HPLC-ECD Determination of the Brain Levels of Biogenic Monoamines and Their Major Metabolites

The levels of monoamines and their metabolites in the brain extracts were measured by HPLC with ECD. The systems used were as follows: a CMA/200 autosampler (CMA/Microdialysis AB, Stockholm, Sweden), a micro LC pump (BAS, city, IN), an LC-4 C ECD (BAS), a Bio-Phase ODS-4 51-6034 column (4.0 × 110 mm; BAS), a CR-6A recorder (Shimadzu, Kyoto, Japan), an LC-26A vacuum degasser (BAS), and a CTO-10A column heater set at 35°C (Shimadzu). The mobile-phase solution consisted of 0.1 M tartaric acid-0.1 M sodium acetate buffer, pH 3.2, containing 0.5 mM EDTA-2Na, 650 μM sodium 1-octane sulfonate, and 5% acetonitrile. The flow rate was 700 μl/min. The concentration of each compound, that is, dopamine (DA), serotonin (5-HT), dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindoleacetic acid (5-HIAA), was calculated by comparison with the internal and external standards. Ratios of metabolite level/monoamine level (DOPAC/DA, 5-HIAA/5-HT) were also calculated as indices of monoamine turnover.

Statistical Analyses

To examine differences in the levels of monoamines and their metabolites (DA, 5-HT, DOPAC, 5-HIAA), and differences in the monoamine turnover ratios (DOPAC/DA, 5-HIAA/5-HT), three-way MANOVA (Wilks's lambda) for housing condition (group-housed, isolation-housed), novelty stress (non-stress, stress), and fluvoxamine ((–), (+)) was conducted on dependent measures followed by the Dunnett test. There were some missing values as follows: group-housed, fluvoxamine (+), stress (n = 7, due to a lack of DA in two animals); isolation-housed, fluvoxamine (+), non-stress (n = 8, due to a lack of DA in one animal). Thus, the total number of animals used for the statistical analyses was 69.

RESULTS

Housing condition significantly altered the dependent measures (F (6, 56) = 6.040, $p < .0001$). Dunnett test revealed the following results: isolation housing significantly increased DOPAC ($p < .01$), 5-HT ($p < .01$), and 5-HIAA ($p < .05$) levels, as well as the DOPAC/DA ratio ($p < .05$), whereas it significantly decreased the 5-HIAA/5-HT ratio ($p < 0$ .05; Figures 2 and 3).

Novelty stress did not significantly alter the dependent measures (F (6,

56) = 0.582, p = .7431). Fluvoxamine significantly altered the dependent measures (F (6, 56) = 18.969, $p < .0001$). Dunnett test revealed the following results: fluvoxamine significantly decreased DA ($p < .05$), DOPAC ($p < .05$), and 5-HIAA ($p < .01$) levels, as well as the 5-HIAA/5-HT ratio ($p < .01$), whereas it significantly increased the 5-HT level ($p < .01$; Figures 2 and 3).

The following interactions were significant: that between housing condition and novelty stress (F (6, 56) 3.630, p = .0041), between housing condition and fluvoxamine (F (6, 56) = 5.746, p = .0001), between novelty stress and fluvoxamine (F (6, 56) = 2.838, p = .0175), and among housing condition, novelty stress, and fluvoxamine (F (6, 56) = 2.676, p = .0236).

Thus, isolation housing significantly increased the levels of DOPAC and 5-HIAA, whereas fluvoxamine led to a significant decrease in those levels. Both isolation housing and fluvoxamine significantly increased the 5-HT level and decreased the 5-HIAA/5-HT ratio. Although fluvoxamine significantly decreased the DA level, isolation housing had no effect on the DA level.

