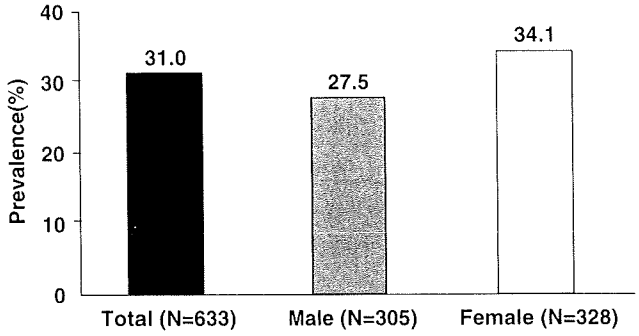

FIGURE 1. Sex difference in the prevalence of IBS among outpatients in Japan. The prevalence of IBS in medical outpatients denoted 31% in Japan. The prevalence of IBS in females tended to be more than that in males ($\chi^2 = 2.92$, $P<0.1$).

($P < 0.01$) or FBD subjects ($P < 0.01$). IBS scores in FBD subjects were significantly higher than those in normal subjects ($P < 0.01$).

Table 2 shows the subgroups of IBS subjects: 42 subjects (21.4%) were IBS-C, 58 subjects (29.6%) were IBS-D, and 96 subjects (49.0%) were IBS-A. The sex ratio (male:female) of the subgroups was as follows: IBS-C (1:1), IBS-D (1:1.52), and IBS-A (1:1.4, Table 2). There was no significant difference in the sex ratio among IBS subgroups.

The prevalence of IBS depending on age is shown in Figure 3. The ages of the subjects ranged from 15 to 96 years old. The proportion of the population depending on age in this study was not significantly different from the proportion of the total population of Japan depending on age given in September 2002 by the Ministry of Health, Labour and Welfare of Japan. The prevalence of IBS

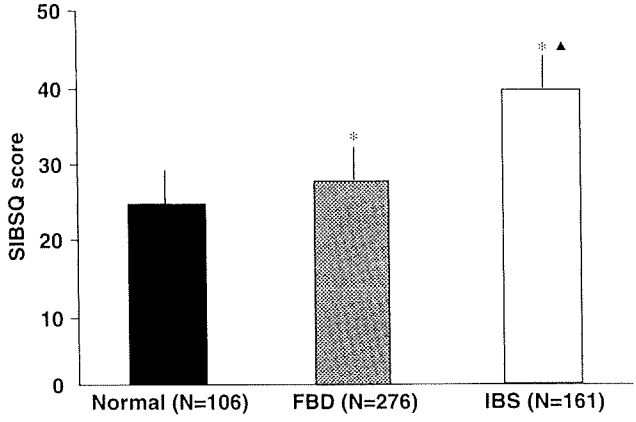

FIGURE 2. The SIBSQ scores of patients with IBS, those with FBD, and normal subjects. A significant difference among the 3 groups of subjects was detected by ANOVA ($F = 17$, $P<0.01$). Post hoc test revealed significant difference between normal subjects and subjects with FBD *($P<0.01$), normal subjects and subjects with IBS *($P<0.01$), and subjects with FBD and those with IBS (▲$P<0.01$).

TABLE 2. IBS Subgroups

	Male (%)	Female (%)	Total
IBS-C	21 (10.7)	21 (10.7)	42 (21.4)
IBS-D	23 (11.7)	35 (17.9)	58 (29.6)
IBS-A	40 (20.4)	56 (28.6)	96 (49.0)
Total	84 (42.8)	112 (57.2)	196 (100)

was relatively high (43% to 56%) among the 10 to 30-year olds, but gradually declined age-dependency and formed a nadir (14%) in patients in their 70-years-old. After 70-years-old, the prevalence of IBS gradually increased again. These changes in the prevalence of IBS were statistically significant ($\chi^2_8 = 20.8$, $P < 0.05$). IBS symptoms in the elderly were independent on the organic comorbidity.

Figure 4 shows the ratio of RIIMQ-defined IBS or FBD among the top 6 common medical diseases in Japanese outpatients. A significantly different distribution of IBS or FBD status was observed among physician-diagnosed medical diseases (ANOVA, $P = 0.01$). The prevalence of subjects with RIIMQ-defined IBS (72%) was significantly high among physician-diagnosed IBS patients ($P < 0.01$). In the other 5 medical diseases, the prevalence of IBS ranged from 12% to 33%. The prevalence of IBS in the remaining various diseases denoted 44%.

Table 3 shows differences in perceived stress, lifestyle, and hospital visiting status among IBS, FBD, and normal subjects. Perceived stress ($P < 0.0001$), meal habits ($P < 0.0001$), and sleep habits ($P < 0.0001$) significantly differed among the 3 groups. IBS patients had significantly more perceived stress, less regular sleep, and meal habits. There was no difference in smoking and drinking behavior or in hospital visiting status among the 3 groups.

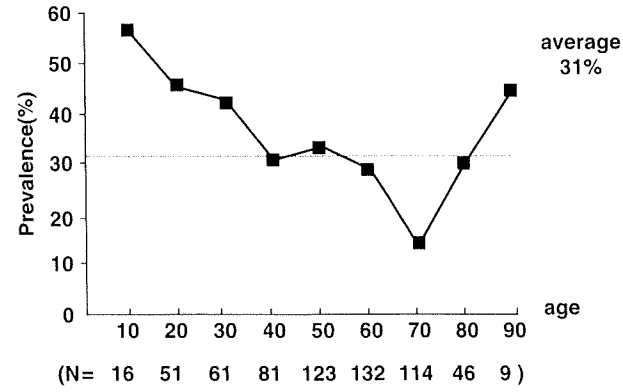

FIGURE 3. The age distribution in the prevalence of IBS among medical outpatients. Changes in prevalence of IBS depending on age were statistically significant ($\chi^2 = 20.8$, $P<0.05$).

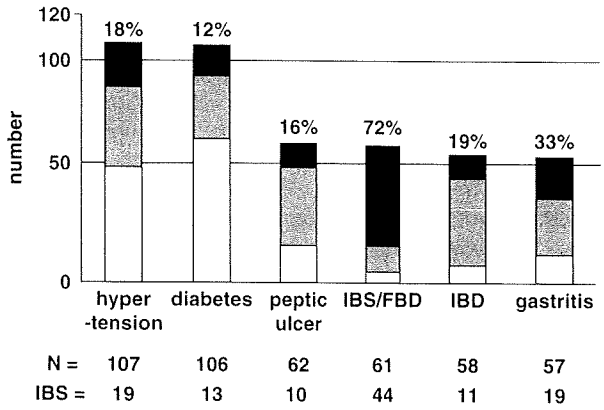

FIGURE 4. Physician's diagnosis and IBS/FBD status. Significantly different distribution of IBS/FBD status was observed among the physician-diagnosed medical diseases (ANOVA, $F=11$, $P<0.01$). RIIMQ-defined IBS was significantly higher in IBS/FBD patients ($P<0.01$). Boxes are indicated as follows: closed box ■; IBS, shaded box ▨; FBD, and open box □; normal.

DISCUSSION

This study is the first national survey of the prevalence of Rome II-defined IBS among medical outpatients in Japan. RIIMQ correctly reflected the validated IBS scores of SIBSQ. In addition, subjects diagnosis based on RIIMQ correctly reflected physician-diagnosed IBS status. Because RIIMQ is available in English-speaking countries,[18,19] the results of this study are comparable with those obtained in those countries.

In this study, the prevalence of IBS in Japanese medical outpatients was 31%. This value is as high as the 30% prevalence of IBS obtained in the United Kingdom.[4] However, the study conducted in the United Kingdom included only patients with GI complaints. It is striking that 31% of Japanese patients who visited hospitals departments of internal medicine during our study were diagnosed with IBS even if the sample patients were not restricted to GI diseases. The rationale for the high prevalence of IBS in medical outpatients in Japan may be the influence of the rapidly increasing westernized eating habits, irregular sleeping habits, and the stressful social life in Japan. Indeed, previous studies have shown that Japanese who have low fiber intake have more GI complaints,[24] increased sleep disorders,[25] and increased psychosocial stress,[26] all of which are associated with IBS.[13,27-28]

Most epidemiologic data of IBS from western countries show that IBS is dominant in females.[1-5] This study also denoted a trend of female dominance. However, an earlier epidemiologic survey in Japan reported that males had a higher prevalence of Rome I-defined IBS than females.[21] As the earlier survey[21] did not use a validated questionnaire for IBS, our study here seems to be close to accurate IBS status in Japan. In addition, the lack of a stronger dominance among females in Japanese IBS subjects may be due to the somewhat weaker sex effect than those in western countries.

In our paper, IBS-A is the remaining IBS other than IBS-C or IBS-D. In the systematic review of bowel habit subtypes of IBS,[29] primary care office-based studies showed IBS-A as the most prevalent group such as 52.4% or so. The prevalence 49% of IBS-A in our study is comparable data with the earlier reports.[29]

The prevalence of IBS depending on age in this study showed results similar to those in the United States and Europe[30,31] even though the age range was slightly higher in this study. The highest prevalence of IBS was obtained among teenagers, then the prevalence decreased with age until the 70s. The prevalence of IBS in the 90s was also high, second only to teenagers. However, the small number of subjects (4 in 9 subjects) was not enough to draw a firm conclusion. On the other hand, when calculating the proportion of people aged 85 years and above to the total Japanese population in accordance with the estimated national population classified by sex and age in September 2002, it is only 2%, which is the same ratio as in this study. In addition, an earlier study has suggested that the prevalence of IBS increases with increased age.[32] Therefore, worldwide prevalence of IBS depending on age may form 2 peaks, first among adolescents and second among the elderly. Although data of high prevalence of IBS in the elderly should be considered carefully, independence of IBS comorbidity with the organic diseases in the elderly suggest the aging over 70 as a potential risk of IBS. Larger survey focusing IBS and the elderly is necessary.

The high (72%) but not maximal (100%) prevalence of RIIMQ-defined IBS-D in physician-diagnosed IBS or FBD is not surprising, because general physicians do not always agree to use Rome II criteria.[22] The prevalence of IBS in patients with various medical diseases (44%)

TABLE 3. Stress/Life/Style and IBS/FBD Status

Items	Choices	Normal (N = 161, %)	FBD (N = 276, %)	IBS (N = 196, %)
Smoking	Yes	26.0	71.7	25.0
	No	73.9	28.2	75.0
Alcohol	No	53.0	50.7	58.6
	Drink sometimes	26.7	27.5	24.5
	Drink everyday	19.9	21.7	18.4
Diet*†	Regular	42.8	38.7	31.9
	Sometimes irregular	50.3	51.8	45.9
	Irregular	6.8	9.4	23.0
Sleeping*†	Regular	29.8	32.9	71.0
	Sometimes irregular	59.0	53.3	49.5
	Irregular	11.2	3.6	29.6
Perceived stress*†	Present	29.2	31.9	55.1
	Absent	70.8	68.1	44.9
Initial visit	Yes	85.7	88.4	85.7
	No	14.3	11.6	14.3

*Compared with normal vs. IBS, $P < 0.0001$.
†Compared with FBD vs. IBS, $P < 0.0001$.

or gastritis (33%) was also very high. In addition, a considerable prevalence of IBS in patients with hypertension (18%) or diabetes mellitus (12%) was detected. These findings suggest that IBS is a hidden medical problem among patients who visit hospitals departments of internal medicine.

Similar to earlier reports,[12,13] this study proved a higher perception of psychosocial stress in patients with IBS. Although earlier reports have also suggested a hypersensitivity to food in IBS patients,[33,34] this study clarified that long-term irregular dietary and sleeping behavior among IBS patients plays a role in the pathogenesis of IBS. Actually, the therapeutic guideline for IBS in the United Kingdom focuses on modification of life style.[27] Whether only behavioral modification, such as stress inoculation, a regular diet and/or regular sleep cycles, may improve the status of IBS is a testable hypothesis.

The sample size (633) of this study is considered to be enough to estimate the prevalence of IBS in outpatients in Japan. It is much more than the number (400) indicated by the theory of sample size determination and the number of subjects (255) published in an earlier study conducted in the United Kingdom.[4] In addition, the response rate (60.6%) in our study was much more higher than that in the earlier report,[4] and the surveyed area covered all districts of Japan. Finally, the number of outpatients consultation during the study was close to the average number of daily practice in Japan.

There are several limitations to this study. First, the patients were enrolled at outpatient departments of internal medicine that belong to the IBS Club. Therefore, the data obtained from the study might be biased by patients who knew physicians' interest in IBS. Second, the collective ratio of subjects who completely responded to the questionnaires was only 61%. However, this ratio was similar to those reported in earlier studies (approximately 60% of total sample).[19,35,36] Therefore, it is possible to compare the results of this study with those of previous studies. Third, we could not discriminate whether IBS symptoms were due to organic GI disorder per se. Further investigation on organic GI symptoms is necessary.

CONCLUSIONS

In this study, we conducted the first national survey of the prevalence of Rome II-defined IBS among outpatients in Japan. We found that the prevalence of IBS in medical outpatients in Japan is 31%. Perceived stress together with irregular dietary and sleeping behavior are suggested to be the main causes of this high prevalence of IBS among medical outpatients in Japan.

