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Modulation of Intestinal Motility in an Adolescent Rat Model of Irritable Bowel Syndrome

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Keywords
Adolescent · Enterochromaffin cell · Irritable bowel syndrome · Neonatal maternal separation · Serotonin

Abstract
Introduction: The pathophysiology of irritable bowel syndrome (IBS) remains unknown. This study aimed to evaluate colonic motility and serotonin system response to restraint stress (RS) among adolescent rats who underwent neonatal maternal separation (NMS) to clarify the features of pathogenesis in adolescents with IBS. ***Methods:*** Male rats were exposed to NMS as chronic stress, and a normally handled (NH) group was used as control. Four groups were created by adding RS as acute stress treatment to the NMS and NH groups. To realize the RS treatment, the subjects were restrained for 1 h at the age of 5 weeks, and hourly fecal pellet discharge was determined. After euthanization and proximal colon intestinal tissue collection, 5-hydroxytryptamine (5-HT) and 5-hydroxytryptamine receptor 3 (5-HT3R) concentrations, enterochromaffin (EC) cell density, and the expression of mRNA-encoding slc6a4 were examined. ***Results:*** The amount of fecal pellet discharge during RS increased significantly in the RS and NMS+RS groups compared with that in the NH and NMS groups, respectively. The 5-HT concentration in the intestinal tissue of rats in the RS and NMS groups increased significantly compared with that of rats in the NH group. EC cell density also increased significantly in the NMS and NMS+RS groups compared with that in the NH and RS groups. However, combined stress did not result in any significant differences in the expression of 5-HT3R and mRNA-encoding slc6a4. ***Conclusions:*** The combination of juvenile and acute stress effectively induced increased 5-HT concentration or EC cell density via the 5-HT pathway in the proximal colon of adolescent rats.

Introduction

Irritable bowel syndrome (IBS) is defined using the Rome IV criteria as a common gastrointestinal motility disorder characterized by recurrent abdominal pain and fecal manifestations, such as diarrhea and constipation without any organic disease [1, 2]. Although the pathophysiology of this disease is still unknown, accumulating evidence indicates that IBS is a multifactorial disease

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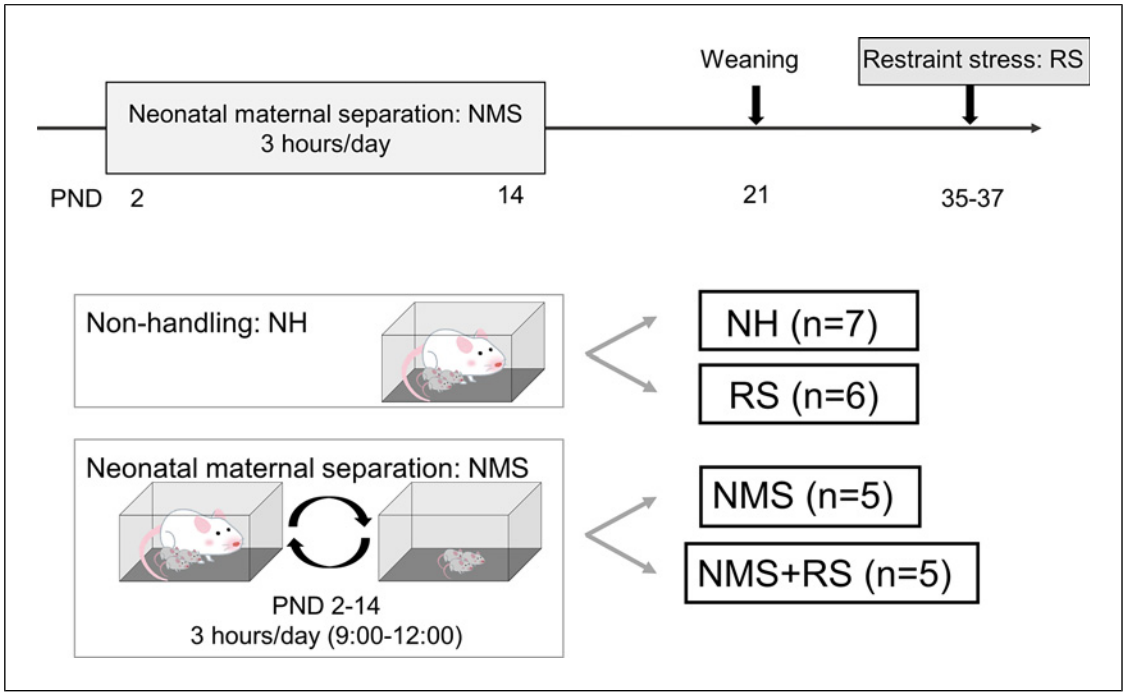