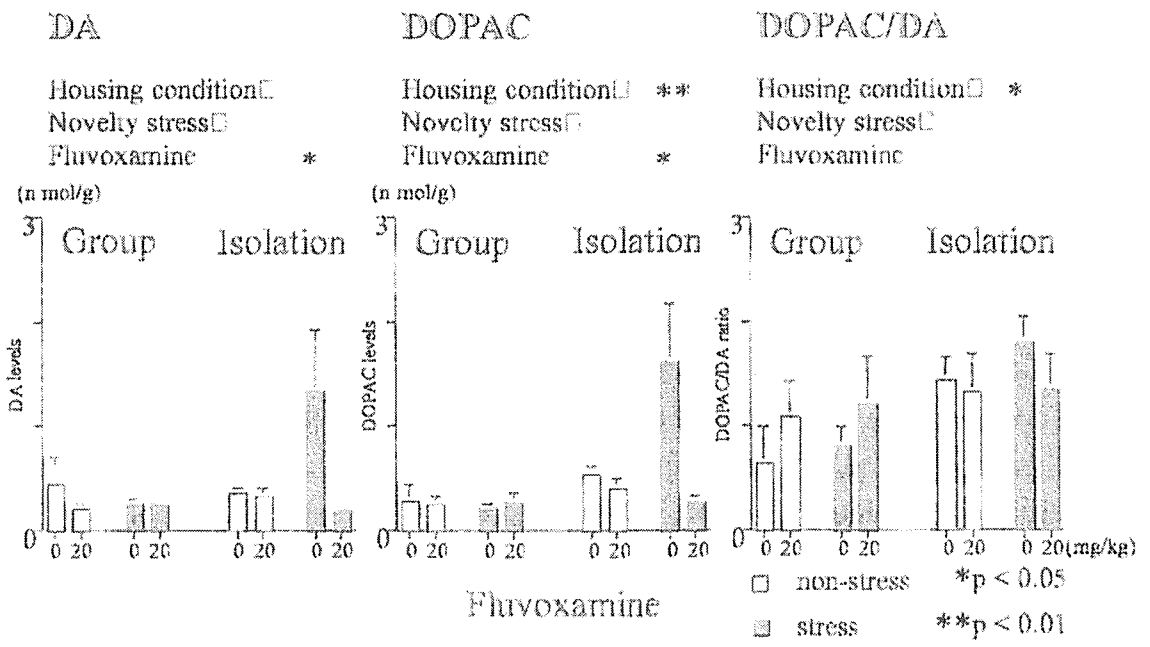

Figure 2. Changes in DA and DOPAC levels, and the DOPAC/DA ratio elicited by housing condition, novelty stress, and fluvoxamine. Group, group housing condition (n = 34); Isolation, isolation housing condition (n = 35). The total number of rats was 69. White bars, non-stress (n = 35); Black bars, stress (n = 34, n = 69 total). Fluvoxamine: 0, 0 mg/kg (n = 36); 20, 20 mg/kg (n = 33, n = 69 total). Each bar indicates the final group division. There were 9 rats in each group, with the following exceptions: 7 rats in group housing, stress, 20 mg/kg fluvoxamine group, and 8 rats in isolation housing, non-stress, 20 mg/kg fluvoxamine group, both due to missing data. Values are shown as the mean ± SEM. Symbols indicate the results of a Dunnett test for housing condition, novelty stress, and fluvoxamine; *$p < .05$, **$p < .01$.

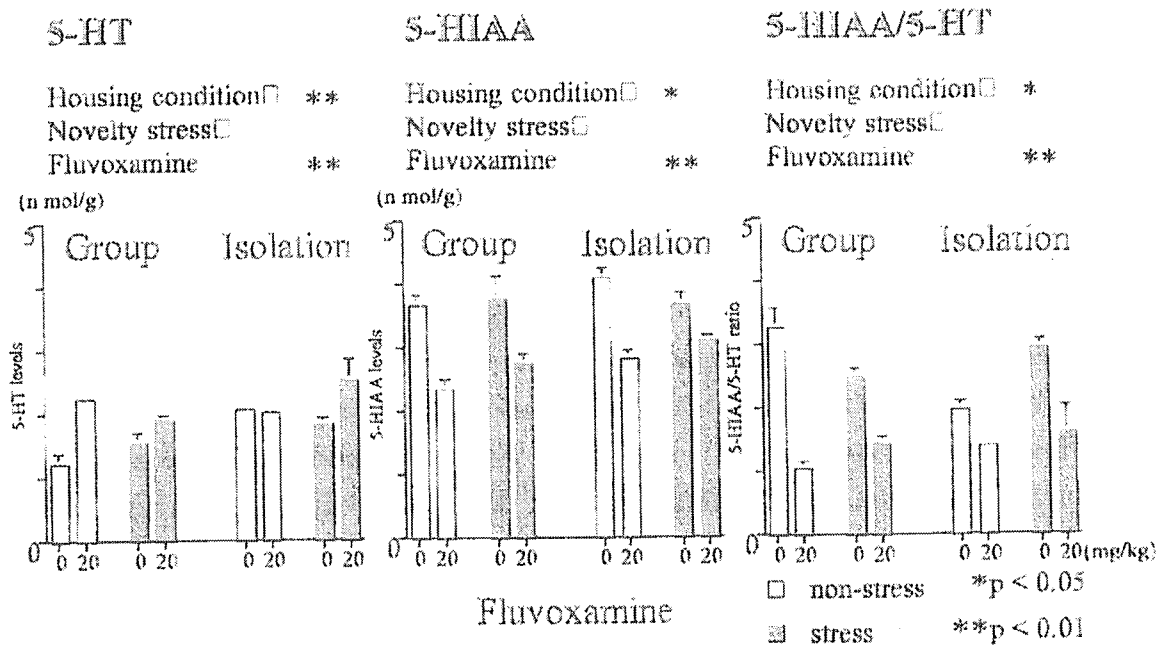

Figure 3. Changes in 5-HT and 5-HIAA levels, and the 5-HIAA/5-HT ratio elicited by housing condition, novelty stress, and fluvoxamine. Group, group housing condition (n = 34); Isolation, isolation housing condition (n = 35). The total number of rats was 69. White bars, non-stress (n = 35); black bars, stress (n = 34, n = 69 total). Fluvoxamine: 0, 0 mg/kg (n = 36); 20, 20 mg/kg (n = 33, n = 69 total). Each bar indicates the final group division. There were 9 rats in each group, with the following exceptions: 7 rats in group housing, stress, 20 mg/kg fluvoxamine group, and 8 rats in isolation housing, non-stress, 20 mg/kg fluvoxamine group, both due to missing data. Values are shown as the mean ±+ SEM. Symbols indicate the results of a Dunnett test for housing condition, novelty stress, and fluvoxamine; $*p <$ .05, $**p <$ .01.

