety-like behavior is likely to involve a decrease in CRH CeA activity (Neugebauer et al., 2020). Thus, the CRH CeA parts of the second hypothesis were also supported.

The ACC has been suggested to be a brain region associated with emotional components of pain and pain control in chronic pain (Apkarian et al., 2005; Zhuo and Kaang, 2015) and IBS (Labus et al., 2009; Traub et al., 1996). IBS patients had abnormal signals in various brain regions, including not only the amygdala but also the ACC during CRD (Tillisch et al., 2011). Acute restraint stress induces c-Fos expression in the ACC (Jankord and Herman, 2008; Radley et al;, 2006) and further increased expression is observed with CRD under restraint (Wang et al., 2009a,b). In this study as well, the ACC was activated by restraint, and further activation was observed with high CRD under the restraint conditions. ACC activation due to CRD was suppressed by injection of high-dose oxytocin antagonist up to

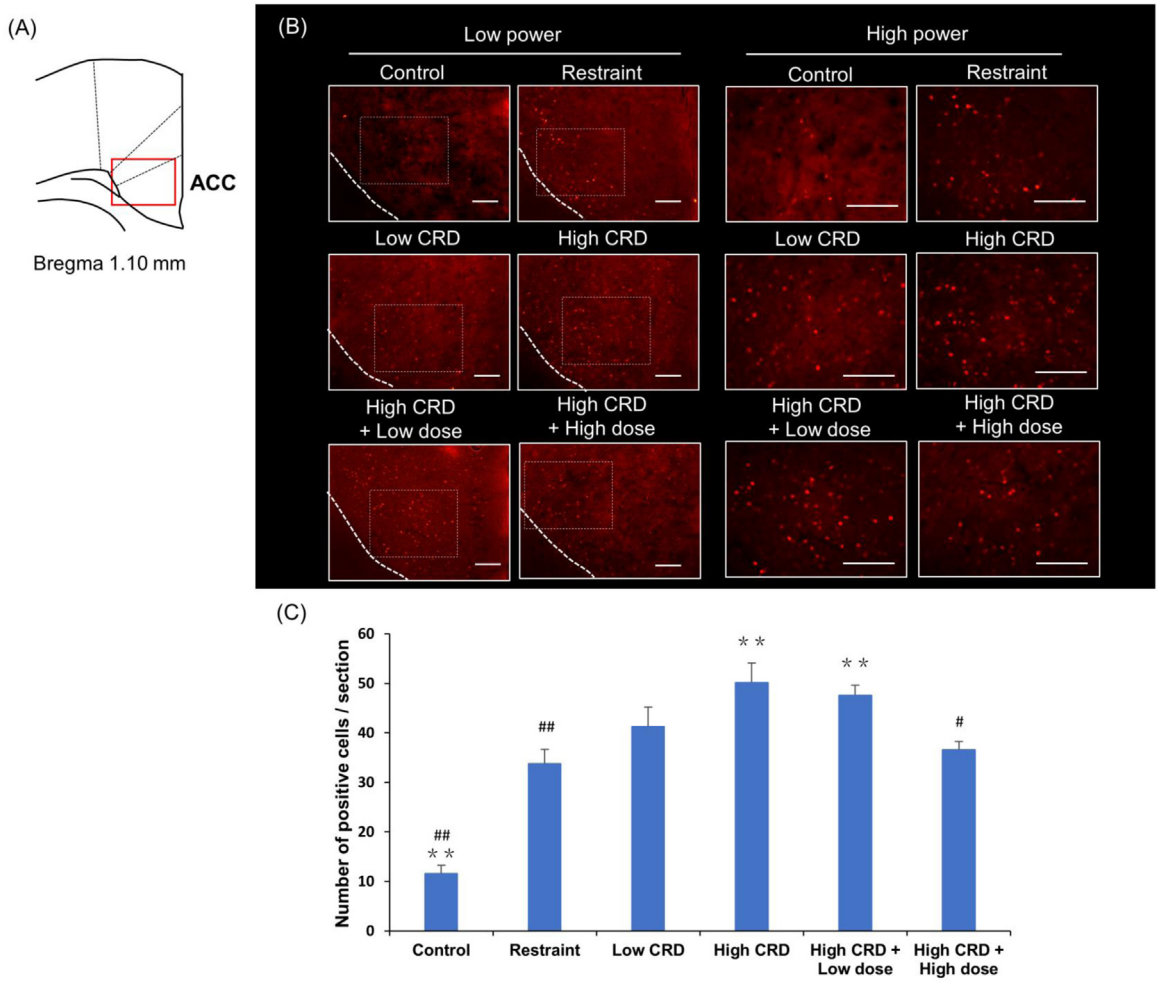

Fig. 8. Immunostaining of c-Fos-positive cells in the anterior cingulate cortex (ACC).
(A) Schematic of the location where positive cells were evaluated. The section shown is about anterior 1.10 mm from bregma. (B) Representative immunostaining using an antibody against c-Fos (red) in the ACC in each group. Scale bar =100 μm (B). (C) The total numbers of c-Fos-positive cells within the ACC were counted. n = 5–7 per group. Data are expressed as mean ± SE and were assessed by one-way ANOVA and by Tukey post hoc test if significant. $^{**}p < 0.01$ vs the restraint group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs the 60-mmHg group.

the activation level of the restraint group. Our results suggest that ACC activation due to CRD is mediated by the oxytocin receptor. Although our results did not prove a role for CRH neurons in the ACC, oxytocin neurons activated by CRD were at least associated with ACC activity. The ACC component of the second hypothesis was thus partially supported.

Interestingly, acute intranasal administration of oxytocin to the mouse activates the ACC and amygdala, with chronic administration downregulating oxytocin receptors in the CeA (Larauche et al., 2009; Pisansky et al., 2017), which is related to anxiety-like behavior (Huang et al., 2014; Peters et al., 2014). Oxytocin enhances amygdala–ACC functional connectivity, suggesting a relationship between social cognition and emotion regulation (Meyer-Lindenberg et al., 2011; Sripada et al., 2013). Thus, the activity of oxytocin-modulated PVN-ACC-amygdala circuitry likely plays a crucial role in gut stimulation-related anxiety. In previous studies, rats exhibited IBS-like symptoms such as gastrointestinal dysmotility, visceral hypersensitivity, and anxiety-like behavior due to chronic exposure to noxious CRD (Saito et al., 2005; Saito-Nakaya et al., 2008). This suggests that chronic stimuli from the colon causes dysregulation of visceral perception-emotional circuits. Considered these previous studies, chronic abnormal signals from the colon may alter oxytocin-modulated PVN-ACC-amygdala circuitry and cause IBS-like symptoms.