Fig. 1. Study design. Male pups were randomly separated into two groups. For the NMS protocol, the rat pups were separated from their mothers for 3 h per day during PNDs 2–14. After weaning on PND 21, siblings were allocated to separate cages until the molecular analysis started. On PNDs 35–37, the NH and NMS groups were divided into two groups: with or without RS. The NH (n = 7) and NMS groups (n = 5) did not receive RS treatment. The other two groups, i.e., the RS (n = 6) and NMS+RS groups (n = 5) received RS treatment. NMS, neonatal maternal separation; PND, postnatal day; NH, normally handled; RS, restraint stress.

caused by interactions among gastrointestinal motility, visceral sensitization, subtle intestinal inflammation, disorders in gut flora, food hypersensitivity, genetic factors, and psychosocial imbalance [3].

5-hydroxytryptamine (5-HT) is a crucial neurotransmitter and parasympathetic signaling molecule that affects the sympathetic and parasympathetic nerves as well as enteric nervous systems, thereby altering visceral perception and motility [4]. 5-HT is biosynthesized and reserved in enterochromaffin (EC) cells of the digestive tract. Studies have indicated that alterations in EC cells and increased 5-HT concentration in the human colon are correlated with IBS complains and other gastrointestinal dysfunctions [5–8]. In addition, both 5-HT3 and 5-HT4 serotonin agonists are well recognized to have a significant impact on IBS manifestations through their diverse effects on visceral analgesia and lower gastrointestinal motility [9]. Consequently, 5-HT signaling cascades are prominent candidates for IBS therapy.

Rat models of neonatal maternal separation (NMS)-triggered visceral hypersensitivity have been successfully established [10]. Given that the characteristics of the model resemble the symptoms of patients with IBS, it is frequently used to investigate the mechanisms of visceral hypersensitivity and evaluate the efficacy of the pharmacological agents in potential IBS treatments. Several studies have described changes in the enteric nervous system in NMS models [11, 12]. The effect of NMS on 5-HT expression in colonic tissues has also been investigated, but inconsistent findings were reported [11, 13–16]. Further, to the best of our knowledge, no study in the literature has evaluated this system in adolescent rats exposed to NMS.

Children with IBS develop recurrent abdominal pain in early childhood, which gradually progresses into severe bowel movement anomalies in adolescence. Compared with adults, children are more susceptible to psychological stress, such as anxiety, depression, and anger. Thus, pediatric IBS has different characteristics, and in the Rome IV criteria, adolescent-onset IBS is classified separately from IBS in adults [1, 2]; however, there are no reports on differences in pathogenesis based on patient maturity. Therefore, in this study, we evaluated the response of the colonic motor and serotonin system to restraint stress (RS) in adolescent rats who underwent NMS, with the aim to clarify the features of pathogenesis in adolescents with IBS.