Whereas isolation housing significantly increased the DOPAC/DA ratio, fluvoxamine did not alter the ratio. Novelty stress did not have any observable effect on monoamine levels, nor were the levels of monoamnine metabolites, or the ratios of monoamine turnover affected. Because the interaction between housing condition and fluvoxamine was significant, fluvoxamine may have suppressed the levels of DOPAC and 5-HIAA, thereby countering the effect of isolation housing, which led to an elevation of these levels.

DISCUSSION

The present study proposed an animal model incorporating two environmental risk factors of human depression (novelty stress, i.e., acute environmental change; and isolation housing, i.e., social isolation). Because the onset of

clinical depression in normally developed and socially adapted humans tends to occur in middle- or older age, the study initiated the isolation housing of rats after the animals reached adulthood. The hippocampus was selected to examine the influence of these two environmental factors because the rat hippocampus is a site in the 5-HT system with great plasticity in response to stress (Gould, 1999; McEwen, 2000); moreover, it is one of the areas of the brain in which neurogenesis persists during adulthood (Gould, 1999). Thus, it was reasoned that neurological changes adapted for the isolation housing condition would occur in the hippocampus of adult rats.

The present study used F344 rats. As mentioned earlier, aging is an important factor in the onset of human depression. Etiological and pathophysiological studies of human depression using animal models are thought to be helpful for clarifying the role of aging. F344 rats have been used in the studies that have applied novelty stress to older animals (Handa et al., 1993, 1996). Thus, the authors' recent study investigating the influence of isolation housing and novelty stress on brain monoamines in old age also used F344 rats (Miura et al., 2002b). The present study used young adult F344 rats to investigate the effects of fluvoxamine, in preparation for further studies investigating aging factors using older aged rats.

Isolation housing increased the DOPAC level and DA turnover (Figure 2), increased 5-HT and 5-HIAA levels, and decreased 5-HT turnover (Figure 3). Novelty stress did not change either the DA system or 5-HT system activity. Fluvoxamine decreased the levels of DA and DOPAC (Figure 2), whereas it increased the 5-HT level, and decreased 5-HIAA levels, as well as the rate of 5-HT turnover (Figure 3). Fluvoxamine may have suppressed DOPAC and 5-HIAA levels, thus countering the effect of isolation housing, which led to an elevation in these levels. Previous studies using rats have reported that isolation housing suppressed presynaptic 5-HT hippocampal function (Jaffe, 1993; Muchimapura et al., 2003; Lapiz et al., 2003) and suppressed stress (restraint stress, inescapable mild footshock)-induced 5-HT release, as measured by in vivo microdialysis (Muchimapura et al., 2002; Lapiz et al., 2003). The present results indicating that isolation housing decreased 5-HT turnover, and that novelty stress had no effect on 5-HT turnover may support findings from studies investigating suppression of presynaptic 5-HT function. Furthermore, isolation housing appeared to result in a loss of 5-HT terminals throughout the CA regions of the hippocampus (Whitaker-Azmitia et al., 2000) and it also reduced synapse-specific protein synaptophysin immunoreactivity in the dentate gyros molecular layer of the hippocampus (Varty et al., 1999). Moreover, isolation housing changed the activity in the postsynap-

tic 5-HT system. Isolation housing has been shown to decrease the level of 5-HT_{1A} mRNA in the dorsal hippocampus and the number of 5-HT_{1A} receptors in the CA1 region of the hippocampus, as compared to an enriched housing condition (Rasmuson et al., 1998). Postsynaptic down-regulation of the 5-HT_{1A} receptors is thought to alter signal transduction, and 5-HT innervation of the DA system is thought to attenuate the activity of DA neurons, thus, in the present study, the loss of 5-HT terminals and postsynaptic 5-HT_{1A} receptor downregulation induced by isolation housing may have resulted in the enhancement of DA turnover because of an attenuation of the inhibitory regulation of DA neuronal activity in a previous electrophysiological study, acute administration of fluvoxamine and other SSRIs attenuated the firing rate of DA neurons in the ventral tegmental areas (VTA) as well as the firing rate of 5-HT neurons in the dorsal raphe nucleus (DRN) (Di Mascio et al., 1998). Further; chronic administration of fluvoxamine (14 days) has been shown to prolong the suppression of CA3 pyramidal neuron firing elicited by the local application of 5-HT, although postsynaptic 5-HT_{1A} receptors in the hippocampus remained normosensitive (Dong et al., 1999). Thus, fluvoxamine was able to suppress the activity of mesolymbic DA neurons as well as the 5-HT system, and isolation housing may have potentiated the inhibition of mesolymbic DA neuronal activity by hypersensitization of the postsynaptic 5-HT receptors (Wright et al., 1991).

Although in previous studies the authors reported that novelty stress significantly changed the levels of monoamines and their metabolites (Miura et al., 2002a, b), novelty stress was not found to significantly alter either of these levels in the present study. These differences in the stress response between present and previous studies may be in part attributed to differences in the stress-session procedures. Here, a non-stress condition was employed in which animals were habituated twice to the novel environment before final exposure, and a stress condition in which they experienced the novel environment without prior habituation. In previous studies, animals in the non-stress group were killed without exposure to the novel environment. It cannot be ruled out that the habituation sessions influenced CNS activity in the non-stress group. For this reason, these differences pose limitations to any direct comparison of the effects of novelty stress in the authors' present and previous studies.