ACKNOWLEDGMENTS

The authors thank Dr Motoyori Kanazawa, Dr Yuka Endo, Dr Naoki Nakaya, Dr Todd Holden, Mr Jeremy Simmons, and Mrs Shigeko Simmons for their help throughout the study.

IBS Club, Japan consists of the council members mentioned in the author group and the allied members as belisted below: Teine Royal Hospital, Furano Kyokai Hospital, Kobayashi Hospital, Akita Social Insurance Hospital, Yonaizawa General Hospital, Tohoku Rosai Hospital, Miyagi Central Hospital, Ohta Nishinouchi Hospital, Nanpuh Hospital, Fukuoka Takano Hospital, Machida Municipal Hospital, Minamitama Hospital, Aichiken Saiseikai Hospital, Kanagawa Prefectural Atsugi Hospital, Fukui Kosei Hospital, Fukui Social Insurance Hospital, Fukui General Hospital, Koto Hospital, Higashisumiyoshi Morimoto Hospital, Osaka City Juso Hospital, Chuden Hospital, Mazda Hospital, JR Hiroshima General Hospital, Saiseikai Fukuoka General Hospital, Takano Hospital, Ouji Hospital, Takeda General Hospital, Morinosato Hospital, Tsusima Municipal Hospital, and Saganino Central Hospital.

REFERENCES

1. Drossman DA, Ritcher JE, Talley NJ, et al. *Functional Gastrointestinal Disorders*. Boston: Little Brown; 1994:1–174.
2. Drossman DA, Whitehead WE, Camilleri M. Irritable bowel syndrome: a technical review for practice guideline development. *Gastroenterology*. 1997;112:2120–2137.
3. Müller-Lissner SA, Bollani S, Brummer RJ, et al. Epidemiological aspects of irritable bowel syndrome in Europe and North America. *Digestion*. 2001;64:200–204.
4. Thompson WG, Heaton KW, Smyth GT, et al. Irritable bowel syndrome in general practice: prevalence, characteristics, and referral. *Gut*. 2000;46:78–82.
5. Donker GA, Foets M, Spreeuwenberg P. Patients with irritable bowel syndrome: health states and use of health care services. *Br J Gen Pract*. 1999;49:787–792.
6. Levy RL, Von Korff M, Whitehead WE, et al. Costs care for irritable bowel syndrome patients in a health maintenance organization. *Am J Gastroenterol*. 2001;96:3122–3129.
7. Silk DB. Impact of irritable bowel syndrome on personal relationships and working practice. *Eur J Gastroenterol Hepatol*. 2001;13:1327–1332.
8. Oliver C, Jean-Francois B. Quality of life in irritable bowel syndrome, effect of therapy. *Eur J Surg Suppl*. 1998;583:81–86.
9. Drossman DA, Patrick DL, Whitehead WE, et al. Further validation of the IBS-QOL: a disease specific quality-of-life questionnaire. *Am J Gastroenterol*. 2000;95:999–1007.
10. Groll D, Vanner SJ, Depew WT, et al. The IBS-36: a new quality of life measure for irritable bowel syndrome. *Am J Gastroenterol*. 2002;97:962–971.
11. Drossman DA, Camilleri M, Mayer EA, et al. AGA technical review on irritable bowel syndrome. *Gastroenterology*. 2002;123:2108–2131.
12. Drossman DA, Sandler RS, McKee DC, et al. Bowel patterns among subjects not seeking health care. Use of a questionnaire to identify a population with bowel dysfunction. *Gastroenterology*. 1982;83:529–534.
13. Whitehead WE, Crowell MD, Robinson JC, et al. Effects of stressful life events on bowel symptoms: subjects with irritable bowel syndrome compared with subjects without bowel dysfunction. *Gut*. 1992;33:825–830.
14. Whitehead WE, Schuster MM. Irritable bowel syndrome: definition of the syndrome and relation to other disorders. Physiological and psychological mechanism. In: Whitehead WE, Schuster MM, eds. *Gastrointestinal Disorders: Behavioral and Physiological Basis of Treatment*. New York: Academic Press; 1985:155–209.
15. Drossman DA, Creed F, Olden KW, et al. Psychosocial aspects of functional gastrointestinal disorders. *Gut*. 1999;45(suppl 2):II25–II30.

16. Thompson WG, Longstreth GF, Drossman DA, et al. Functional bowel disorders and functional abdominal pain. *Gut*. 1999;45 (suppl 2):II43–II47.
17. Thompson WG, Longstreth GF, Drossman DA, et al. Functional bowel disorders and functional abdominal pain. In: Drossman AD, Talley NJ, Corazziari ETNJ, et al, eds. *Rome II The Functional Gastrointestinal Disorders*. 2nd ed. Boston: McLean, Degnon Associates; 2000:351–432.
18. Drossman DA, Talley NJ, Whitehead WE, et al. Research diagnostic questions for functional gastrointestinal disorders: Rome II Modular Questionnaire: Investigator and Respondent Forms. In: Drossman DA, Corazziari E, Tallen NJ, et al, eds. *Rome II The Functional Gastrointestinal Disorders*. 2nd ed. Boston: McLean, Degnon Associates; 2000:669–688.
19. Thompson WG, Irvine EJ, Pare P, et al. Functional gastrointestinal disorders in Canada: first population-based survey using Rome II criteria with suggestions for improving the questionnaire. *Dis Dig Sci*. 2002;47:225–235.
20. Maruyama S, Morimoto K. Effects of long work hours on life-style, stress and quality of life among intermediate Japanese managers. *Scand J Work Environ Health*. 1996;22:353–359.
21. Itasaka T, Takahashi T. The epidemiological investigation for irritable bowel syndrome using a group examination. *Ther Res*. 1996; 17:4059–4064.
22. Shinozaki M, Kanazawa M, Sagami Y, et al. Validation of the Japanese version of the Rome II modular questionnaire and irritable bowel syndrome severity index. *J Gastroenterol*. 2006;41:491–494.
23. Endo Y, Yoshizawa M, Fukudo S, et al. Panic disorder in irritable bowel syndrome. *Jpn J Psychosom Med*. 2000;40:339–346.
24. Nakaji S, Sugawara K, Saito D, et al. Trends in dietary fiber intake in Japan over the last century. *Eur J Nutr*. 2002;41:222–227.
25. Ohida T, Osaki Y, Doi Y, et al. An epidemiologic study of self-reported sleep problems among Japanese adolescents. *Sleep*. 2004; 27:978–985.
26. Fujino Y, Mizuno T, Tokui N, et al. Prospective cohort study of stress, life satisfaction, self-rated health, insomnia, and suicide death in Japan. *Suicide Life Threat Behav*. 2005;35:227–237.
27. Jones J, Boorman J, Cann P, et al. British society of gastroenterology guideline for the management of the irritable bowel syndrome. *Gut*. 2000;47(suppl II):1–19.
28. Vege SS, Locke GR III, Weaver AL, et al. Functional gastrointestinal disorders among people with sleep disturbances: a population-based study. *Mayo Clin Proc*. 2004;79:1501–1506.
29. Guilera M, Balboa A, Mearin F. Bowel habit subtypes and temporal patterns in irritable bowel syndrome: systematic review. *Am J Gastroenterol*. 2005;100:1174–1184.
30. Saito Y, Schoenfeld P, Locke GR III. The epidemiology of irritable bowel syndrome in North America: a systematic review. *Am J Gastroenterol*. 2002;97:1910–1915.
31. Agreus L, Talley NJ, Svardsudd K, et al. Identifying dyspepsia and irritable bowel syndrome: the value of pain or discomfort, and bowel habit descriptors. *Scand J Gastroenterol*. 2000;35:142–151.
32. Agreus L, Svardsudd K, Talley NJ, et al. Natural history of gastroesophageal reflux disease and functional abdominal disease: a population-based study. *Am J Gastroenterol*. 2001;96: 2905–2914.
33. Locke GR III, Zinsmister AR, Talley NJ, et al. Risk factors for irritable bowel syndrome: role of analgesics and food sensitivities. *Am J Gastroenterol*. 2000;95:157–165.
34. O'Leary C, Wieneke P, Buckley S, et al. Celiac disease and irritable bowel type symptoms. *Am J Gastroenterol*. 2002;97: 1463–1467.
35. Talley NJ, Boyce PM, Jones M. Is the association between irritable bowel syndrome and abuse explained by neuroticism? A population based study. *Gut*. 1998;42:47–53.
36. Locke GR III, Talley NJ, Nelson DK, et al. Helicobacter pylori and dyspepsia: a population-based study of organism and host. *Am J Gastroenterol*. 2000;95:1906–1913.

Neurogastroenterol Motil (2008) **20**, 1147–1156 doi: 10.1111/j.1365-2982.2008.01151.x

Corticotropin-releasing hormone receptor 1 antagonist blocks colonic hypersensitivity induced by a combination of inflammation and repetitive colorectal distension

K. SAITO-NAKAYA, R. HASEGAWA, Y. NAGURA, H. ITO & S. FUKUDO

Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

Abstract *Gastroenteritis is one of the risk factors for developing irritable bowel syndrome (IBS). However, the precise mechanism of postinfectious IBS is still unknown. We tested the hypothesis that a combination of previous inflammation and repetitive colorectal distention (CRD) makes the colon hypersensitive and that treatment with a corticotropin-releasing hormone receptor 1 (CRH-R1) antagonist blocks this colonic hypersensitivity. Rats were pretreated with vehicle or 2,4,6-trinitrobenzene sulphonic acid (TNBS) 6 weeks before CRD. For the CRD experiment, the colorectum was distended once a day for six consecutive days. The CRH-R1 antagonist (CP-154,526, 20 mg* kg^{-1}*) or vehicle was injected subcutaneously 30 min before CRD. Visceral perception was quantified as visceromotor response (VMR) using an electromyograph. For histological examination, the rats were killed on the last day of CRD experiment, and haematoxylin and eosin-staining of colon segments was performed. Although from the first to the third day of CRD, VMRs increased in both the vehicle-treated rats and TNBS-treated rats, they were significantly higher in TNBS-treated rats than those in vehicle-treated controls. On the fifth day of CRD, however, VMRs in the vehicle-treated rats were significantly greater than those in TNBS-treated rats. Pretreatment of rats with CP-154,526 significantly attenuated the increase in VMR induced by repetitive CRD with previous inflammation. Finally, we found that repetitive CRD and repetitive CRD after colitis induced visceral inflammation. These results indicate that a combination of previous inflammation and repetitive CRD induces visceral hypersensitivity and that a CRH-R1 antagonist attenuates this response in rats.*

Keywords *corticotropin-releasing hormone receptor 1, inflammation, repetitive colorectal distension, sensitization, visceral hypersensitivity.*

Abbreviations: CRD, colorectal distention; CRH-R1, corticotropin-releasing hormone receptor 1; EMG, electromyographic; GABA, γ-amino butyric acid; IBS, irritable bowel syndrome; PI-IBS, postinfectious IBS; TNBS, 2,4,6-trinitrobenzene sulphonic acid; VMR, visceromotor response.

INTRODUCTION

Irritable bowel syndrome (IBS), a prototypic functional gastrointestinal disorder,[1,2] is generally accompanied by hypersensitivity to rectal[3,4]/colonic[5] distention and increased intestinal reactivity to psychosocial stressors.[6–8] Because IBS has an estimated prevalence of 3–22% of the population,[9–11] it is believed that common life events account for the sensitization process in IBS patients. Several prospective studies have indicated that a substantial proportion of patients (7–33%) with acute bacterial gastroenteritis develop IBS symptoms that persist for many months.[12–14] Although there are many possible causes of IBS, two prospective studies using non-infected controls have indicated that bacterial gastroenteritis is one of the main risk factors for developing IBS, which is known in this case as postinfectious IBS (PI-IBS).[15,16] Moreover, individual susceptibility to psychosocial stressors, such as depression, anxiety and somatization, before acute gastroenteritis is a strong predictor of PI-IBS.[12,13,16]

Various types of stress are known to increase parameters associated with colonic inflammation. For instance, acute cold-restraint stress and water-avoidance stress have been shown to enhance ionic and

Address for correspondence
Shin Fukudo MD, PhD, Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
Tel: +81 22 717 8214; fax: +81 22 717 8161;
e-mail: sfukudo@mail.tains.tohoku.ac.jp
Received: 6 June 2007
Accepted for publication: 30 April 2008

macromolecular permeability.[17,18] In addition, chronic water-avoidance stress has been reported to induce barrier dysfunction, bacterial adhesion and penetration into enterocytes, and hyperplasia and activation of mast cells.[19–21] Moreover, a previous study has indicated that previous colitis makes the colon more vulnerable to stress.[22] Indeed, 6 weeks after 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis, restraint stress caused a significant increase in myeloperoxidase activity in TNBS-treated rats but not in the stressed controls.[22] Other studies have shown that chronic water-avoidance stress and wrap-restraint stress aggravate TNBS-induced colitisa[23] and that acute or previous inflammation induces colon hypersensitive to colorectal distention (CRD).[24–27] These animal observations are consistent with some of the main symptoms displayed by IBS patients and suggest that rats with previous TNBS-induced colitis can be used as a model of PI-IBS.