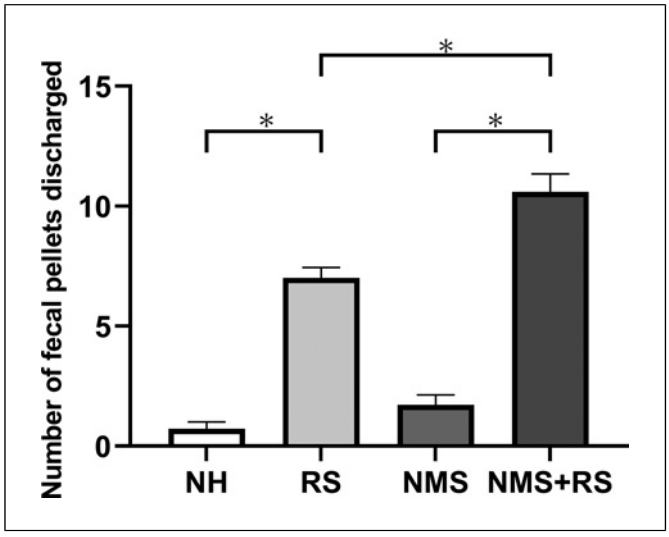

Fig. 2. Comparison of colon motility among the NH (n = 7), NMS (n = 5), RS (n = 6), and NMS+RS (n = 5) groups. Colon motility was analyzed by measuring hourly fecal pellet discharge with restraint in the RS and NMS+RS groups or with isolation in the NH and NMS groups. Data are presented as mean ± SEM. *p < 0.05. NH, normally handled; NMS, neonatal maternal separation; RS, restraint stress; SEM, standard error of the mean.

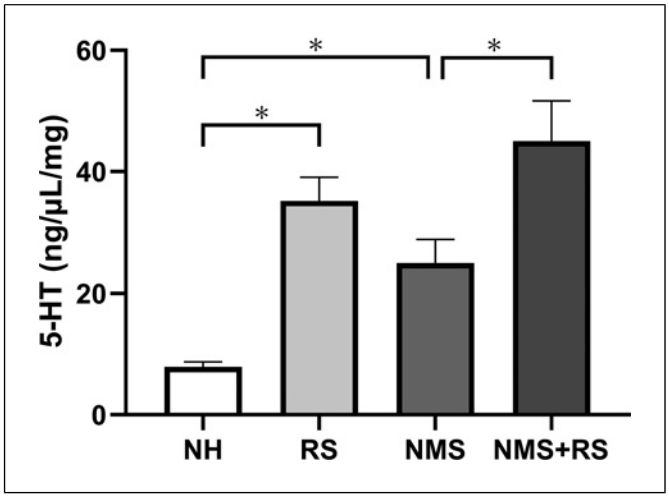

Fig. 3. Comparison of 5-HT expression in colonic tissue among the NH (n = 7), RS (n = 6), NMS (n = 5), and NMS+RS (n = 5) groups. Data are presented as mean ± SEM. *p < 0.05. 5-HT, 5-hydroxytryptamine; NH, normally handled; RS, restraint stress; NMS, neonatal maternal separation; SEM, standard error of the mean.

Materials and Methods

Animals and NMS

Pregnant female Sprague-Dawley rats were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) on gestational day 20 and were housed individually in standard polypropylene cages containing wood chip bedding material. They were maintained on a 12:12-h light-dark cycle, with free access to food and water. In the animal laboratory, the temperature was maintained at 24°C, with humidity set at 55 ± 15%. The animal care and experimental protocols were approved by the Institutional Review Board of our university (approval No. 310185). Further, all procedures were performed in accordance with the relevant guidelines and regulations. This study was also conducted in compliance with the "Animal Research: Reporting of In Vivo Experiments" guidelines.

The study design is shown in Figure 1. The NMS procedure has been described in a previous study [10]. From day 2 to day 14 after birth, rat pups were separated from their mothers for 3 h a day. Separation was performed daily between 9:00 and 12:00. The dams of the separated litters remained in their home cages during the 3-h pup isolation period. Normally handled (NH) pups were allowed to remain undisturbed in their cages with their maternal parent. We used only male pups to avoid hormonal cycle-induced variations, and the rat pups were weaned 21 days after the neonatal period, with 4–5 pups per cage. Only 5-week-old male adolescent rats weighing approximately 130.4 g (mean weight 132.5 ± 27.2 g in the NH group, n = 13, and 127.8 ± 7.3 g in the NMS group, n = 10) were utilized for this study.