In experiments employing isolation housing, it is crucial that the animals have no social contact. It has been considered that the handling of animals during the exchange of cages or injection of drugs may compromise, to some degree, the integrity of the isolation, and thereby effect the results of the

experiment (Holson et al., 1991; Krebs-Thomson et al., 2001). The present study used hanging-type cages with wire-mesh bottoms to minimize the influence of handling and social experience. However, the use of these cages may itself have constituted a stressor. Thus, the experimental procedure for simulating isolation housing has a limitation in this regard.

The present study investigated the influence of isolation housing on neurochemical responses to novelty stress in the hippocampus of adult rats. In addition, it considered the effects of fluvoxamine on these responses. The results suggest that isolation housing increased both the DOPAC level as well as DA turnover, increased 5-HT and 5-HIAA levels, and decreased 5-HT turnover. Fluvoxamine decreased the levels of DA and DOPAC, increased the levels of 5-HT, and decreased both the 5-HIAA levels and 5-HT turnover. Because the interaction between housing condition and fluvoxamine was significant, fluvoxamine appears to have counteracted the increase in the levels of DOPAC and 5-HIAA elicited by isolation housing. Because in almost all previous studies investigating the effects of isolation housing, isolation was initiated soon after weaning, the present data cannot be directly compared with the data from previous studies. However, the present results suggest that isolation housing initiated in adulthood did alter both DA and 5-HT neuronal activity The present animal model simulated two major environmental risk factors of human depression. This model elicited neurochemical changes that were partially reversed by fluvoxamine. Additional investigations of dose response to SSRIs, the time course of recovery, and effects in older animals will be needed to further demonstrate the usefulness of this model.

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Classical conditioned response of rectosigmoid motility and regional cerebral activity in humans

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Abstract *The relationship between the central processes of classical conditioning and conditioned responses of the gastrointestinal function is incompletely understood in humans. We tested the hypothesis that the rectosigmoid motility becomes conditioned with anticipatory painful somatosensory stimulus and that characteristic brain areas become activated during anticipation. In nine right-handed healthy male subjects, a loud buzzer (CS, conditional stimulus) was paired with painful transcutaneus electrical nerve stimulation to the right hand (unconditional stimulus). Rectosigmoid muscle tone measured by the barostat as the intrabag volume, phasic contractions of the bowel measured as the number of phasic volume events (PVEs), and regional cerebral blood flow assessed by positron emission tomography (PET), were measured before and after conditioning. Following conditional trials, the bag volume after CS alone did not show significant changes between before and after the stimulus, but the number of PVEs after 2-minute interval of the CS alone was significantly greater than that before the stimulus ($P < 0.05$). The PET data showed the conditioning elicited significant cerebral activation of the prefrontal, anterior cingulate, parietal and insula cortices ($P \leq 0.001$, uncorrected). Rectosigmoid motility can be conditioned with increase in phasic contractions in humans.*

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INTRODUCTION

In classical or Pavlovian conditioning, the conditional stimulus (CS), which is a neutral stimulus paired with an uncomfortable unconditional stimulus (US) previously, comes to elicit behavioural and physiological responses and the US alone.[1–3] This learning process provides a model to understand anticipatory reports of pain and anticipatory gastrointestinal symptoms in situations that are not objectively threatening or painful.[4]

Little is known about the process of anticipatory response in gastrointestinal motility in humans. Physical and psychological actual stress induces significant changes in gastrointestinal motility, which includes smooth muscle tone and phasic contractions of the gastrointestinal tract.[5] Patients with irritable bowel syndrome (IBS) show greater responses with abnormal patterns in the duodenal and colonic motility than healthy subjects during stress.[6] The studies of the Pavlovian conditioning paradigm in the animal model revealed that the anticipatory stimulus elicited the same gastrointestinal responses as a delivered actual stimulus.[7] In this model, the CS caused a significant increase in colonic spike burst frequency compared to basal values after repeated foot shock.[7] Moreover, epidemiological studies revealed that posttraumatic stress disorder (PTSD)[8] and a history of sexual or physical abuse,[9] which tend to be accompanied with anticipatory fear/anxiety,[10] had a high prevalence in patients with IBS. These phenomena suggest that central enhancement induced by associative learning may affect changes in gastrointestinal function.[11]

Classical conditioning is considered to be a model to understand anticipatory responses to aversive events, which is an essential component of how the brain–gut interaction develops in functional gastrointestinal disorders. Recently, central process of anticipatory responses has been investigated by several paradigm of brain imaging studies.[12–15] In spite of research progress of brain imaging studies, there have been few studies to observe conditioned response in both brain and gastrointestinal motility function. It has been established that conditioned response can be observed by pairing a painful somatosensory stimulus with a neutral stimulus.[16] In this study, we tested the following hypothesis: (i) the rectosigmoid motility becomes conditioned with increasing smooth muscle tone and increasing number of phasic contractions in humans and (ii) characteristic brain areas become activated during anticipation regardless of the stimulus intensity.

METHODS

Subjects

Nine right-handed healthy male subjects (mean age 24 ± 1 years; 19–29 years) were recruited from Tohoku University Campus in Sendai, Japan. All participants were free of gastrointestinal complaints and had not taken any medications within 4 weeks prior to testing. Each participant in this study underwent a medical history evaluation and was given a physical examination. Written informed consent was obtained from all participants, and this study was approved by the Tohoku University Ethics Committee.