Recent advances in neuroscience have clearly conceptualized the idea that information from visceral organs is fundamental for understanding emotional processing.[28] In this concept, CRD has been considered as a unique stressor that triggers interoceptive stress response.[29] Although corticotropin-releasing hormone (CRH) and its receptor 1 (CRH-R1) are known to play an important role in colonic response to acute stress[30–34] and in visceral inflammatory responses,[17,18] little is known about the role of CRH-R1 in the response to repetitive CRD. We have previously reported that chronic CRD induces increased fecal pellet output in response to novel environment stressor in rats.[35] This response was not accompanied by elevation of plasma adrenocorticotropic hormone release and anxiety, but interestingly was blocked by a CRH-R1 antagonist.[35] Based on these findings, we wished to examine the effects of a combination of previous inflammation and repetitive CRD on visceral perception and the role of CRH-R1 in the ensuing responses. In this study, we tested the hypothesis that combination of previous inflammation and repetitive CRD makes the colon hypersensitive and that pretreatment with a CRH-R1 antagonist blocks this colonic hypersensitivity. We also tested the hypothesis that the sensitization pattern varies among the days of repetitive CRD.

MATERIALS AND METHODS

Animals

Male Wistar rats (n = 24) weighing 180–210 g were purchased from Charles River Breeding Laboratories Inc. (Yokohama, Japan). The rats were housed under controlled illumination (12 : 12-h light/dark-cycle starting 8:00 AM) and temperature (23 ± 1 °C) with free access to food and water. This study was approved by the Ethics Committee of Laboratory Animals, Tohoku University.

Drugs

The specific CRH-R1 antagonist CP-154,526 (*N*-butyl-*N*-[2,5-dimethyl-7-(2,4,6,trimethylphenyl)-7H-pyrrolo-[2,3-d]pyrimidin-4-yl]-*N*-ethylamine; Pfizer, Croton, CT, USA) was kept at room temperature and dissolved in a mixture of 5% dimethyl sulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA), 5% cremophor El (Sigma) and 90% saline.

Induction of colitis

Induction of colitis and recovery from it were performed as described elsewhere.[22,23] In brief, rats were lightly anaesthetized with ether, and a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus and its tip was advanced to 8 cm proximal to the anus verge. 2,4,6-Trinitrobenzene sulphonic acid dissolved in 50% ethanol was instilled into the colon through the cannula (30 mg in a volume of 0.25 mL). Following TNBS instillation, the animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillate. Control rats were instilled with 0.25 mL of 50% ethanol instead of TNBS/ethanol. After induction of colitis, rats were allowed to recuperate for 6 weeks before testing.

Surgical procedure

Rats were deeply anaesthetized with pentobarbital sodium (50 mg kg^{-1}) administered intraperitoneally. The electrodes of an electromyograph (EMG; Star Medical, Tokyo, Japan) were then stitched into the external oblique musculature for elecromyogram recording. Electrode leads were tunneled subcutaneously and exteriorized at the nape of the neck for future access. After surgery, rats were housed individually and allowed to recuperate for 6 days before testing.

Colorectal distention

For CRD, rats were restrained in a tube cage 3 min before EMG recording. A 7- to 8-cm-long polyethylene bag was inserted into the colorectum through the anus, and anchored by taping the balloon catheter to the base

of the tail. The pressure was monitored and kept constant by a pressure controller-timing device (Distender Series II; G & J Electronics, Toronto, ON, Canada). Rats were distended with noxious phasic CRD (80 mmHg, 20 s, 1-min interstimulus interval, 15 times), and their response was quantified as visceromotor response (VMR) characterized by contraction of the abdominal and hindlimb musculature. Electromyographic activity produced by contraction of the external oblique musculature was quantified by recording the number of discharges crossing a preset voltage threshold (baseline), as described previously.[36] The preset voltage threshold was determined by data collected 20 s before each distention. EMG recordings were collected and analysed using 8 STAR version 6.0–19.2 for Windows software (Star Medical). An increase in the total number of counts during distention over baseline was taken as response.

Experimental protocol

Repetitive CRD experiments, in which the colorectum of each rat was distended once a day for six consecutive days, started 6 weeks after instillation of TNBS or vehicle. The rats were divided into three groups; repetitive distention alone (n = 7), repetitive distention after colitis (n = 5), and repetitive distention after colitis and treatment with CRH-R1 antagonist (n = 7) (Fig. 1). CRH-R1 antagonist (CP-154,526, 20 mg kg^{-1}) or vehicle was injected subcutaneously 30 min before CRD. For histological examination, the rats were killed on the last day of CRD experiment and colonic segments were obtained from the distal colon (7–8 cm from the anus). Non-stressed rats (n = 5), kept under the same housing conditions as the stressed rats, were used as control.

Histological examination

A 1-cm-long piece of the colon was fixed in 10% formalin neutral buffer solution for at least 24 h, embedded in paraffin, and haematoxylin and eosin (H&E)-stained. Tissues from five to seven rats per group were scrutinized, one tissue section per rat, and, for each tissue, 10 contiguous non-overlapping areas above the muscularis mucosae were examined. Neutrophils, eosinophils and intraepithelial lymphocytes were identified, and cells in coded sections were counted and expressed as number of cells per mm^2 or number of cells per 100 cells.

Statistical analysis

All data are expressed as the mean ± standard error (SE). Mean value of the initial three (first, second and third) recordings on the first day was set as 100% VMR. Each datum was calculated as percentage VMR. Percentage VMR to each stimulus was first analysed and then the mean value for each day was calculated. Statistical significance was evaluated using ANOVA (one- and 2-way, repeated measures) and the *post hoc* test. A probability level of 0.05 or less was considered to be statistically significant.

RESULTS

Effects of previous inflammation on repetitive colorectal distention-induced visceromotor response

Significant period effect (F = 3.5, P < 0.001) and group × period interaction (F = 4.7, P < 0.001) were detected by two-way ANOVA on percentage VMR

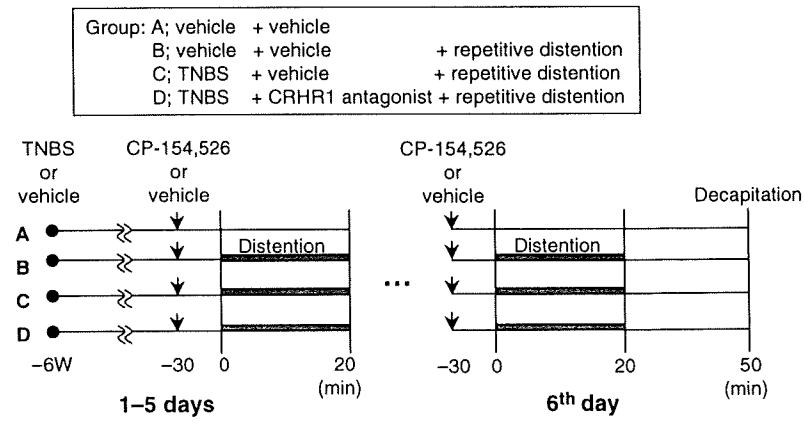

Figure 1 Timeline for colonic inflammation and repetitive colorectal distention (CRD) in rats. ▬ Colorectal distention (80 mmHg, 20 s, 15 times).

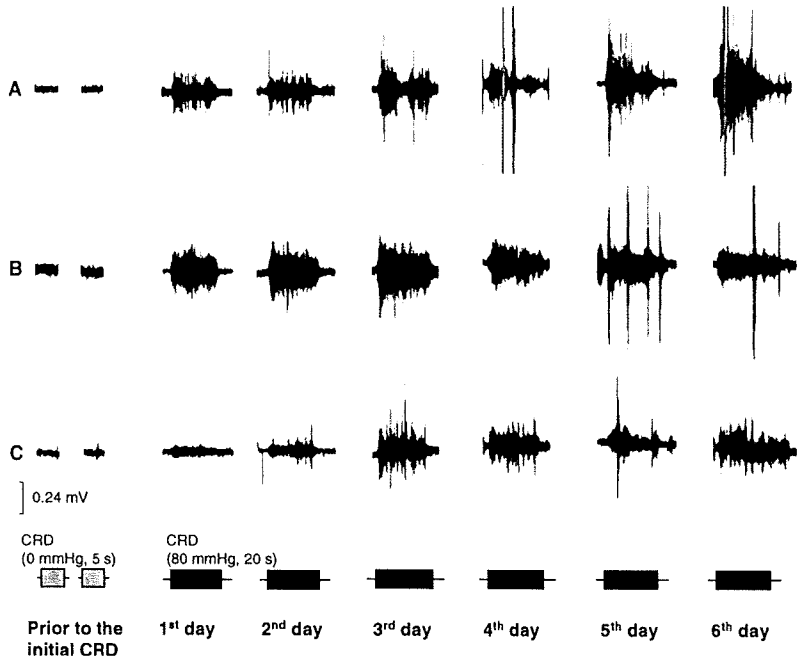

Figure 2 Visceromotor response represented as electromyographic activity prior to the initial distension (0 mmHg, 5 and 15 s before colorectal distention (CRD)) and following repetitive CRD (80 mmHg, 20 s duration). (A) Repetitive CRD alone, (B) repetitive CRD with previous inflammation and (C) repetitive CRD with previous inflammation and CP-154,526 treatment.

(Fig. 2). However, no significant group effect was detected by two-way ANOVA on percentage VMR (Fig. 3). In the rats subjected to repetitive CRD alone, VMR on the fifth and sixth days of CRD was significantly higher than that on the first, second, third and fourth days of CRD ($P < 0.001$) (Fig. 3). In TNBS-treated rats, in contrast, VMR on the third day of CRD was significantly higher than that on the first, second, fourth, fifth and sixth days of CRD ($P < 0.05$). Similarly, VMR on the first, second and third days of CRD was significantly higher than that on the fourth day of CRD ($P < 0.01$) (Fig. 3). On the first day of CRD, VMR in the initial three recordings did not significantly differ between the vehicle-treated rats (group A, Fig. 3) and TNBS-treated rats (group B, Fig. 3). However, repetitive CRD produced significantly robust contractions of the abdominal musculature in both the vehicle-treated rats ($P < 0.01$) and TNBS-treated rats ($P < 0.01$), although TNBS-treated rats showed significantly greater response than the vehicle-treated rats ($P < 0.05$). Although on the second day of CRD VMR in the vehicle-treated rats significantly increased with repeated stimuli ($P < 0.01$), it did not significantly differ from that recorded in TNBS-treated rats. On the third day of CRD, VMR in TNBS-treated rats was significantly greater than that in the vehicle-treated rats ($P < 0.01$). On the fourth day of CRD, however, VMR did not significantly differ between the vehicle-treated rats and TNBS-treated rats. On the fifth day of CRD, VMR in the vehicle-treated rats was, in contrast to the initial 3 days of CRD, significantly greater than that in TNBS-treated rats ($P < 0.05$). Finally on the sixth day of CRD, VMR did not significantly differ between the vehicle-treated rats and TNBS-treated rats.

Effects of specific CRH-R1 antagonist on previous inflammation-induced visceral hypersensitivity

Significant group effect (TNBS-treated rats and TNBS-/CRH-R1 antagonist-treated rats, $F = 12.6$, $P < 0.01$), period effect ($F = 1.5$, $P < 0.01$) and group × period interaction ($F = 2.9$, $P < 0.001$) were detected by two-way ANOVA on percentage VMR (Fig. 3). In the rats treated with TNBS and the CRH-R1 antagonist CP-154,526, VMR on the third, fourth, fifth and sixth days of CRD was significantly higher than that on the first and second days of CRD ($P < 0.05$) (Fig. 3). On the first day of CRD, VMR in the initial three recordings did not significantly differ between TNBS-treated rats (1655.6 ± 146.5 counts $20\ s^{-1}$) and TNBS-/CRH-R1 antagonist-treated rats (1695.6 ± 98.3 counts $20\ s^{-1}$). From the first to the third day of CRD, VMR in TNBS-/CRH-R1 antagonist-treated rats was significantly lower than that in TNBS-treated rats ($P < 0.001$). However, from the fourth to the sixth day of CRD, VMR did not significantly differ between TNBS-treated rats and TNBS-/CRH-R1 antagonist-treated rats.