Study Design

We investigated the combination effect of NMS and RS on the role of 5-HT in colonic motility and colonic tissue in adolescent rats. The rats were separated into four groups immediately after birth. The rats in the NH (n = 7) and NMS (n = 5) groups did not receive RS treatment, while those in the other two groups, i.e., the RS (n = 6) and NMS+RS groups (n = 5), received RS treatment. Fecal pellet discharge was collected from the cages, and the rats were euthanized after RS treatment. For all experimental rats, proximal colonic tissue samples were collected for EC cell studies and 5-HT expression analysis after RS or isolation and at 35–37 postnatal days.

Restraint Stress

RS treatment was performed using a modified protocol from a previous study [16]. Briefly, rats were isolated in cylindrical holders (KN-325-C-3; Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 1 h; the isolation was performed only once. Rats in the NH and NMS groups underwent 1-h isolation in individual cages without restraint.

Measurement of Fecal Pellet Discharge

Each rat was treated once with 1 h of RS or isolation, and the number of fecal pellets discharged per hour for either treatment was measured.

Tissue Preparation

After calculating the amount of fecal pellet discharge, the rats were euthanized using diethyl ether, and approximately 3 cm of the proximal colon was promptly sampled. The distal part of the proximal colon was frozen at −80°C for 5-HT and 5-hydroxytryptamine receptor 3 (5-HT3R) analysis. The middle part was preserved in RNA-later (Applied Biosystems, Waltham, MA, USA; Life Technologies, Carlsbad, CA, USA) and stored at −80°C for the assessment of mRNA-encoding slc6a4 expression.

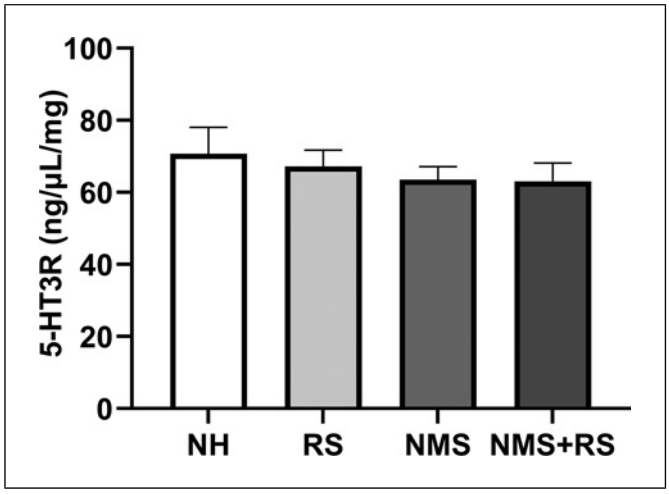

Fig. 4. Comparison of tissues stained for 5-HT3R in the proximal colon among the NH (n = 7), RS (n = 6), NMS (n = 5), and NMS+RS (n = 5) groups. The concentrations of 5-HT3R in the proximal colon tissue samples were not significantly different, regardless of the stress. Data are presented as mean ± SEM. 5-HT3R, 5-hydroxytryptamine receptor 3; NH, normally handled; RS, restraint stress; NMS, neonatal maternal separation; SEM, standard error of the mean.

Approximately 1-cm sections of proximal lesions of the terminal ileum and proximal colon were fixed in 4% paraformaldehyde and examined histologically.

Analysis of 5-HT and 5-HT3R in the Proximal Colonic Tissue

A 1 cm-long section of the proximal colon was recovered and maintained at −80°C until use. The tissue samples were homogenized in phosphate-buffered saline containing protease inhibitors at a 50 mg/mL concentration. Thereafter, 5-HT and 5-HT3R concentrations in the supernatant of the tissue homogenates were measured using enzyme-linked immunosorbent assay, in accordance with the manufacturer's instructions (5-HT; ImmuSmol, Bordeaux, France; 5-HT3R; MyBioSource, San Diego, CA, USA).

Measurement of mRNA-Encoding slc6a4 Expression

The amount of mRNA-encoding slc6a4 was quantified using real-time polymerase chain reaction (PCR). Specifically, TaqMan probe-based quantitative reverse transcription-PCR was performed using cDNA synthesized from full-thickness segments of proximal colonic tissue samples for RNA preparation (high-volume cDNA reverse transcription kit; Applied Biosystems) using a 7500 Fast Real-Time PCR system (Applied Biosystems). The expression of *slc6a4* (assay number Rn00564737_m1) was normalized to that of *β-actin* (assay number Rn00667869_m1) using the standard curve method.