There are several strengths and limitations to this study. The first strength is that We used mice as the experimental animal. Despite the small size of their brains and difficult CRD and VMR experiments, the experiments were successfully completed. Mice have many advantages regarding exploration of the neuronal circuit and associated molecules. The second strength is the identification of intracerebral cells and peptides that are strongly associated with visceral nociception and anxiety.

In contrast, the first limitation is that we did not identify the neural circuit of oxytocin neurons and CRH or other neurons that specifically control visceral nociception and anxiety. A further study of the neural circuit should be conducted. The second limitation is that we could not completely rule out antagonism of vasopressin receptors via the administration of L-368,899 (Manning et al., 2012). However, L-368,899 was selected as the antagonist specific enough to oxytocin receptors without action to vasopressin receptors (Williams et al., 1994). Practically, L-368,899 is proving to be useful as a research tool (Manning et al., 2012). The third limitation is that we should have examined the effect of L-368,899 in the restraint group to determine the exact role of oxytocin neural activation by CRD. The 4th limitation is that we presented no differential counting of oxytocin neurons between parvocellular and magnocellular regions. The 5th limitation is that higher magnification photographs were lacking along with pho-

tographs for quantification. The 4th and 5th limitations should be clarified in the future studies such as using Crh-IRES-Cre mutant mice for visualization of peptidergic neurons with high resolution (Wamsteeker Cusulin et al., 2013). The 6th limitation is that this study was based on normal mice. In this study, visceral hypersensitivity as a common characteristic of IBS was induced by acute stress and oxytocin receptor antagonist. IBS pathophysiology also includes the dysregulation of intestinal fluid secretion, increased epithelial permeability of the intestine, low grade inflammation, altered composition of the gut microbiota, and visceral hypersensitivity (Enck et al., 2016). Thus, running this experiment on mice with chronic visceral hypersensitivity as an animal model of IBS (Osteen et al., 2016) may produce some different results. Further research into the role of oxytocin in normal and pathophysiological visceral pain and/or anxiety is warranted.

In conclusion, this series of experiments in mice demonstrated that intraperitoneal injection of L-368,899, an oxytocin receptor antagonist, increases anxiety-related behavior and visceral hypersensitivity, mimicking the cardinal features of IBS. CRD activates oxytocin neurons in the PVN, which likely inhibits CRH neurons in the CeA with activation of ACC neurons.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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