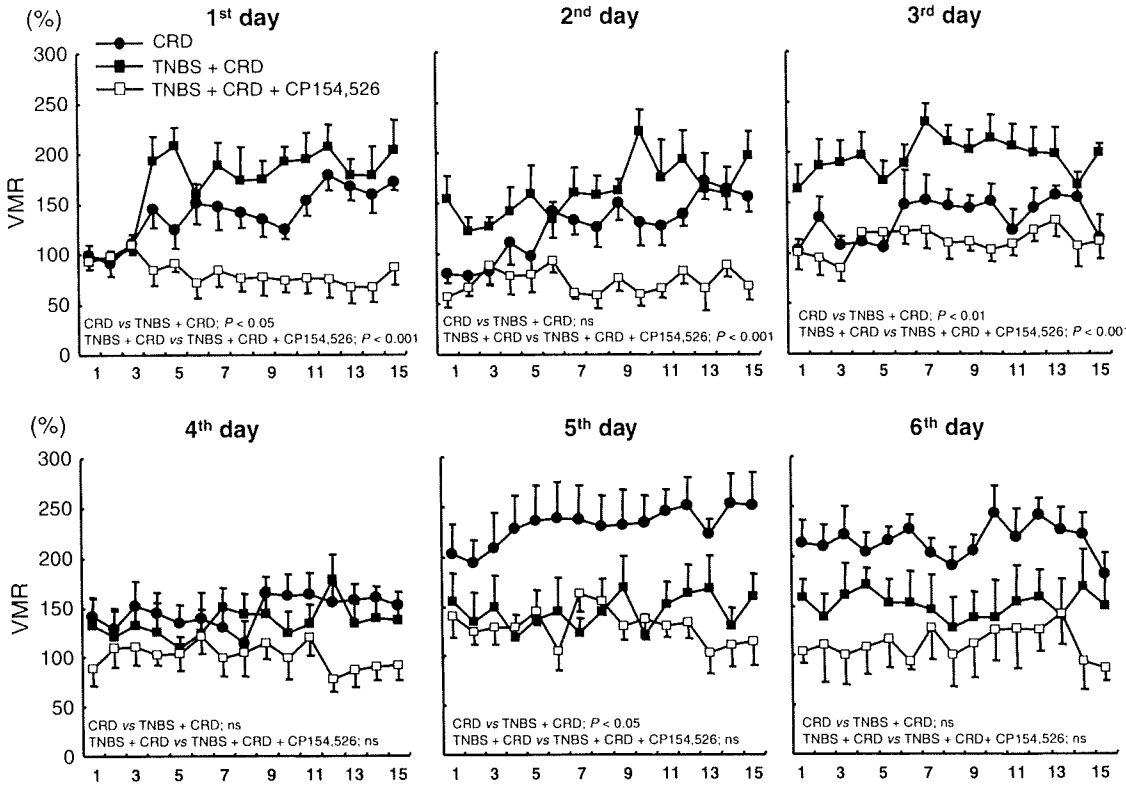

Figure 3 Effects of repetitive colorectal distention (CRD) alone, repetitive CRD with previous inflammation, and repetitive CRD with previous inflammation and CP-154,526 on visceromotor response. Numbers on the abscissa mean ordinal number of stimulation with 80 mmHg for 20 s. Data are expressed as the mean ± SE (n = 5–7 rats per group). P-value for group effect was determined by two-way ANOVA.

Effects of repetitive CRD and repetitive CRD after colitis: histological examination

Light microscopic photomicrographs of H&E-stained colon segments taken on the sixth day of CRD showed colonic inflammation in both the rats that received repetitive CRD rats and those that were treated with TNBS (Fig. 4B,C). However, no overt colonic inflammation was observed in the non-stressed rats and TNBS-/CRH-R1 antagonist-treated rats (Fig. 4A,D). In rats that received repetitive CRD, CRD exposure for 6 days induced a rise in the numbers of neutrophils, eosinophils and intraepithelial lymphocytes compared with the non-stressed rats (Table 1). On the other hand, in TNBS-treated rats, CRD exposure for 6 days induced a rise in the number of intraepithelial lymphocytes compared with the non-stressed rats, and a significant decrease in the number of neutrophils and eosinophils compared with the repetitive CRD rats. Finally, in TNBS-/CRH-R1 antagonist-treated rats, the numbers of neutrophils, eosinophils and intraepithelial lymphocytes did not differ from those in the TNBS-treated rats. There was a significant decrease in the numbers of neutrophils and eosinophils in TNBS-/CRH-R1 antagonist-treated rats when compared with the repetitive CRD rats.

DISCUSSION

This is the first to demonstrate that combination of previous inflammation and repetitive CRD makes the colon hypersensitive. Moreover, the time course of colonic sensitization by repetitive visceral stimulation has clearly been clarified in this study. The most important finding of this study is that treatment with a specific CRH-R1 antagonist dramatically attenuated visceral hypersensitivity induced by a combination of previous inflammation and repetitive CRD.

Previous inflammation makes the colon vulnerable to stress and increases parameters associated with colonic inflammation.[22,23] In addition, it has been reported that inflammation induces visceral

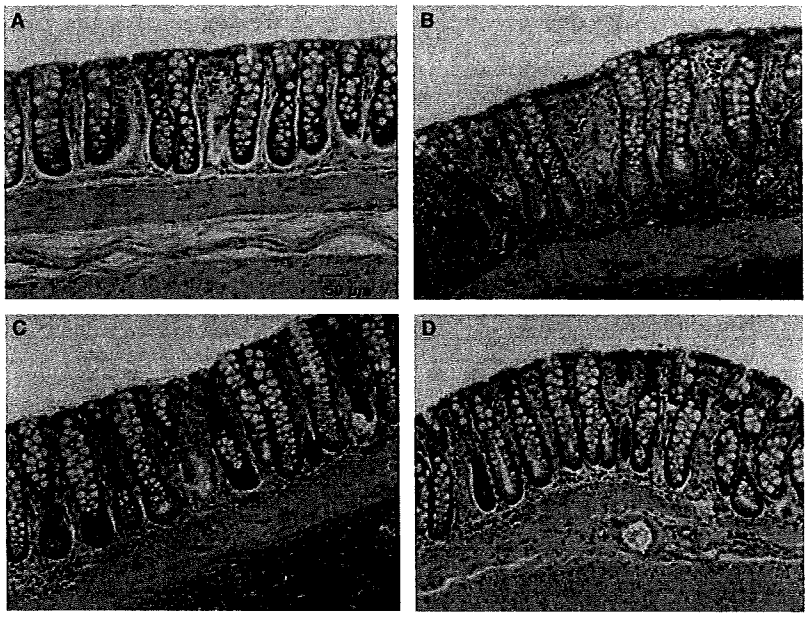

Figure 4 Effects of repetitive colorectal distention (CRD) alone and repetitive CRD with previous inflammation on colonic inflammation. (A) Non-repetitive CRD, (B) repetitive CRD alone, (C) repetitive CRD with previous inflammation and (D) repetitive CRD with previous inflammation and CP-154,526 treatment.

Table 1 Effects of repetitive CRD and repetitive CRD after colitis on cells-count in H&E-stained colon segments

	Neutrophils (cells mm^{-2})	Eosinophils (cells mm^{-2})	IEL (cells per 100 cells)
Non-stressed	25 (2)	30 (6)	4 (1)
Repetitive CRD	40 (3)[a]	46 (4)[a]	8 (1)[a]
TNBS-treated	29 (2)[b]	23 (3)[b]	8 (1)[a]
TNBS-/CRH-R1 antagonist-treated	22 (3)[b]	24 (3)[b]	8 (1)[a]

CRD, colorectal distention; IEL, intraepithelial lymphocytes; TNBS, 2,4,6-trinitrobenzene sulphonic acid; CRH-R1, corticotropin-releasing hormone receptor 1. The rats were killed on the last day of CRD experiments, and haematoxylin and eosin (H&E)-staining of colon segments was performed. Tissues from five to seven rats per group were scrutinized, one tissue section per rat, and, for each tissue, 10 contiguous non-overlapping areas above the muscularis mucosae were examined. Values are shown as mean (SEM). [a]$P < 0.05$ compared with non-stressed rats. [b]$P < 0.01$ compared with rats that received repetitive CRD rats.

sensitization and hyperalgaesia, and that TNBS-, mustard oil- or acetic acid-induced colitis increases VMR to phasic or tonic CRD.[24–27] Increased pain sensitivity after acute colitis has been suggested to be due to sensitization of peripheral and/or central nociceptors.[37–39] At the peripheral level, gut tissue injury has been shown to induce the release of prostaglandins and bradykinin, which increase sensitivity of afferent nerve terminals.[38,39] Prostaglandins and bradykinin are also known to stimulate the release of histamine, serotonin, nerve growth factor and prostanoids from afferent nerves. These events sensitize the endings of afferent nerve terminals and increase response to painful stimuli.[38,39] At the central level, inflammation increases pain signal transmission in the dorsal horn of the spinal cord and/or in the brain.[38,39] In this study, sensitization in TNBS-treated rats continued for the first 3 days, but was not observed after the fourth day of CRD. The sensitization for the first 3 days seems to be similar to that observed in patients with PI-IBS. We reported that acute CRD induces anxiety-like behaviours in rats.[35] More anxious patients suffered from IBS after acute gastroenteritis.[12] Because rats pretreated with CRH-R1 antagonist did not exhibit colonic sensitization, the present rat model at least in part provides a possible mediator of colonic hypersensitivity in PI-IBS. Acute inflammation has been shown to generate hyperalgesia, whereas repetitive inflammation, which involves lymphocytic infiltration into the mucosal and submucosal tissue accompanied by μ-opioid receptor and β-endorphin upregulation, provides an antinociceptive input that restores normal visceral perception in mice.[40] Patients with active colitis have been reported to exhibit reduced tolerance to balloon distension of the rectum, whereas patients with chronic or quiescent colitis exhibit normal or increased tolerance to distension.[41] These differences have been attributed to changes in descending spinal modulation of sensory input from the periphery during chronic inflammation.[41] This could partly explain the switch in visceral sensitivity that occurred on day 4 in repetitively distended TNBS-treated rats. On the other

hand, detection of visceral hypersensitivity after intestinal inflammation may depend on the programme of viscera stimulation. Accordingly, no difference in VMR was observed between TNBS-treated and TNBS-untreated rats at the initial three stimuli on the first day of CRD, but VMR increased from the fifth distention in the TNBS-treated rats. In addition, no robust differences between the two groups were observed on the initial days of testing. This may be due to the fact that after induction of colitis, rats were allowed to recuperate for 6 weeks, a period reported to be sufficient for recovery from TNBS-induced colonic inflammation.[22] It has also been reported that 6 weeks after administration of TNBS, mild restraint stress for three consecutive days causes a significant increase in myeloperoxidase activity in TNBS-treated rats, but not in the stressed controls.[22] This is the first study that measures VMR under almost the same experimental protocol. There is a possibility that inflammation was induced by stimulation on the first day and differences in VMR appeared after the second day. However, this is only a hypothesis as no histological assessment was conducted on the first and second days of stimulation. Further studies are necessary to clarify the mechanisms behind these phenomena.

Repetitive distention of the sigmoid colon has been reported to induce visceral hypersensitivity[42] and increased contractions of the descending colon[5] in patients with IBS. However, the precise mechanism of visceral hypersensitivity after repetitive visceral stimulation is still unknown. In this study, from the first to the third day of CRD, rats treated with TNBS were the most sensitized, while on the fifth day of CRD, rats that received CRD alone were the most responsive. Repetitive noxious CRD induces colonic plasma extravasation, suggesting minor colonic inflammation.[43–45] In addition, experimental stress can be an initiating factor in intestinal inflammation by impairing mucosal defense against luminal bacteria via mast cell degranulation.[19,46] Moreover, repetitive CRD as an interoceptive stressor can on one hand reactivate colonic inflammation[23] and lead to decrease in sensitivity after a few days, and on the other hand activate other pathways, such as mast cell degranulation, and increase colonic permeability, leading to hyperalgesia. Inflammation can also be responsible for remodelling of the primary afferent neurons innervating the colon, which could explain hypersensitivity to repetitive CRD.[47] Another possible explanation of this phenomenon is that repetitive CRD induces changes in molecular components of the brain–gut axis. Indeed, repetitive noxious CRD has been shown to increase Fos immunoreactivity in the spinal cord[45,48] via *N*-methyl-D-aspartate receptors.[45] Previous studies have also shown that many molecules, including T-type Ca^{2+}channels, γ-amino butyric acid (GABA), $GABA_B$ receptors, opioids and CRH are involved in the control of visceral perception.[35,38,39,49,50] However, the hypothesis that dynamic changes in nociceptive and antinociceptive molecules may account for visceral hypersensitivity after repetitive visceral stimulation remains to be verified.

In this study, treatment with a specific CRH-R1 antagonist attenuated visceral hypersensitivity induced by a combination of previous inflammation and repetitive CRD. We have previously elucidated the role of CRH in visceral hypersensitivity[51] and shown that administration of non-specific CRH receptor antagonist reduces abdominal pain evoked by repetitive electrical stimulation of the rectum in patients with IBS.[34] We have also proved that pretreatment with CRH-R1 antagonist attenuates CRD-induced hippocampal noradrenaline release and visceral perception in rats.[35] In agreement with these results, others have shown that a CRH-R1 receptor antagonist abolishes the activation of locus coeruleus neurons induced by colorectal distension in rats.[52] Other evidence on the other hand contrasts the role of CRH-R1 and CRH-R2 in visceral nociception, i.e. CRH-R1 is involved in pronociception of visceral pain, whereas CRH-R2 is related to antinociception.[53,54] Stress-induced colonic hypersensitivity has been reported to be mediated by CRH and CRH-R1.[55,56] Besides, it has been shown that peripheral CRH may also play an important role in intestinal hypersecretion and inflammation induced by *Clostridium difficile* toxin A and that CRH-R1 antagonists inhibit this response.[57] Activation of CRH-R1 causes pro-inflammatory responses, whereas stimulation of CRH-R2 provokes anti-inflammatory effects.[51,58] In our study, histological findings in animals treated with a CRH-R1 antagonist support this notion. Further studies on CRH and this peptide family, and on CRH receptor subtypes during sensitization (e.g. from first to third day of CRD in TNBS-treated rats) or recovery (e.g. from fourth to sixth day of CRD in TNBS-treated rats) processes in the brain–gut axis are warranted. The results of this study suggest that among the key molecules relating to visceral perception, at least CRH and CRH-R1 play a key role in visceral hypersensitivity caused by a combination of previous inflammation and repetitive CRD.