Measurement of rectosigmoid function

The experiment was performed after a fasting period of at least 9 h. The subjects were placed in supine position and were instructed not to move during each session because of positron emission tomography (PET) scanning at the same time. A computer-driven barostat (Synectics Visceral Stimulator; Synectics, Stockholm, Sweden) was used to assess the rectosigmoid function.[17–19] A polyethylene bag (diameter, 9 cm; length, 9 cm; volume, 0–500 mL), which was tightly fixed at both ends to a catheter, was inserted into the rectosigmoid colon of each subject and placed with distal end of the bag 10 cm from the anal verge 30 min before the study. The biomechanical properties of the bag were determined by pressure–volume measurements with the bag outside of a subject (*ex vivo*; Fig. 1). At volumes of less than 430 mL, the bag itself did not contribute to resistance to inflation.

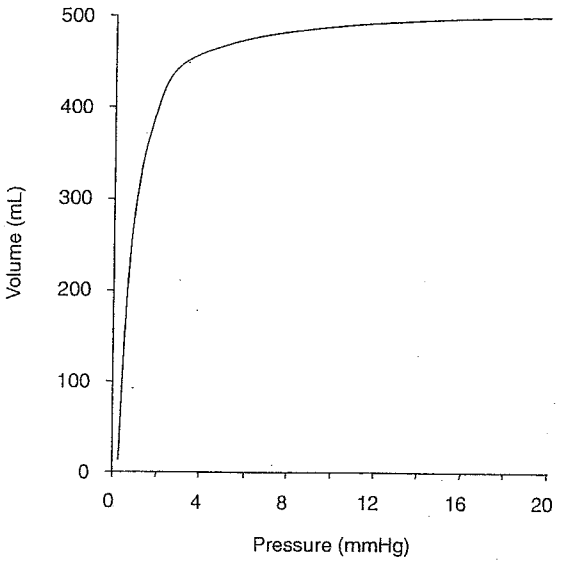

Figure 1 Barostat bag compliance measured *ex vivo*. The pressure–volume curve demonstrated operation in the low-elastance portion for operating volumes <430 mL. In this range, the bag itself did not contribute to resistance to inflation, assuring that barostat measurements reflect the mechanical characteristics of the surrounding tissue.

Before the protocol, an initial distension in which the balloon pressure was increased from 0 to 40 mm Hg in 2 mm Hg steps at 10-second intervals was performed to reduce variability in compliance and to confirm the reproducibility. Thereafter rectal compliance was assessed by graded inflation until the first painful sensation or a maximal pressure of 40 mm Hg in the same way of the initial distension. During the protocol, the intra-operating pressure of the barostat bag was kept constant at 10 mm Hg as one of the standard methods for measuring colonic tone.[19] On the other hand, there is the other standard method to consider the minimal distending pressure as the intra-operating pressure.[17–19] However, no subjects in the present study showed that each minimal distending pressure (median 8 mm Hg; 6–8 mm Hg) exceeded the operating pressure. Besides, the operating pressure was much lower compared with the threshold of the first painful sensation (median 30 mm Hg; 22 to >40 mm Hg), which showed reproducibility in each subject.

Measurement of brain activation

Using a similar technique, which we have described in the previous report,[20] regional cerebral blood flow (rCBF) was measured. Subjects were instructed to lie on their back in the PET scanner and to minimize head

movement and keep their eyes closed during the scanning (for 70 s). Using a $^{68}Ge/^{68}Ga$ radiation source, transmission scans were carried out prior to PET scanning. [^{15}O]-labelled water (Tohoku University Cyclotron Radioisotope Center) was injected into the right arm vein 10 s before the beginning of each stimulus session. Ten seconds later, the radioactivity in the brain reached a plateau and an increase in rCBF was detected by the PET scanning as an index of neural activity evoked by the stimulus. As shown in Fig. 2, five scans of rCBF in each subject were measured using PET scanner in three-dimensional sampling mode (HEADTOME V SET-2400W, Shimadzu, Kyoto, Japan).[21] The scanner produced 63 horizontal slices with a separation of 3.125 mm, an axial field of view of 200 mm, an in-plane resolution of 590 mm, a full width at half maximum (FWHM) and an axial resolution of 3.9 mm FWHM. To ensure that radioactivity levels in each subject returned to baseline before starting a new scan, a 10-minute interval was given between successive scans.

Protocol

The protocol for the present study is shown in Fig. 2. There were three sessions; preconditioning, conditional and postconditioning trials. Subjects were exposed seven times to a loud buzzer (500 Hz with an intensity of 87 dB) lasting 1 s and being followed by a 9 s break. This sequence served as the CS. For the first sequence, only the CS tones were administrated as a preconditioning trial.

The US, which followed the CS during the conditional trials and a part of the postconditioning trials, was composed of transcutaneous electrical nerve stimulations (TENS; OG GIKEN AUDIO TREATER EF-501, Okayama, Japan) delivered to the back of the right hand at a frequency of 15 Hz with two different levels of intensity (7 or 4 mA). The US started just after each tone was finished and the stimulus period lasted 70 s (Fig. 2). After three sets of the CS or the postconditioning CS sequence, high-mA TENS was applied as the US. After the postconditioning CS sequence, low-mA TENS was applied as weak US. After the pre- or postconditioning CS-alone sequence, the US was not applied. In the postconditioning session, stimulus intensities of 0 (sham), 4 and 7 mA were given in random order.