EC Cell Assay

Colonic tissue samples were fixed in 4% paraformaldehyde at room temperature (~20°C) for 24 h. Subsequently, 4 μm-thick serial tissue sections were prepared from formalin-fixed and paraffin-embedded samples. EC cells were then identified via 5-HT staining. In brief, the cells were incubated with anti-5-HT antibody (1:10; Thermo Fisher Scientific, Rockford, IL, USA) and then stained using an iVIEW™ DAB Detection Kit (Ventana Medical Systems, Oro Valley, AZ, USA) and hematoxylin counterstain II (Ventana Medical Systems). The automatic immunostainer (BenchMark™; Ventana Medical Systems) was utilized for 5-HT staining in accordance with the manufacturer's instructions. Positive staining was defined as the cytoplasm of EC cells stained brown. The extent of colonic tissue in each image was evaluated using a KS400 Image Analyzer System (Carl Zeiss AG, Jena, Germany); the number of positive cells in a 1-mm^2 area of the colonic sections of each rat was counted. The average for six serial areas was defined as the EC cell density.

Statistical Analysis

Data were assessed for normality and plotted as the mean ± standard error of the mean. Graphs were generated using GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were performed using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan). The significance of the differences between groups was determined using Kruskal-Wallis test followed by Steel-Dwass test. Statistical significance was defined as $p < 0.05$.

Results

Colonic Motility

Colonic motility was analyzed by measuring the amount of hourly fecal pellet discharge with restraint in the RS and NMS+RS groups or with isolation in the NH and NMS groups. The amount of fecal pellet discharge was significantly higher in the RS and NMS+RS groups than in the NH and NMS groups ($p < 0.05$; Fig. 2). It was also significantly higher in the NMS+RS group than in the RS group ($p < 0.05$; Fig. 2).

5-HT and 5-HT3R Concentration in the Proximal Colon

The effects of NMS and/or RS on 5-HT and 5-HT3R concentrations in the proximal colonic tissue were evaluated using enzyme-linked immunosorbent assay, which showed that the concentration of 5-HT in the RS or NMS groups increased significantly compared with that in the NH group ($p < 0.05$; Fig. 3). The NMS+RS group also showed increased 5-HT concentration compared with the NMS group. However, the NMS+RS group did not show a significant increase in 5-HT concentration compared with the RS group. Furthermore, no significant differences in 5-HT3R concentration in the proximal colonic tissues were observed regardless of stress (Fig. 4).