As indicated by our histological examination of H&E-stained colonic segments, repetitive CRD induced colonic inflammation. The number of intraepithelial lymphocytes was increased in all groups except the non-stressed group. However, the numbers of neutrophils and eosinophils were increased only in the

vehicle-treated groups. It is known that TNBS induces acute colitis, notably transmural inflammatory cellular infiltration associated with a Th1-dominated cytokine profile.[59] On the other hand, under Th2-associated inflammatory conditions, a marked increase in eosinophils occurs not only in the lamina propria but also in Peyer's patches.[60] Therefore, rats with repetitive CRD appear to show a different quality of inflammation from rats with repetitive CRD after TNBS. Further studies are needed to clarify the precise mechanisms underlying these phenomena. Moreover, data from Collins group clarified that transient colitis induced by TNBS full recovers and there is no inflammation in the mucosa.[22] However, TNBS is known to induce transmural inflammation in the colon.[59] Therefore, still there is possibility that sensitization of primary afferent neurons may be due to the sustained inflammatory changes in the muscular layer. This possibility and relating molecule should be clarified in the future study.

A limitation of this study is that the short accommodation time before CRD may be responsible for variability of data. It can be difficult to perform CRD without restraining the rats, although the results of the study may be, to a certain degree, influenced by this practice. Both types of studies have been conducted; those where animals are restrained prior to CRD and those where animals are not restrained.[44,61–64] Because the intensity and frequency of CRD differ from study to study, it is difficult to compare directly these studies. However, no report provides direct evidence of the effects of animals' habituation to restrain prior to testing on CRD outcome.

In conclusion, our results show that a combination of previous inflammation and repetitive CRD induces colonic hypersensitivity and that CRH-R1 antagonist attenuates this response in rats. These results can help clarify the mechanisms underlying PI-IBS and would be useful in the development of treatment for IBS patients who suffer from visceral hypersensitivity.

GRANT SUPPORT

This research was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Grant-in-Aid for Scientific Research from the Ministry of Health, Welfare, and Labor of Japan.

REFERENCES

1 Drossman DA. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377–90.
2 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480–91.
3 Mertz H, Naliboff B, Munakata J, Niazi N, Mayer EA. Altered rectal perception is a biological marker of patients with irritable bowel syndrome. *Gastroenterology* 1995; **109**: 40–52.
4 Whitehead WE, Holtkotter B, Enck P *et al.* Tolerance for rectosigmoid distention in irritable bowel syndrome. *Gastroenterology* 1990; **98**: 1187–92.
5 Fukudo S, Kanazawa M, Kano M *et al.* Exaggerated motility of the descending colon with repetitive distention of the sigmoid colon in patients with irritable bowel syndrome. *J Gastroenterol* 2002; **37**(Suppl. 14): 145–50.
6 Fukudo S, Suzuki J. Colonic motility, autonomic function, and gastrointestinal hormones under psychological stress on irritable bowel syndrome. *Tohoku J Exp Med* 1987; **151**: 373–85.
7 Welgan P, Meshkinpour H, Beeler M. Effect of anger on colon motor and myoelectric activity in irritable bowel syndrome. *Gastroenterology* 1988; **94**: 1150–6.
8 Fukudo S, Nomura T, Muranaka M, Taguchi F. Brain-gut response to stress and cholinergic stimulation in irritable bowel syndrome. A preliminary study. *J Clin Gastroenterol* 1993; **17**: 133–41.
9 Drossman DA, Li Z, Andruzzi E *et al.* U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569–80.
10 Boyce PM, Koloski NA, Talley NJ. Irritable bowel syndrome according to varying diagnostic criteria: are the new Rome II criteria unnecessarily restrictive for research and practice? *Am J Gastroenterol* 2000; **95**: 3176–83.
11 Saito YA, Locke GR, Talley NJ, Zinsmeister AR, Fett SL, Melton LJ III. A comparison of the Rome and Manning criteria for case identification in epidemiological investigations of irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 2816–24.
12 Gwee KA, Graham JC, McKendrick MW *et al.* Psychometric scores and persistence of irritable bowel after infectious diarrhoea. *Lancet* 1996; **347**: 150–3.
13 Gwee KA, Leong YL, Graham C *et al.* The role of psychological and biological factors in postinfective gut dysfunction. *Gut* 1999; **44**: 400–6.
14 Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. *BMJ* 1997; **314**: 779–82.
15 Rodriguez LA, Ruigomez A. Increased risk of irritable bowel syndrome after bacterial gastroenteritis: cohort study. *BMJ* 1999; **318**: 565–6.
16 Mearin F, Perez-Oliveras M, Perello A *et al.* Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology* 2005; **129**: 98–104.
17 Soderholm JD, Yates DA, Gareau MG, Yang PC, MacQueen G, Perdue MH. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. *Am J Physiol (Gastrointest Liver Physiol)* 2002; **283**: G1257–63.
18 Saunders PR, Santos J, Hanssen NP, Yates D, Groot JA, Perdue MH. Physical and psychological stress in rats

enhances colonic epithelial permeability via peripheral CRH. *Dig Dis Sci* 2002; **47**: 208–15.
19 Soderholm JD, Yang PC, Ceponis P *et al.* Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 2002; **123**: 1099–108.
20 Santos J, Benjamin M, Yang PC, Prior T, Perdue MH. Chronic stress impairs rat growth and jejunal epithelial barrier function: role of mast cells. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G847–54.
21 Velin AK, Ericson AC, Braaf Y, Wallon C, Soderholm JD. Increased antigen and bacterial uptake in follicle associated epithelium induced by chronic psychological stress in rats. *Gut* 2004; **53**: 494–500.
22 Collins SM, McHugh K, Jacobson K *et al.* Previous inflammation alters the response of the rat colon to stress. *Gastroenterology* 1996; **111**: 1509–15.
23 Million M, Tache Y, Anton P. Susceptibility of Lewis and Fischer rats to stress-induced worsening of TNB-colitis: protective role of brain CRF. *Am J Physiol* 1999; **276**: G1027–36.
24 Burton MB, Gebhart GF. Effects of intracolonic acetic acid on responses to colorectal distension in the rat. *Brain Res* 1995; **672**: 77–82.
25 Gschossmann JM, Adam B, Liebregts T *et al.* Effect of transient chemically induced colitis on the visceromotor response to mechanical colorectal distension. *Eur J Gastroenterol Hepatol* 2002; **14**: 1067–72.
26 Palecek J, Willis WD. The dorsal column pathway facilitates visceromotor responses to colorectal distention after colon inflammation in rats. *Pain* 2003; **104**: 501–7.
27 Bercik P, Wang L, Verdu EF *et al.* Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. *Gastroenterology* 2004; **127**: 179–87.
28 Critchley HD, Wiens S, Rotshtein P, Ohman A, Dolan RJ. Neural systems supporting interoceptive awareness. *Nat Neurosci* 2004; **7**: 189–95.
29 Naliboff BD, Berman S, Chang L *et al.* Sex-related differences in IBS patients: central processing of visceral stimuli. *Gastroenterology* 2003; **124**: 1738–47.
30 Mönnikes H, Schmidt BG, Raybould HE, Taché Y. CRF in the paraventricular nucleus mediates gastric and colonic motor response to restraint stress. *Am J Physiol* 1992; **262**: G137–43.
31 Taché Y, Martinez V, Million M, Rivier J. Corticotropin-releasing factor and the brain-gut motor response to stress. *Can J Gastroenterol* 1999; **13**(Suppl. A): 18A–25A.
32 Maillot C, Million M, Wei JY, Gauthier A, Taché Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* 2000; **119**: 1569–79.
33 Fukudo S, Nomura T, Hongo M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. *Gut* 1998; **42**: 845–9.
34 Sagami Y, Shimada Y, Tayama J *et al.* Effect of a corticotropin releasing hormone receptor antagonist on colonic sensory and motor function in patients with irritable bowel syndrome. *Gut* 2004; **53**: 958–64.
35 Saito K, Kasai T, Nagura Y, Ito H, Kanazawa M, Fukudo S. Corticotropin-releasing hormone receptor 1 antagonist blocks brain-gut activation induced by colonic distention in rats. *Gastroenterology* 2005; **129**: 1533–43.
36 Coutinho SV, Meller ST, Gebhart GF. Intracolonic zymosan produces visceral hyperalgesia in the rat that is mediated by spinal NMDA and non-NMDA receptors. *Brain Res* 1996; **736**: 7–15.
37 Gebhart GF. Peripheral contributions to visceral hyperalgesia. *Can J Gastroenterol* 1999; **13**(Suppl.): 37A–41A.
38 Scholz J, Woolf CJ. Can we conquer pain? *Nat Neurosci* 2002; **5**(Suppl.): 1062–7.
39 Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 2000; **288**: 1765–8.
40 Verma-Gandhu M, Verdu EF, Bercik P *et al.* Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. *Gut* 2007; **56**: 358–64.
41 Mayer EA, Berman S, Suyenobu B *et al.* Differences in brain responses to visceral pain between patients with irritable bowel syndrome and ulcerative colitis. *Pain* 2005; **115**: 398–409.
42 Munakata J, Naliboff B, Harraf F *et al.* Repetitive sigmoid stimulation induces rectal hyperalgesia is patients with irritable bowel syndrome. *Gastroenterology* 1997; **112**: 55–63.
43 Traub RJ, Pechman P, Iadarola MJ, Gebhart GF. Fos-like proteins in the lumbosacral spinal cord following noxious and non-noxious colorectal distention in the rat. *Pain* 1992; **49**: 393–403.
44 Traub RJ, Herdegen T, Gebhart GF. Differential expression of c-fos and c-jun in two regions of the rat spinal cord following noxious colorectal distention. *Neurosci Lett* 1993; **160**: 121–5.
45 Zhai QZ, Traub RJ. The NMDA receptor antagonist MK-801 attenuates c-Fos expression in the lumbosacral spinal cord following repetitive noxious and non-noxious colorectal distention. *Pain* 1999; **83**: 321–9.
46 Gue M, Del Rio-Lacheze C, Eutamene H, Theodorou V, Fioramonti J, Bueno L. Stress-induced visceral hypersensitivity to rectal distension in rats: role of CRF and mast cells. *Neurogastroenterol Motil* 1997; **9**: 271–9.
47 Zhou Q, Caudle RM, Price DD, Valle-Pinero AYD, Verne GN. Selective up-regulation of NMDA-NR1 receptor expression in myenteric plexus after TNBS induced colitis in rats. *Mol Pain* 2006; **2**: 3.
48 Kozlowski CM, Green A, Grundy D, Boissonade FM, Bountra C. The 5-HT_3 receptor antagonist alosetron inhibits the colorectal distention induced depressor response and spinal c-fos expression in the anaesthetized rat. *Gut* 2000; **46**: 474–80.
49 Riedel W, Neeck G. Nociception, pain, and antinociception: current concepts. *Z Rheumatol* 2001; **60**: 404–15.
50 Kim D, Park D, Choi S *et al.* Thalamic control of visceral nociception mediated by T-type Ca^{2+}channels. *Science* 2003; **302**: 117–9.
51 Fukudo S, Saito K, Sagami Y, Kanazawa M. Can modulating corticotropin releasing hormone receptors alter visceral sensitivity? *Gut* 2006; **55**: 146–8.
52 Kosoyan HP, Grigoriadis DE, Tache Y. The CRF(1) receptor antagonist, NBI-35965, abolished the activation of locus coeruleus neurons induced by colorectal distension and intracisternal CRF in rats. *Brain Res* 2005; **1056**: 85–96.
53 Nijsen M, Ongenae N, Meulemans A, Coulie B. Divergent role for CRF1 and CRF2 receptors in the modulation of visceral pain. *Neurogastroenterol Motil* 2005; **17**: 423–32.

54 Million M, Wang L, Wang Y *et al.* CRF2 receptor activation prevents colorectal distension induced visceral pain and spinal ERK1/2 phosphorylation in rats. *Gut* 2006; **55**: 172–81.
55 Schwetz I, Bradesi S, McRoberts JA *et al.* Delayed stress-induced colonic hypersensitivity in male Wistar rats: role of neurokinin-1 and corticotropin-releasing factor-1 receptors. *Am J Physiol (Gastrointest Liver Physiol)* 2004; **286**: G683–91.
56 Schwetz I, McRoberts JA, Coutinho SV *et al.* Corticotropin-releasing factor receptor 1 mediates acute and delayed stress-induced visceral hyperalgesia in maternally separated Long-Evans rats. *Am J Physiol (Gastrointest Liver Physiol)* 2005; **289**: G704–12.
57 Wlk M, Wang CC, Venihaki M *et al.* Corticotropin-releasing hormone antagonists possess anti-inflammatory effects in the mouse ileum. *Gastroenterology* 2002; **123**: 505–15.
58 Hsu SY, Hsueh AJ. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat Med* 2001; **7**: 605–11.
59 Neurath M, Fuss I, Strober W. TNBS-colitis. *Int Rev Immunol* 2000; **19**: 51–62.
60 Rothenberg ME, Mishra A, Brandt EB, Hogan SP. Gastrointestinal eosinophils. *Immunol Rev* 2001; **179**: 139–55.
61 Su X, Joshi SK, Kardos S, Gebhart GF. Sodium channel blocking actions of the kappa-opioid receptor agonist U50,488 contribute to its visceral antinociceptive effects. *J Neurophysiol* 2002; **87**: 1271–9.
62 Coelho AM, Vergnolle N, Guiard B, Fioramonti J, Bueno L. Proteinases and proteinase-activated receptor 2: a possible role to promote visceral hyperalgesia in rats. *Gastroenterology* 2002; **122**: 1035–47.
63 Traub RJ, Silva E, Gebhart GF, Solodkin A. Noxious colorectal distention induced-c-Fos protein in limbic brain structures in the rat. *Neurosci Lett* 1996; **215**: 165–8.
64 Saito K, Kanazawa M, Fukudo S. Colorectal distention induces hippocampal noradrenaline release in rats. *Brain Res* 2002; **947**: 146–9.