The PET scanning was performed at the resting period as a background, and the pre- and postconditioning trials for each subject (five injections per scans, see Fig. 2). Each combination of the stimulus (the CS with/without the US) with break (10-second duration) was repeated seven times because the PET technique requires a 70-second recording window for each scan. The intra-bag pressure of barostat was kept at 10 mm Hg to measure changes in the bag volume in the rectosigmoid colon.

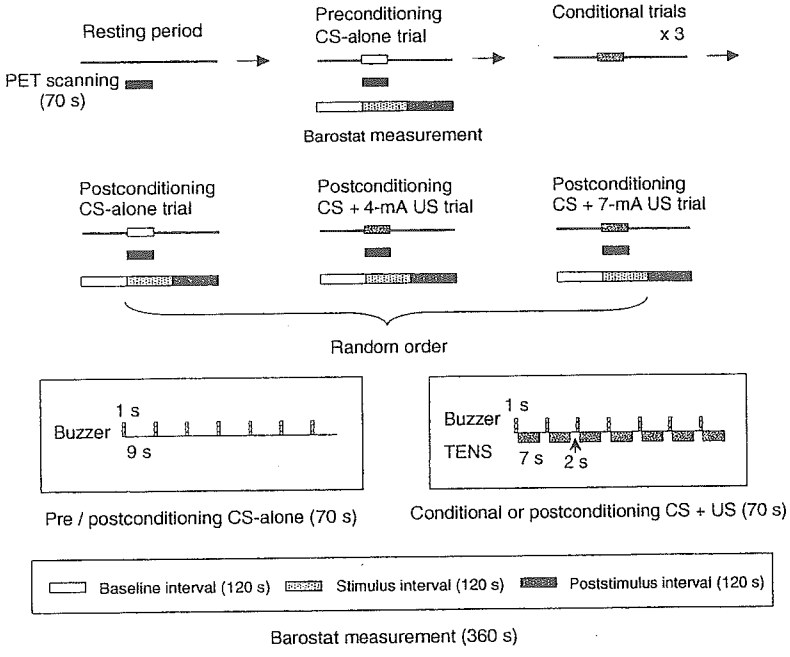

Figure 2 The protocol in this study. Simple tones of buzzer horn were used as conditional stimulus (CS) and following transcutaneus electrical stimulation (TENS) to the right hand were used as unconditional stimulus (US). Only the CS tones were administrated as a preconditioning trial. After three 70-second sequences of conditional trials in which the CS was paired with the US, three additional test sequences were presented in random order; they consisted of the CS presented alone, CS paired with 7-mA US, or CS paired with 4-mA US as postconditioning trials. Subjects were exposed seven times to a loud buzzer in each trial. The US was started just after each tone was finished (no overlap). PET scanning was performed at the resting period as a background, and the pre- and postconditioning trials.

The subjects were also asked in the scanner to verbally rate the intensity of overall anxiety on a 0–10 point scale, with 0 representing no anxiety and 10 being the most anxious, before and after all series of the sessions.

Analysis

The intrabag volume in the rectosigmoid colon was measured continuously and its variations were visually analysed. Mean bag volume over each 2-minute interval served as a measure of muscle tone, and number of phasic volume events (PVEs), served as a measure of phasic contractions according to the reported standard method.[17,18] In the present study, 2-minute interval for the analysis of barostat measurement was selected not to fail to observe changes in the rapid volume waves.[17] To control for occasional, minor changes in colorectal tone, the volume had to differ more than 10% from the baseline tone occurring at a frequency of 1–4 min^{-1} to be characterized as a change[17] (see Fig. 3). Movement artifacts were defined as sudden changes in bag volume that did not continue for more than 15 s and/or did not differ more than 10% from baseline;[17] these artifacts were excluded from data analysis. Changes in the bag volume or number of PVEs from each 2-minute baseline interval just before the stimulus (baseline interval) to each 2-minute interval just after the beginning of the stimulus (stimulus interval), and each following 2-minute interval (poststimulus interval), were considered to represent the colorectal wall reactivity to the CS with/without the US (Fig. 3). The paired Student's *t*-test or Wilcoxon's rank-sum test was used for comparing the rectosigmoid function in the 2-minute baseline, stimulus and poststimulus intervals of each trial. Alpha level was set at 5% for these statistical analyses.

The PET data were transferred to a super computer (NEC SX-4/128H4, Tokyo, Japan) at the computer centre of Tohoku University through the optical network. The image reconstruction of all brain area was carried out using the three-dimensional filtered back projection algorithm.[22] The PET image data were analysed using standard software (Statistical Parametric Mapping; SPM99, The Welcome Department of Cognitive Neurology, London) according to the method of Friston *et al.*[23] All brain slices were analysed. The PET images were realigned, spatially normalized and