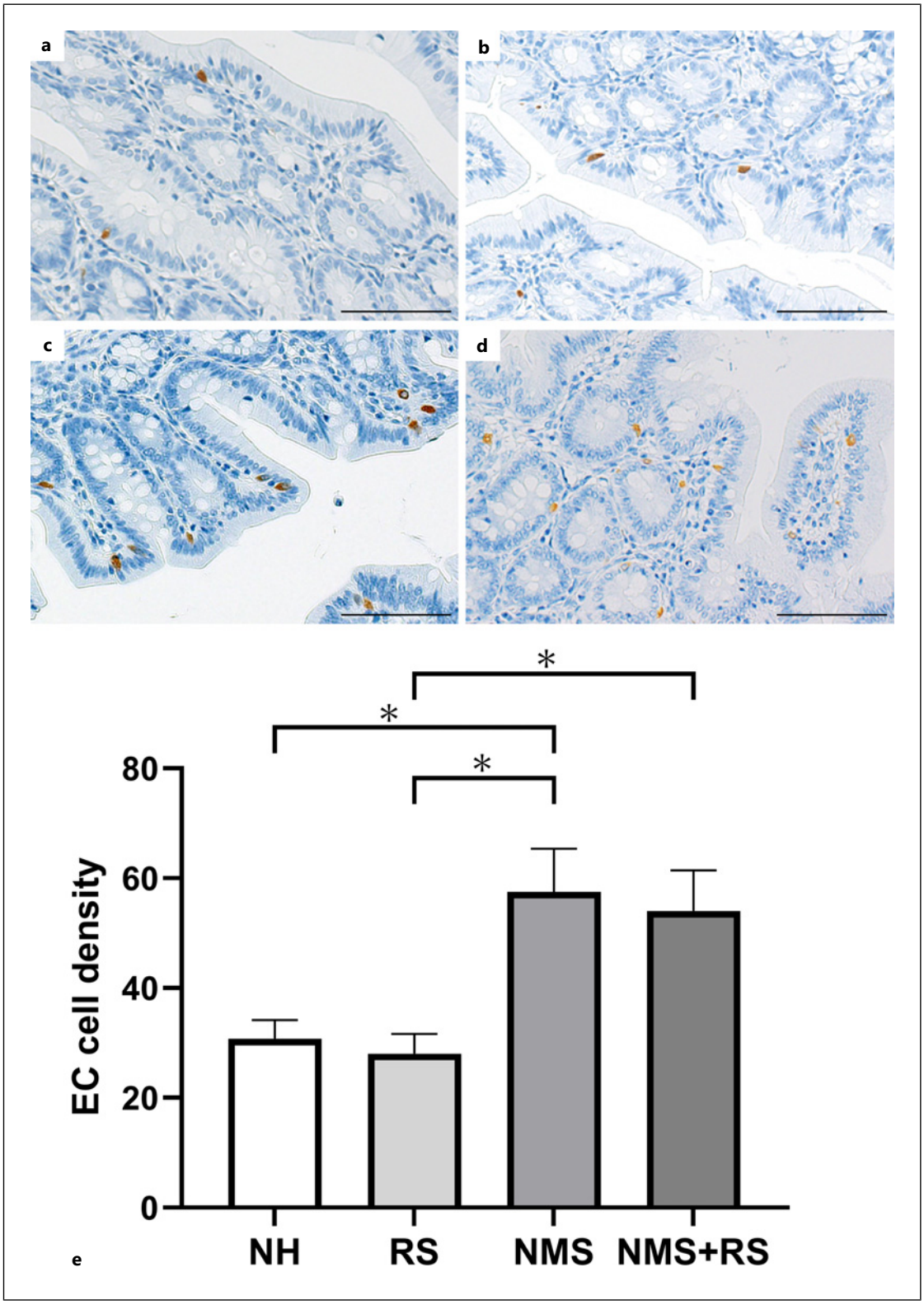

Fig. 5. Characteristic EC cell staining and EC cell density in the proximal colon of rats in the NH ($n = 7$) (**a**), RS ($n = 6$) (**b**), NMS ($n = 5$) (**c**), and NMS+RS ($n = 5$) (**d**) groups. **e** Comparison of the EC cell counts among the NH ($n = 7$), RS ($n = 6$), NMS ($n = 5$), and NMS+RS ($n = 5$) groups. EC cell density was calculated based on the number of EC cells per mm^2 of proximal colon mucosa. Data are presented as mean ± SEM. *$p < 0.05$. EC, enterochromaffin cell; NH, normally handled; RS, restraint stress; NMS, neonatal maternal separation; SEM, standard error of the mean.