Increased Brain Histamine H1 Receptor Binding in Patients with Anorexia Nervosa

Masahiko Yoshizawa, Manabu Tashiro, Shin Fukudo, Kazuhiko Yanai, Atsushi Utsumi, Michiko Kano, Masako Karahasi, Yuka Endo, Joe Morisita, Yasuhiro Sato, Masasi Adachi, Masatosi Itoh, and Michio Hongo

Background: The central histaminergic neuron system modulates various brain functions, including eating behavior. We hypothesized that women have higher density of histamine H1 receptor (H1R) in the limbic system than men and that the density of central H1R is increased in patients with anorexia nervosa (AN).

Methods: Subjects were 12 female AN patients, 12 healthy female subjects, and 11 healthy male subjects. Positron emission tomography with H1R radioligand [^{11}C]doxepin was performed on all subjects and regions of interest based analysis was conducted to evaluate brain H1R binding potential (BP). Abnormal eating behavior, depression, and anxiety of subjects were evaluated using the Eating Attitude Test-26 (EAT-26), Self-Rating Depression Scale (SDS), and State-Trait Anxiety Inventory (STAI), respectively.

Results: Binding potential of [^{11}C]doxepin in female subjects was significantly higher than that in male subjects at the following brain sites: amygdala, hippocampus, medial prefrontal cortex, orbitofrontal cortex, and temporal cortex. Anorexia nervosa patients showed significantly higher BP of [^{11}C]doxepin in the amygdala and lentiform nucleus than the control female subjects. In AN patients, BP of [^{11}C]doxepin in the amygdala and thalamus negatively correlated with EAT-26 scores. There was a significant negative correlation between BP of [^{11}C]doxepin and SDS or STAI scores in the amygdala, anterior cingulate cortex, and orbitofrontal cortex of AN patients.

Conclusions: These findings support the hypothesis that women have higher H1R density in the limbic system than men and suggest that AN patients may have higher expression of H1R in the limbic brain, particularly in the amygdala.

Key Words: Amygdala, anorexia nervosa, doxepin, histamine, histamine H_1 receptors, positron emission tomography

Anorexia nervosa (AN) is a behavioral disorder characterized by fear of becoming obese, refusal to maintain a minimally normal body weight, disturbance of body image, and denial of the seriousness of the current low body weight (1). Anorexia nervosa occurs mainly in adolescent or young adult females (1). Anorexia nervosa patients start a self-imposed diet with chronic starvation featuring continuous strengthening symptoms and AN signs. The cause and progression of AN may involve biological vulnerability with dysfunction in the central neuron system (CNS) being one of the most important causation factors.

Several candidates including monoamines (serotonin, dopamine, and norepinephrine) (2–4) and neuropeptides (corticotropin-releasing hormone, neuropeptide-Y, peptide-YY, cholecystokinin, beta-endorphin, and leptin) (5–8) have been reported to be involved in the pathogenesis of AN. These neurotransmitters and neuropeptides may collectively play some roles in various features of AN; however, it is unlikely that only one of them is responsible for all features of AN.

Recently, a number of functional neuroimaging studies in AN patients have been conducted (9–15). At rest, single-photon emission computed tomography (SPECT) has shown hypoperfusion of regional cerebral blood flow (rCBF) in the medial prefrontal and anterior cingulate cortices and hyperperfusion of rCBF in the thalamus and amygdala-hippocampus complex (9). In addition, positron emission tomography (PET) has revealed a generally reduced brain glucose metabolism, although higher than the normal glucose metabolism has been observed in the inferior frontal cortex and basal ganglia (10). Moreover, functional magnetic resonance imaging has shown that the prefrontal and anterior cingulate cortices, amygdala, and the parahippocampal gyrus are activated by unpleasant presentation to elicit symptom-related brain processes (11–13). Under recovery conditions, rCBF showed hypoperfusion in the temporal, parietal, occipital, and orbitofrontal cortices in a SPECT study (14), and a recent PET study has shown that serotonin 2A (5-HT2A) receptor binding was reduced in the amygdala, hippocampus, and cingulate cortex (15). These findings suggest that dysfunction of the limbic brain with dysregulation of neurotransmitters/neuromodulators may play a role in the pathophysiological features of AN.

Central histaminergic neurons are located exclusively in the tuberomammillary nuclei of the hypothalamus (16) and their neuronal fibers project extensively into the limbic system and neocortex (17–19). The central histaminergic neuron system modulates various physiological functions such as wakefulness, sleep-awake cycle, fluid balance, body temperature, cardiovascular control, appetite control, stress-related hormone release, learning, memory, aggressive behavior, and emotion (20,21). It has been reported that histamine decreases food intake via H1 receptors (H1Rs) in the ventromedial hypothalamus and the paraventricular nucleus of the hypothalamus (20,21). The H1Rs are mainly postsynaptically located and are present particularly in the cortex and limbic areas, including the hypothalamus, amygdala, and hippocampus (22). Using PET with [^{11}C]doxepin, a potent radioligand for H1R, it has been shown that H1R binding

From the Departments of Psychosomatic Medicine (MY, SF, AU, MKan, MKar, YE, JM, YS, MA, MH), Behavioral Medicine (SF, MKan), Pharmacology (KY), and Comprehensive Medicine (MH), Tohoku University School of Medicine; SS30 Health Care Center (MY); and Cyclotron and Radioisotope Center (MT, MI), Tohoku University, Sendai, Japan.

MY and MT contributed equally to this work.

Address reprint requests to Shin Fukudo, M.D., Ph.D., 2-1 Seiryo, Aoba, Sendai 980-8574, Japan; E-mail: sfukudo@mail.tains.tohoku.ac.jp.

Received December 28, 2007; revised August 4, 2008; accepted August 4, 2008.

0006-3223/09/$36.00
doi:10.1016/j.biopsych.2008.08.012

BIOL PSYCHIATRY 2009;65:329–335

is abundant in the prefrontal, temporal, and cingulate cortices of the human brain (23).

Central histaminergic activity is increased by food intake after starvation (24,25). Also, dehydration has been reported to increase the synthesis and release of histamine in the hypothalamus (26). Moreover, H1R concentration has been shown to be inversely correlated with food intake, particularly low protein diets (27,28). In addition, the central histaminergic neuron system is affected by various stressors (29–32) and other central appetite modulators, such as leptin (21,33,34), orexin-A, and neuropeptide-Y (35). These findings suggest the alteration of central histaminergic activity in AN patients. Alterations in the central histaminergic neuron system in some psychiatric disorders such as depressive disorder (36) and schizophrenia (37) have also been reported; however, there has been no report on AN to date.

The central histaminergic neuron system shows sex differences. These differences include H1R densities (male < female) (38), suppressive effect of histidine on food intake (male < female) (39), stress-related hypothalamic histamine release (40) in rat, and arousal-reducing effects of H1R-antagonist in mouse (male < female) (41). A study of human cerebrospinal fluid (CSF) suggests that central histaminergic activity is higher in women than in men (42). These sex differences may partially explain why women are more prone to suffer from AN than men.

We tested the following major hypotheses in this study: 1) women have higher H1R density in the limbic system than men, and 2) the density of central H1R is increased in AN patients. Furthermore, we additionally hypothesized that the central histaminergic activity in AN patients is proportional to abnormal eating behaviors and/or negative emotion.

Methods and Materials

Subjects

Twelve female AN patients, 11 healthy age-matched male volunteers, and 12 healthy age-matched female volunteers were enrolled in this study. Subject screening and diagnosis were conducted by medical doctors (with a diploma from the Japanese Society of Psychosomatic Medicine) in Tohoku University Hospital based on psychiatric interview performed according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision* (DSM-IV-TR) criteria (1). All patients were of the restricting type, had no complications, never had any other neuropsychiatric disorders, were drug-free, and received no high-calorie infusion throughout the study. The healthy control subjects, who were recruited through advertisement, never suffered from eating problems, substance abuse, menstrual problems, or physical, neurological, or psychiatric disorders. Psychometric tests including Eating Attitudes Test-26 (EAT-26) (43), Self-Rating Depression Scale (SDS) (44), and State-Trait Anxiety Inventory (STAI) (45) were used to quantify eating attitude, depression, and anxiety, respectively. The Japanese versions of all three tests have already been validated (46–48). The characteristics of each group of subjects are shown in Table 1. Anorexia nervosa patients showed significantly lower body mass index (BMI), higher EAT-26 scores, higher depression scores, and higher anxiety scores than the control subjects. Before the start of the study, all subjects gave a written informed consent. This study was performed in accordance with the policy of the Declaration of Helsinki and was approved by the Ethics Committee of Tohoku University School of Medicine (No. 2000-42).

Table 1. Characteristics of Study Subjects

	Control Male Subjects (n = 11)	Control Female Subjects (n = 12)	AN Patients (n = 12)
Age (years)	21.8 ± 1.3	22.3 ± 2.5	23.4 ± 2.8
BMI	20.4 ± 1.3	20.3 ± 1.1	14.7 ± 1.7[a,b]
Duration of illness (years)(range)	0 ± 0 (0)	0 ± 0 (0)	5.2 ± 2.0 (3–9)
EAT-26 score	3 ± 3.7	1.9 ± 2.7	22.3 ± 13.4[c,d]
SDS score	37 ± 7.2	35 ± 6.2	50 ± 8.1[e,f]
STAI-state score	40.5 ± 9.5	38.8 ± 9.5	49.6 ± 11.5[g,h]
STAI-trait score	46.5 ± 8.9	41.1 ± 11.2	52.1 ± 11.5[i,j]
Estradiol (pg/mL)		67 ± 51.7	13.2 ± 7.3[k]

One-way ANOVA for three group difference and post hoc analysis using the Tukey's test between two groups were performed. Values are expressed as mean ± SD.

AN, anorexia nervosa; ANOVA, analysis of variance; BMI, body mass index; EAT-26, Eating Attitudes Test-26; SDS, Self-Rating Depression Scale; STAI, State-Trait Anxiety Inventory.

[a] $p < .001$ versus control male subjects.
[b] $p < .001$ versus control female subjects.
[c] $p < .001$ versus control male subjects.
[d] $p < .001$ versus control female subjects.
[e] $p < .001$ versus control male subjects.
[f] $p < .001$ versus control female subjects.
[g] ns versus control male subjects.
[h] $p < .05$ versus control female subjects.
[i] ns versus control male subjects.
[j] $p < .05$ versus control female subjects.
[k] $p < .01$ versus control female subjects.

PET Tracer

Positron emission tomography experiments were conducted at the Cyclotron and Radioisotope Center, Tohoku University, Sendai, Japan. Doxepin, a tricyclic antidepressant, was C-11 labeled and used as a PET tracer. To verify the specific binding of [^{11}C]doxepin to H1R, the characteristics of the binding of doxepin to brain tissues were examined using H1R gene knockout mice (49,50). Doxepin has two saturable binding sites with high and low affinities in wild-type mice. However, only negligible labeling of doxepin was observed in H1R null mice. These data demonstrate clearly that the high affinity component of doxepin binding is associated with H1R. Moreover, a study, which compared the binding ability of a serotonin transporter among several antidepressants, has shown that doxepin possesses the weakest binding ability for a serotonin transporter (51). [^{11}C]doxepin was prepared by [^{11}C]methylation of desmethyl doxepin with [^{11}C]methyl triflate as described previously (52,53). The radiochemical and chemical purities of the ligand were more than 99% and 97%, respectively, and its specific radioactivity at the time of injection was 93.52 ± 50.12 GBq/μmol (2528 ± 1355 mCi/μmol). The injected dose and cold mass of [^{11}C]doxepin were 117.0 ± 23.0 MBq (3.16 ± .62 mCi) and 1.86 ± 1.64 nmol, respectively. [^{11}C]doxepin radiological dose was calculated based on a previous paper on radiological exposure (54).

PET Measurement

All subjects underwent magnetic resonance imaging (MRI) of the brain before PET scanning. No major abnormalities on brain MRI were observed in any subject. All subjects were right-handed, never had long-term treatment with antihistamines, and were free of any medication for at least 2 weeks prior to PET scanning. Before PET scanning, alcohol and nicotine were forbidden for 1 week and caffeinated beverages were not

allowed for at least 1 day. All subjects finished their meals 4 hours before PET scanning. Taking the menstrual cycle into consideration, PET scanning in control female subjects was performed during the follicular phase (within a week after the last menstruation).