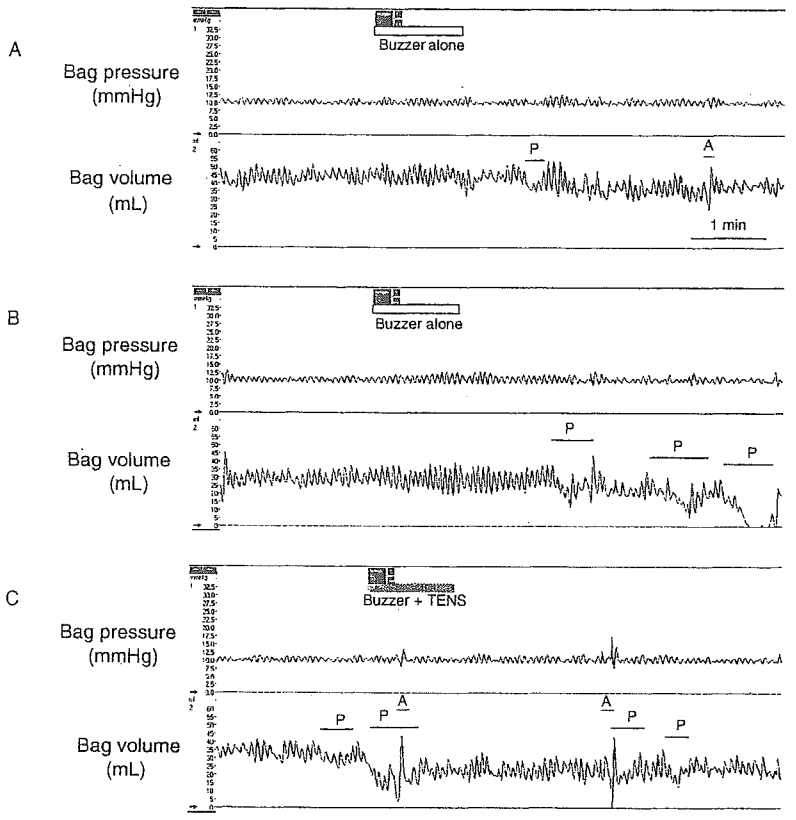

Figure 3 Examples of tracings of the barostat. Changes in the rectosigmoid bag volume were measured during the preconditioning trial (top; A), the postconditioning CS-alone trial (middle; B) and the postconditioning CS/US trial (bottom; C) using the barostat. The tracings that were obtained from one of the healthy 26-year-old male subjects were shown as the intrabag pressure (at the top) and the intrabag volume (at the bottom), respectively. Phasic volume events (P) were considered to be minor changes in colorectal tone, which differed more than 10% from the baseline tone. Artifacts (A) were considered to be sharp waves, that were parenthetically observed and that did not continue for more than 15 s and/or did not differ more than 10% from baseline tone.

transformed into an approximate Talairach–Tournoux stereotactic space, 3D Gaussian filtered (FWHM; 13 mm) and proportionally scaled to account for global confounders. The size of each voxel was set at $2 \times 2 \times 2$ mm. A *t*-test was used to compare rCBF differences between the pre- and postconditioning CS-alone trials as a primal analysis for the effect of the conditioning. In addition, rCBF during the post-conditioning CS with high- or low-mA TENS trial was compared with that during the preconditioning CS-alone trial as secondary analyses for the effect of both nociception and conditioning. As an additional analysis, brain regions manifesting linear correlations to the mean bag volumes or the number of PVEs on the barostat measurement were also examined using simple regression analysis in SPM99. According to the reported methods of the 3D brain imaging studies,[22,23] we set alpha equal to 0.1% (uncorrected for multiple comparisons) as the region of significant differences. The region, which showed the significant activity correlations, was identified on the basis of Talairach co-ordinates.

RESULTS

No subjects had a history of functional/organic gastrointestinal disorders, psychiatric/psychological disorders or physical/sexual abuse. No abnormality was found on physical examination including failure to anal relaxation with a rectal digital examination in each subject. All subjects completed the full protocol. All the subjects reported pain to the right hand and different given stimulus intensities during the post-conditioning buzzer (CS) with high- or low-mA stimulus (US) trials. They did not report any pain or discomfort to the right hand in the buzzer-alone test trials. The buzzer with TENS or the buzzer alone did not induce any gastrointestinal symptoms. The anxiety scores did not show a significant change between before and after the protocol [median 4 (0–10) *vs* 2 (0–10), $P > 0.1$].

Assessment of rectosigmoid function

The rectosigmoid pressure and volume measurements were recorded at each distension step in the bag were plotted as mean values for the subjects in Fig. 3. The mean bag volume during 2-minute baseline interval was not significantly different among the sessions before and after the conditioning (Table 1). Example of actual barostat traces during the pre/postconditioning CS-alone (buzzer-alone) trials and the postconditioning CS with the US (buzzer with TENS) trials are shown in Fig. 4. In the postconditioning CS + high-mA US trial, the mean bag volume during 2-minute poststimulus

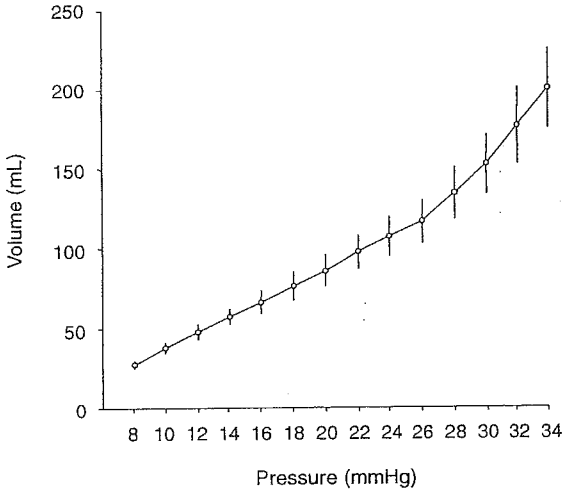

Figure 4 Pressure–volume curve reflecting the rectal compliance during intermittent isobaric distension in the subjects. The curve represents the mean value of the barostat bag volume (±SE) for each step of distension.