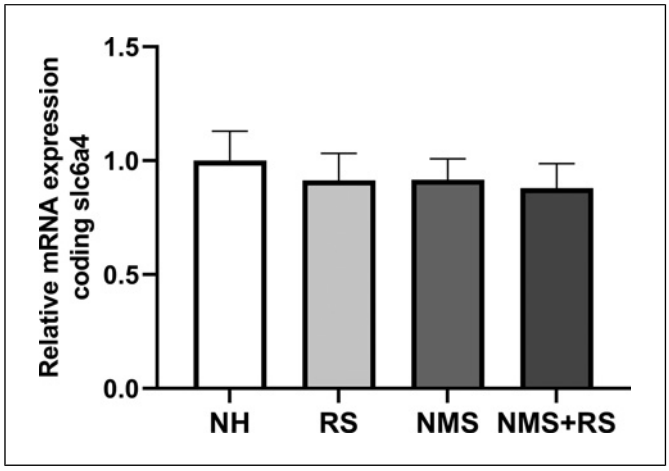

Fig. 6. Comparison of the expression of the mRNA-coding slc6a4 in the proximal colon among the NH (n = 7), RS (n = 6), NMS (n = 5), and NMS+RS (n = 5) groups. The expression amount of mRNA-coding slc6a4 in the proximal colon was not significantly different, regardless of the stress. Data are presented as mean ± SEM. slc6a4, serotonin transporter; NH, normally handled; RS, restraint stress; NMS, neonatal maternal separation; SEM, standard error of the mean.

EC Cell Density of the Proximal Colonic Mucosa

The immunostaining images for 5-HT were shown in Fig. 5a–d. EC cell density was assessed based on the number of EC cells per 1 mm^2 in the proximal colon. As shown in Figure 5e, the NMS and NMS+RS groups showed significantly increased EC density compared with the NH and RS groups, respectively ($p < 0.05$). However, the RS group did not show any increase in EC cell density compared with the NH group.

mRNA-Encoding slc6a4 Expression

Altered serotonin transporter (SERT) expression in the colon can contribute to the abdominal hypersensitivity and abnormal colonic motility associated with IBS [17]. Therefore, we evaluated the level of mRNA-encoding slc6a4 (which encodes SERT) in adolescent rats. We normalized the amount of *slc6a4* mRNA in the proximal colonic tissue to that of *β-actin*, and found that it was not significantly different, regardless of stress (Fig. 6).

Discussion

In this study, we examined the combined effects of acute RS and NMS on the 5-HT system of the intestinal mucosa in adolescent rats. We found that RS in adolescent rats induced colonic hypermotility that increased with the 5-HT concentration in the proximal colon, but there was no change in EC cell density. NMS in adolescent rats resulted in no change in colonic hypermotility but significantly increased the 5-HT concentration owing to hyper-EC cell density in the proximal colon. Adolescent rats with NMS were predisposed to respond strongly to RS, resulting in colonic motility disorders with increased 5-HT concentrations; however, EC cell density in the proximal colon was not affected.

NMS is a well-established stress model for adults. Low adrenocortical activity and reduced reactivity to stressors on postnatal days 4–14 result in a normal stress response [18]. Postnatal NMS can cause lifelong hyperactivity of the hypothalamic-pituitary-adrenal axis and its response to stressors [19], and individuals with NMS are known to exhibit IBS-like hyperkinetic and visceral irritability [20, 21]. The serotoninergic pathway is affected by NMS. Reportedly, the concentration of 5-HT in the frontal cortex and the expression of 5HT-1A, 1B, and 2A in the parietal cortex and/or the hippocampus are increased in NMS [22, 23]. Moreover, serotoninergic neurons in the raphe nucleus and spinal cord are activated in NMS models [20, 21]. In rat experiments, the effects of the treatment with the mast cell inhibitor, resveratrol, were enhanced by pretreatment with a 5-HT-1A agonist, indicating the involvement of 5-HT-1A receptors in the pathogenesis of stress-induced visceral sensitization [24]. Several studies have described changes in the enteric nervous system in NMS models [21, 22]; however, no reports have evaluated the system in adolescent rats exposed to NMS.

Our study is the first to evaluate the intestinal nervous system in adolescent rats exposed to RS and NMS. In adolescent rats, RS-induced colonic hypermotility that increased with the 5-HT concentration in the proximal colon, which showed no change in EC cell density. These findings are consistent with those of a previous study in adult rats, which showed that acute RS stimulates vagal nerves innervating the proximal colon via central CRF1 receptors, increasing 5-HT release from EC cells in the proximal colon [25]. In contrast, NMS in adolescent rats resulted in a significant increase in the 5-HT concentration and EC cell density in the proximal colon without a similar change in colonic hypermotility. A previous study showed that NMS induces the expansion of intestinal stem cells and their differentiation toward secretory lineages, including EC and Paneth cells, leading to EC hyperplasia and increased serotonin production [16].