Positron emission tomography scanning was performed for 90 min (11:00–12:30 or 15:00–16:30). Subjects were resting awake with closed eyes in the supine position. To maintain arousal throughout the scanning period, the subjects were confirmed to be awake every 15 min. Subjects were positioned in a SET2400W (Shimadzu, Kyoto, Japan) scanner so that the transaxial slices were parallel to the orbitomeatal line, and a 7-min long $^{68}Ge/^{68}Ga$ transmission scan was started for tissue attenuation correction. The subjects were then scanned to detect high-energy photon emission (511 keV) from [^{11}C]doxepin injected intravenously via the median cubital vein. The scanner collected 63 simultaneous transverse slices with a spatial resolution of 4 mm (transaxial) and 4.5 mm (axial) full-width at half-maximum in the center of the field of view. The sensitivity for a 20-cm cylindrical phantom was 48.6 kcps $kBq^{-1}mL^{-1}$ (1.8 Mcps $\mu Ci^{-1}mL^{-1}$) in the three-dimensional mode (55). Dynamic PET images were obtained for 90 min (22 sequential scans; 6 scans × 90 sec, 7 scans × 180 sec, 6 scans × 300 sec, and 3 scans × 600 sec) after [^{11}C]doxepin injection.

Image Analysis

Positron emission tomography dynamic images, after being corrected for tissue attenuation, were reconstructed using a filtered backprojection algorithm. The reconstructed PET images were co-registered to the identical stereotaxic brain coordinate system using its own MRI-T1 image as a reference. The MRI images were obtained using a 1.5 Tesla magnetic resonance scanner (HiSpeed, Ver9.1, General Electric Inc., Waukesha, Wisconsin) at Sendai Seiryo Clinic, Sendai, Japan. T1-weighted images (Vascular time of flight [TOF] spoiled gradient recalled [SPGR]: repetition time [TR]/echo time [TE]: 50/2.4 msec, fractional anisotropy [FA]: 45°, number of excitations: 1, matrix size: 256 × 256, and spatial resolution: x,y,z = .86, .86, 20.0 mm) were collected from all subjects. Regions of interest (ROIs) were first placed on the cerebellum. Information from the ROIs was automatically copied onto the co-registered PET images to obtain time activity curves (TACs). Cerebellar TAC was used as input function to calculate parametric brain images of the binding potential (BP) of [^{11}C]doxepin based on the graphical analysis method introduced by Logan *et al.* (56). The applicability of this method to a human study with [^{11}C]doxepin has been confirmed (57). Finally, brain BP images were created.

Region of interest based analysis was conducted to evaluate brain H1R-BP, minimizing the effects of possible brain atrophy using original BP brain images instead of using transformed BP brain images into standardized brain space. Regions of interest were placed on the following brain regions: the medial prefrontal cortex (MPC; Brodmann's area [BA] 9,10), lateral prefrontal cortex (LPC; BA 44,45,46,47), orbitofrontal cortex (Orb; BA 11), temporal cortex (TC; BA 21,22/41,42), parietal cortex (PC; BA 1,2,3/39,40), occipital cortex (OC; BA 17,18,19), anterior cingulate cortex (ACC; BA 24/32), posterior cingulate cortex (PCC; BA 23/31), insula (Ins), thalamus (Th), caudate nucleus (CN), lentiform nucleus (LN), amygdala (Am), and hippocampus (Hi). All subjects were assigned a number, and imaging data were analyzed in a blinded manner.

Data Analysis and Statistics

Data are expressed as mean ± standard deviation (SD). Region of interest based comparisons of BP of [^{11}C]doxepin were compared between the three groups using one-way analysis of variance (ANOVA) followed by Bonferroni correction (58). To minimize the effect on repeated comparisons, we adjusted the significance levels (total of p value < .05). Thereafter, post hoc analyses between the two groups were performed using the Tukey test only in the brain areas with a significant difference in BP of [^{11}C]doxepin as determined by one-way ANOVA. In AN patients, correlations between BP of [^{11}C]doxepin and eating

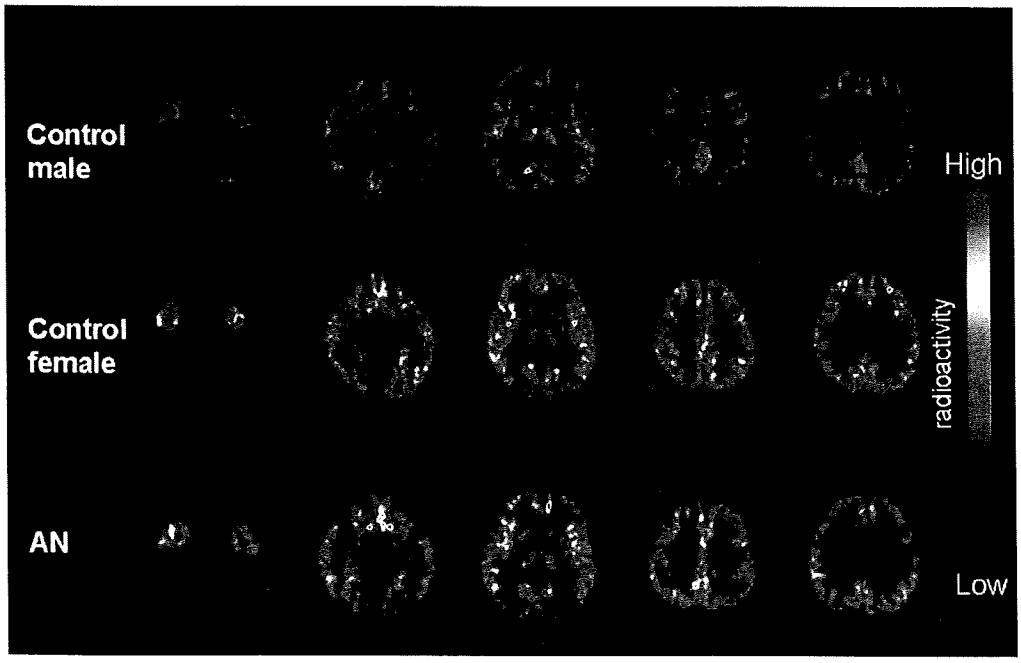

Figure 1. Brain distribution of [^{11}C]doxepin radioactivity in control subjects and AN patients. AN, anorexia nervosa.

Table 2. ROI-Based Comparisons of BP of [^{11}C]Doxepin by One-Way ANOVA

Anatomical Area	Brodmann's Area	*p* Value
L-Medial Prefrontal Cortex	9,10	.028
R-Orbitofrontal Cortex	11	.001
L-Orbitofrontal Cortex	11	.001
R-Temporal Cortex	21,22,41,42	.004
L-Caudate Nucleus		.009
L-Lentiform Nucleus		.001
R-Amygdala		<.001
L-Amygdala		.005
R-Hippocampus		<.001
L-Hippocampus		<.001

Analyses of the three groups were performed by one-way ANOVA.
ANOVA, analysis of variance; BP, binding potential; L, left; R, right; ROI, region of interest.

attitude or psychological status were analyzed using Spearman's rank correlation test; $p < .05$ was considered significant. The SPSS statistical software package for Windows 11.5 (Japanese version, SPSS Japan, Inc., Tokyo, Japan) was used for all statistical analyses.

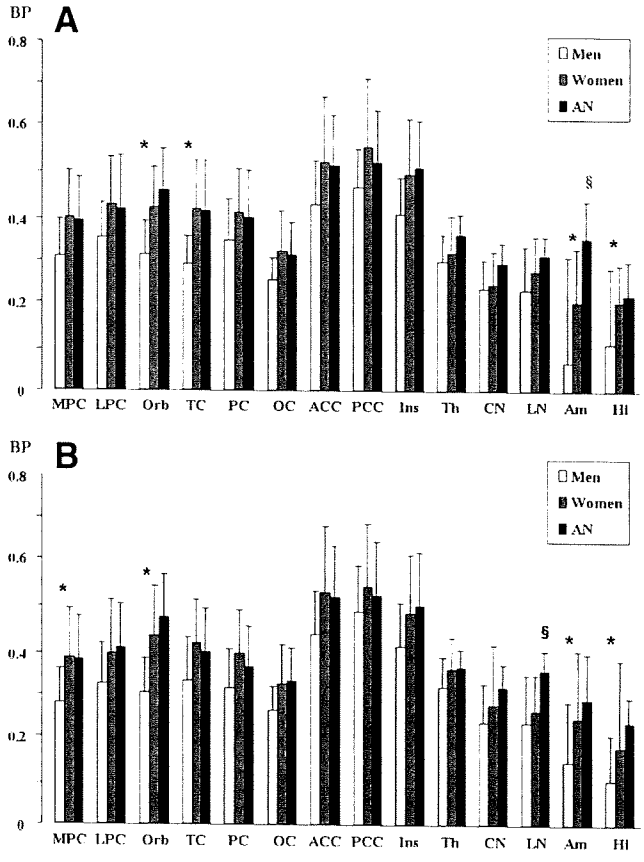

Figure 2. ROI-based comparisons of BP of [^{11}C]doxepin in the right **(A)** and left **(B)** cerebral hemisphere. One-way ANOVA and post hoc analysis using the Tukey's test were performed. *Men versus women ($p < .05$). §Women versus AN ($p < .05$). ACC, anterior cingulate cortex; Am, amygdala; AN, anorexia nervosa; ANOVA, analysis of variance; BP, binding potential; CN, caudate nucleus; Hi, hippocampus; Ins, insula; LN, lentiform nucleus; LPC, lateral prefrontal cortex; MPC, medial prefrontal cortex; OC, occipital cortex; Orb, orbitofrontal cortex; PC, parietal cortex; PCC, posterior cingulate cortex; ROI, region of interest; TC, temporal cortex; Th, thalamus.

Results

ROI-Based Comparisons of BP of [^{11}C]Doxepin Between the Three Groups

Binding potential of [^{11}C]doxepin showed a significant difference in some brain areas between the three groups as determined by one-way ANOVA (Table 2, Figure 1, Figure 2).

Post Hoc Comparisons of BP of [^{11}C]Doxepin

Male Versus Female Subjects. Binding potential of [^{11}C]doxepin in control female subjects was significantly higher than that in control male subjects in the left (L)-MPC, right (R)-Orb, L-Orb, R-TC, R-Am, L-Am, R-Hi, and L-Hi. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in control female subjects than in male subjects (Table 3, Figure 2).

Female Subjects Versus AN Patients. Binding potential of [^{11}C]doxepin in AN patients was significantly higher than that in control female subjects in L-LN and R-Am. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in AN patients than in control female subjects (Table 3, Figure 2).

Male Subjects Versus AN Patients. Binding potential of [^{11}C]doxepin in AN patients was significantly higher than that in control male subjects in R-Orb, L-Orb, R-TC, L-CN, L-LN, R-Am, L-Am, R-Hi, and L-Hi. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in AN patients than in male subjects (Table 3).

Correlation Between BP of [^{11}C]Doxepin and Eating Behavior or Psychological Status in AN Patients. There was no area where BP of [^{11}C]doxepin significantly correlated with BMI or length of illness.

EAT-26. There was a significant negative correlation between BP of [^{11}C]doxepin and EAT-26 scores in R-Th ($r = -.596$, $p < .05$), L-Th ($r = -.667$, $p < .05$), and L-Am ($r = -.646$, $p < .05$). On the other hand, there was no area with significant positive correlation between BP of [^{11}C]doxepin and EAT-26 scores.

Table 3. ROI-Based Comparisons of BP of [^{11}C]doxepin by Post Hoc Analysis Using the Tukey's Test

	Anatomical Area	Brodmann's Area	*p* Value
	L-Medial prefrontal cortex	9,10	.042
	R-Orbitofrontal cortex	11	.019
	L-Orbitofrontal cortex	11	.011
Men < Women	R-Temporal cortex	21,22,41,42	.006
	R-Amygdala		.021
	L-Amygdala		.047
	R-Hippocampus		.001
	L-Hippocampus		.002
Women < AN	L-Lentiform nucleus		.036
	R-Amygdala		.004
	R-Orbitofrontal cortex	11	.001
	L-Orbitofrontal cortex	11	.001
	R-Temporal cortex	21,22,41,42	.013
	L-Caudate nucleus		.007
Men < AN	L-Lentiform nucleus		.001
	R-Amygdala		<.001
	L-Amygdala		.004
	R-Hippocampus		<.001
	L-Hippocampus		<.001

Post hoc comparisons were made using the Tukey's test.
AN, anorexia nervosa; BP, binding potential; L, left; R, right; ROI, region of interest.

SDS. There was a significant negative correlation between BP of [^{11}C]doxepin and SDS scores in R-Orb ($r = -.581$, $p < .05$), R-ACC ($r = -.704$, $p < .05$), and R-Am ($r = -.809$, $p < .01$). On the other hand, there was no area with significant positive correlation between BP of [^{11}C]doxepin and SDS scores.

State Anxiety of STAI. There was a significant negative correlation between BP of [^{11}C]doxepin and state anxiety scores of STAI in L-MPC ($r = -.608$, $p < .05$), R-Orb ($r = -.615$, $p < .05$), R-ACC ($r = -.678$, $p < .05$), and R-Hi ($r = -.671$, $p < .05$). On the other hand, there was no area with significant positive correlation between BP of [^{11}C]doxepin and STAI-state scores.

Trait Anxiety of STAI. There was a significant negative correlation between BP of [^{11}C]doxepin and trait anxiety scores of STAI in R-Orb ($r = -.698$, $p < .05$), L-PC ($r = -.681$, $p < .05$), R-ACC ($r = -.754$, $p < .01$), and R-Am ($r = -.733$, $p < .01$). On the other hand, there was no area with significant positive correlation between BP of [^{11}C]doxepin and STAI-trait scores.

Discussion

The first point that can be drawn from our results is that the histaminergic neuron system in the human brain is different between male and female subjects. In fact, female subjects had significantly higher BP of [^{11}C]doxepin than male subjects in several brain areas, and there was no area where BP of [^{11}C]doxepin was significantly lower in female subjects than in male subjects. In support of this finding, a human CSF study has shown that women have higher levels of histamine metabolites than men (42). In addition, animal studies have also shown sex differences in the central histaminergic neuron system. In particular, it has been reported that H1R density is greater in female rats than in male rats (38), that the suppressive effect of histidine on food intake is greater in female rats than in male rats (39), that hypothalamic histamine release in normal and stressed rats is affected by sex (40), and that female mice are more sensitive to the arousal-reducing effect of pyrilamine, an H1R antagonist, than male mice (41). These findings together with the present results suggest that central histaminergic activity is higher in female subjects than in male subjects in both animals and humans. One possible reason for this difference is the presence of ovarian steroids in female subjects. As mentioned above, H1R density has been reported to be higher in female rats than in male rats (38). However, ovariectomy decreases H1R density and estradiol replacement reverses this effect in female rats (59). In female animals, food restriction induces higher histaminergic activity and reduces food intake (24,25,27,28). Furthermore, estradiol has been reported to facilitate histamine-induced excitation of ventromedial hypothalamus neurons (60). Thus, female animals may adapt better to starvation through the central histaminergic neuron system than male animals. Until now, CNS disturbances seen in AN were mainly considered to be secondary changes due to chronic starvation. However, in the present study, higher BP of [^{11}C]doxepin in several brain areas was observed in normal female subjects. The risk of developing AN may be increased by not only the social background that women want to be thin because people tend to admire a thin figure but also biological vulnerability associated with central histaminergic activity.

The second point is that AN patients showed significantly higher BP of [^{11}C]doxepin in the amygdala and lentiform nucleus than healthy female subjects, and there was no area where BP of [^{11}C]doxepin was significantly lower in AN patients than in control female subjects. These findings are unique to AN patients. However, it is difficult to assume that increased BP of [^{11}C]doxepin in the amygdala and lentiform nucleus in AN patients is the cause or result of AN. Actually, previous functional neuroimaging studies have shown altered rCBF, glucose metabolism, and brain area activation in AN patients (9–13). These alterations cannot be discriminated as the cause or result of AN. In our previous study, chronic food deprivation-induced stress in rats, which can be an AN model, reduced central H1R density (32). In addition, a human PET study in patients with depressive disorder, which is one of the representative stress-related disorders, has shown lower BP of [^{11}C]doxepin in several brain areas in these patients than in healthy control subjects, and there were no brain areas where BP of [^{11}C]doxepin was higher in these patients than in healthy control subjects (36). The decreased H1R density and BP of [^{11}C]doxepin in these studies have been explained by the sustained release of endogenous histamine and the downregulation of H1R as a consequence of endogenous ligands. Low plasma estradiol reduces brain H1R (59), and AN patients in this study showed lower plasma estradiol (Table 1). Therefore, it was predicted that BP of [^{11}C]doxepin in AN patients would be decreased. However, the present results show the opposite. If the increased BP of [^{11}C]doxepin in the amygdala is the cause of AN, female subjects with higher H1R in the amygdala may be more susceptible to AN. The amygdala certainly plays an important role in emotional responses (61–63), and histamine facilitates anxiety via H1R in the rat amygdala (64). The increased BP of [^{11}C]doxepin in the amygdala of AN patients may also be a result of AN. Central histaminergic activity is increased by food intake after starvation (24,25), and H1R concentration is increased by feeding low-protein diets (27,28). Starvation and feeding in AN patients may facilitate the increase in H1R concentration in the amygdala. Whatever the final mechanism or explanation is, the higher BP of [^{11}C]doxepin in the amygdala of AN patients is a novel finding. Further studies, particularly in patients who have recovered from AN, are needed to confirm whether higher BP of [^{11}C]doxepin is the cause or result of AN.

Binding potential of [^{11}C]doxepin in the orbitofrontal cortex, amygdala, and hippocampus was higher in female subjects and AN patients than in male subjects. These brain areas may play important roles in the central modulation of eating behavior because they are parts of the limbic system controlling emotion, cognition, and decision making (61,62,63,65). Previous functional neuroimaging studies in AN patients have also demonstrated disturbances in the limbic brain (9–13). Moreover, in patients after recovery from AN, 5-HT2A receptor binding was reduced in the limbic brain (15), and serotonin 1A (5-HT1A) receptor binding in the limbic brain positively correlated with a measure of anxiety (66). These findings, together with the present findings, suggest that dysfunction of the limbic brain with dysregulation of neurotransmitters/neuromodulators may play a role in the pathophysiological features of AN. These are very interesting results associated with the characteristics observed in AN patients, such as distorted cognition and emotional changes to food and body image. Therefore, the central histaminergic neuron system may play a role in AN, not only through stimulation of the satiety center, but also through activation of advanced psychological systems.

However, our additional hypothesis that BP of [^{11}C]doxepin in AN patients is proportional to abnormal eating behavior and/or negative emotion is unlikely. Rather, BP of [^{11}C]doxepin in AN patients is negatively correlated with abnormal eating behavior and/or negative emotion. These results were unexpected. In fact,

there were cases in which clinical evaluation of eating behavior and psychological status did not correspond to the results of the questionnaires, particularly in the EAT-26 results where the subjects did not show their eating attitude and psychological status. As there are traits such as lack of consciousness of disease and distorted cognition in AN patients, evaluation of the patient's characteristics using self-rating questionnaires has limitations. However, the relationship between H1R and severity of negative emotion in AN may be similar to that in depressive disorder (36). Indeed, our previous PET study in patients with depressive disorder demonstrated that BP of [^{11}C]doxepin decreases in proportion to SDS scores in the frontal and cingulate cortices (36). The actual relationship between BP of [^{11}C]doxepin in the brain and emotional/behavioral symptoms of AN needs further investigation.

There are several limitations of this study. First, the small number of subjects limits statistical power. The present number of AN patients (n = 12) cannot disprove the firm conclusion regarding the relationship between BP of [^{11}C]doxepin and emotional/behavioral symptoms. Second, comparisons among many ROIs also have statistical limitations. These may increase the risk of having type I errors. Third, we cannot completely rule out the possibility of the effect of brain atrophy on the results in AN patients. This is because the relationship between brain atrophy and brain H1R has not been sufficiently clarified to date. Finally, the results are not all encompassing to enable clear explanation of the specific increase in BP of [^{11}C]doxepin in the amygdala and lentiform nucleus in AN patients. Binding potential may reflect changes in receptor density (B_{max}) and/or receptor affinity (K_D). A previous animal study showed that the B_{max} of H1R was higher in female rats than in male rats, while $K_D$remained unchanged (38). Higher BP of [^{11}C]doxepin in human female subjects may induce increased B_{max} of H1R in the brain. However, we could not specifically conclude which abnormalities in B_{max} and/or in K_D values were more attributable to the increased BP observed in AN patients. Further studies, such as those employing larger samples, focusing on specific brain regions (e.g., amygdala), using patients who have recovered from AN, or carrying out postmortem binding assays in the brain of AN patients, are needed to arrive at a definitive conclusion.

In conclusion, the present study demonstrates that female subjects have higher BP of [^{11}C]doxepin in the limbic system than male subjects and that AN patients have higher BP of [^{11}C]doxepin in the amygdala and lentiform nucleus than normal female subjects. These findings suggest that the central histaminergic neuron system may play an important role in the pathophysiology of AN.

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology and Grants-in-Aid from the Ministry of Health, Welfare, and Labor, Japan.

We appreciate the technical assistance of Mr. M. Miyake and S. Watanuki in the positron emission tomography (PET) studies.

The authors reported no biomedical financial interests or potential conflicts of interest.

1. American Psychiatric Association (2000): *Diagnostic and Statistical Manual for Mental Disorders, 4th ed., Text Revision.* Washington, DC: American Psychiatric Press.
2. Kaye WH, Ebert MH, Raleigh M, Lake R (1984): Abnormalities in CNS monoamine metabolism in anorexia nervosa. *Arch Gen Psychiatry* 41: 350–355.
3. Kaye WH, Gwirtsman HE, George DT, Ebert MH (1991): Altered serotonin activity in anorexia nervosa after long-term weight restoration: Dose elevated cerebrospinal 5-hydorxyindoleacetic acid level correlate with rigid and obsessive behavior? *Arch Gen Psychiatry* 48:556–562.
4. Brewerton TD (1995): Toward a unified theory of serotonin dysregulation in eating and related disorders. *Psychoneuroendocrinology* 20:561–590.
5. Kaye WH, Berrettini WH, Gwirtsman HE, Gold PW, George DT, Jimerson DC, *et al.* (1989): Contribution of CNS neuropeptide (NPY, CRH, and beta-endorphin) alterations to psychophysiological abnormalities in anorexia nervosa. *Psychopharmacol Bull* 25:433–438.
6. Gendall KA, Kaye WH, Altemus M, McConaha CW, La Via MC (1999): Leptin, neuropeptide Y, and peptide YY in long-term recovered eating disorder patients. *Biol Psychiatry* 46:292–299.
7. Baranowska B, Radzikowska M, Wasilewska-Dziubainska E, Roguski K, Borowiec M (2000): Disturbed release of gastrointestinal peptides in anorexia nervosa and in obesity. *Diabetes Obes Metab* 2:99–103.
8. Baranowska B, Wolinska-Witort E, Wasilewska-Dziubinska E, Roguski K, Chmielowska M (2001): Plasma leptin, neuropeptide Y(NPY) and galanin concentrations in bulimia nervosa and in anorexia nervosa. *Neuro Endocrinol Lett* 22:356–358.
9. Takano A, Shiga T, Kitagawa N, Koyama T, Katoh C, Tsukamoto E, *et al.* (2001): Abnormal neuronal network in anorexia nervosa studied with I-123-IMP SPECT. *Psychiatry Res* 107:45–50.
10. Delvenne V, Goldman S, De Maertelaer V, Lotstra F (1999): Brain glucose metabolism in eating disorders assessed by positron emission tomography. *Int J Eat Disord* 25:29–37.
11. Ellison Z, Foong J, Howard R, Bullmore E, Williams S, Treasure J (1998): Functional anatomy of calorie fear in anorexia nervosa [letter]. *Lancet* 352:1192.
12. Seeger G, Braus DF, Ruf M, Goldberger U, Schmidt MH (2002): Body image distortion reveals amygdale activation in patients with anorexia nervosa–a functional magnetic resonance imaging study. *Neurosci Lett* 326:25–28.
13. Uher R, Murphy T, Brammer MJ, Dalgleish T, Phillips ML, Ng VW, *et al.* (2004): Medial prefrontal cortex activity associated with symptom provocation in eating disorders. *Am J Psychiatry* 161:1238–1246.
14. Råstam M, Bjure J, Vestergren E, Uvebrant P, Gillberg IC, Wentz E, *et al.* (2001): Regional cerebral blood flow in weight-restored anorexia nervosa: A preliminary study. *Dev Med Child Neurol* 43:239–242.
15. Frank GK, Kaye WH, Meltzer CC, Price JC, Greer P, McConaha C, *et al.* (2002): Reduced 5-HT2A receptor binding after recovery from anorexia nervosa. *Biol Psychiatry* 52:896–906.
16. Watanabe T, Taguchi Y, Shiosaka S, Tanaka J, Kubota H, Terano Y, *et al.* (1984): Distribution of the histaminergic neuron system in the central nervous system of rats: A fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. *Brain Res* 295:13–25.
17. Inagaki N, Yamatodani A, Ando-Yamamoto M, Tohyama M, Watanabe T, Wada H (1988): Organization of histaminergic fibers in the rat brain. *J Comp Neurol* 273:283–300.
18. Panula P, Pirvola U, Auvinen S, Airaksinen MS (1989): Histamine-immunoreactive nerve fibers in the rat brain. *Neuroscience* 28:585–610.
19. Airaksinen MS, Paetau A, Paljärvi L, Reinikainen K, Riekkinen P, Suomalainen R, *et al.* (1991): Histamine neurons in human hypothalamus: Anatomy in normal and Alzheimer diseased brains. *Neuroscience* 44:465–481.
20. Brown RE, Stevens DR, Hass HL (2001): The physiology of brain histamine. *Prog Neurobiol* 63:637–672.
21. Masaki T, Yoshimatsu H (2006): The hypothalamic H1 receptor: A novel therapeutic target for disrupting diurnal feeding rhythm and obesity. *Trends Pharmacol Sci* 25:279–284.
22. Martinez-Mir MI, Pollard H, Moreau J, Arrang JM, Ruat M, Traiffort E, *et al.* (1990): Three histamine receptors (H1, H2 and H3) visualized in the brain of human and non-human primates. *Brain Res* 526:322–327.
23. Yanai K, Watanabe T, Yokoyama H, Meguro K, Hatazawa J, Itoh M, *et al.* (1992): Histamine H1 receptors in human brain visualized in vivo by [11C]doxepin and positron emission tomography. *Neurosci Lett* 137: 145–148.
24. Oishi R, Itoh Y, Nishibori M, Saeki K (1987): Feeding-related circadian variation in tele-methylhistamine levels of mouse and rat brains. *J Neurochem* 49:541–547.