Our analysis of tissue samples collected immediately after 1 h of acute stress showed an increase of 5-HT concentration in the NMS+RS and RS groups. Interestingly,

Bian et al. [14] showed that 1 h of water avoidance stress as acute stress had no effect on colonic motility 24 h after acute stress and on the 5-HT concentration in the proximal colon after 24 h. Therefore, we supposed that the change in the 5-HT concentration in the proximal colon caused by 1 h of acute stress may not persist.

We detected no differences in the expression of 5-HTR in both the NMS and NH groups similar to several previous studies, which showed no significant differences in 5-HT3 receptor mRNA expression level between NMS rats and normal rats [11, 26]. Therefore, we guessed that it is unlikely for hyper-colonic motility to result from increased affinity of 5-HT to receptors.

Bian et al. [15] showed increased SERT expression in adult rats who had undergone NMS. In adolescents who underwent NMS in our study, there was no increase in the expression amount of mRNA-encoding slc6a4, which encodes SERT; however, it is necessary to assess protein expression as it is unclear whether this difference between adolescent and adult rats resulted from differences in maturity.

This study had some limitations that need to be addressed by future studies. First, we used hourly fecal pellet discharge in the RS and NMS groups to assess colonic motility as a symptom of IBS. Nonetheless, intestinal hypersensitivity is one of the major symptoms of IBS; therefore, stress-induced hypersensitivity should be evaluated under the same conditions. Second, to confirm colonic contractions, the direct assessment of intestinal peristalsis is recommended using analytic methods such as electric field stimulation using ex vivo colon tissue; however, direct motility was not assessed in the present study. As a surrogate for the assessment of direct bowel motility, the number of bowel movements was assessed. Third, we assessed only the 5-HT system of the proximal colon because a previous study showed that the proximal colon has a higher 5-HT concentration and is more sensitive to stress than the distal colon [14]. Nonetheless, the 5-HT mechanism in other parts of the intestinal tract, including the distal small intestine or distal colon, should be evaluated to clarify the mechanism of 5-HT involvement in IBS development. Fourth, in this study, we compared the symptoms of IBS in adulthood and the characteristics of the serotonin mechanism with those previously reported. Adult rat models should be established and examined under the same conditions to evaluate the mechanisms of IBS development in adolescents. Fifth, it has been reported that 5-HT3R genotypes are associated with IBS onset. However, in this study, we did not investigate the genotype of the receptor. Thus, further studies are required in this regard. Additionally, it will be very interesting to compare the symptoms and serotonergic mechanisms of rats expressing the IBS susceptibility gene with those of wild-type rats by performing NMS or RS.

Finally, IBS development is influenced by factors other than those in the 5-HT pathway; consequently, further studies on other factors such as mucosal permeability and intestinal microbiota need to be considered. In conclusion, our study demonstrated that in the proximal colon of adolescent rats, a combination of NMS stress and acute RS effectively induced intestinal motility deficits via the 5-HT pathway, which did not differ from the findings noted in adult rats.

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Statement of Ethics

The animal care and experimental protocols were approved by the Institutional Review Board of our university (approval No. 310185). All procedures were performed in accordance with the relevant guidelines and regulations, and this study was conducted in compliance with the "Animal Research: Reporting of In Vivo Experiments" guidelines.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

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Author Contributions

R.K., T.K., and M.S. formulated the ideas, research goals and aims, and design of the methodology, and wrote and presented the published work, specifically writing the initial draft; R.K., T.K., M.S., and T.I. curated data and conducted formal analysis; R.K., N.I., K.T., N.A., M.S., and K.J. conducted the research and investigations, specifically performing the experiments and collecting data and

evidence; Y.O. and T.S. were entrusted with leadership responsibility for the research, ensuring planning and execution and providing mentorship to the core team; T.K. and T.S. performed critical review, commentary, or revision. All authors have read and approved the final manuscript.

Data Availability Statement

All analyses relevant to the study are included in this manuscript. All data requests should be submitted to the corresponding authors for consideration.

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