Table 1 Mean bag volume and number of PVEs in the rectosigmoid colon during pre- and postconditioning trials

	Mean bag volume (mL)			Number of PVEs (min^{-1})		
	Baseline interval	Stimulus interval	Poststimulus interval	Baseline interval	Stimulus interval	Poststimulus interval
CS alone/preconditioning	43 ± 8	41 ± 8	40 ± 8	0 (0–1.5)	0.5 (0–1.5)	0.5 (0–1.5)
CS alone/postconditioning	36 ± 11	36 ± 11	34 ± 13	0 (0–2)	0.5 (0–3)	1 (0–2.5)*
CS + US (4 mA)/postconditioning	48 ± 20	44 ± 15	38 ± 11	0.5 (0–1.5)	1 (0.5–2)	1 (0.5–2)*
CS + US (7 mA)/postconditioning	65 ± 29	63 ± 30	47 ± 18*	0 (0–1.5)	1 (0–3)	1 (0–2.5)*

CS, conditional stimulus; US, unconditional stimulus; PVEs, phasic volume events.
The duration of each interval is 2 min. Data were shown as mean ± SE or median with range. *$P < 0.05$ *vs* baseline interval (Student's *t*-test or Wilcoxon's rank-sum test).

interval was significantly smaller than that during 2-minute baseline interval ($P < 0.05$, Table 1). In the preconditioning trial and the postconditioning CS-alone and CS + low-mA US trials, the mean bag volume during the stimulus or poststimulus intervals did not show significant difference compared to that during each baseline interval. Thus, no conditioned effect was demonstrated for rectosigmoid muscle tone.

In the postconditioning CS-alone trial, the number of PVEs during the 2-minute poststimulus interval was significantly greater than that during the immediately preceding 2-minute baseline interval ($P < 0.05$, Table 1). The number of PVEs during the poststimulus intervals were significantly greater than those during the baseline intervals in the postconditioning CS + low-mA US ($P < 0.05$) and CS + high-mA US ($P < 0.05$) trials, respectively. There were no significant differences in the number of PVEs in the preconditioning trial (Table 1). These data support a conditioning effect for colonic phasic contractions.

Assessment of central activation

The average PET data from all the subjects showed the conditioning elicited significant activation of the left lateral prefrontal, right anterior cingulate, bilateral parietal cortices, right insula, right pons and left cerebellum ($P \leq 0.001$, uncorrected; Table 2 and Fig. 5) when comparing rCBF differences between pre- and postconditioning CS-alone trials of PET images. Comparing the postconditioning CS with 7-mA US with the preconditioning CS-alone trials, there was significant more activation of the bilateral primary sensory, left frontal, temporal, posterior cingulate and occipital cortices and bilateral pons ($P \leq 0.001$, uncorrected; Table 3). Comparing the postconditioning CS with 4-mA US with the preconditioning CS-alone trials, there was significant more activation of the left primary sensory, prefrontal, anterior cingulate, parietal and primary motor cortices, bilateral precentral gyrus, left putamen and left pons ($P \leq 0.001$, uncorrected; Table 4).

For the postconditioning CS-alone trial, there were no significant correlations between rCBF in any region and the mean bag volumes or the number of PVEs.

Table 2 Areas of rCBF significantly increased in the postconditioning CS-alone trial compared to the preconditioning CS-alone trial ($P \leq 0.001$, uncorrected)

Area (Broadmann area)	Hemisphere	Talairach co-ordinate			Z-score
		x	Y	Z	
Prefrontal cortex (46)	Left	−42	40	10	4.1
Anterior cingulate cortex (32)	Right	14	34	30	3.4
Insula (13)	Right	36	2	−4	3.8
Parietal cortex (40)	Left	−34	−32	40	3.6
	Right	54	−40	30	3.2
Pons*	Right	14	−26	−42	3.4
	Right	12	−14	−28	3.8
Cerebellum	Left	−28	−54	−40	3.2

rCBF, regional cerebral blood flow.
*Two different activated areas in the right pons were discriminated.

DISCUSSION

In the present study, the loud buzzer used prior to conditioning as a conditioned stimulus (CS) did not cause any alteration in rectosigmoid motility. However, following a series of conditional trials in which the buzzer was paired with painful electrical stimulation to the right hand, the buzzer-alone elicited increases in the phasic contractions of the rectosigmoid colon, which were similar to those seen following the conditioned stimulus plus the US. This provides evidence for Pavlovian conditioning of phasic motor responses. However, we did not find evidence for conditioning of the tonic motor response (barostat volume) or subjective pain; following conditional trials, the CS alone did not elicit changes in barostat volumes or reports of any gastrointestinal symptoms in the healthy subjects.

Little has been known about the anticipatory motor response in digestive system in humans. Previously, Naliboff *et al.*[15] and our group[20] reported that healthy human subjects reported slight unpleasantness/pain in response to sham distention of the colon after actual painful distention, and Mertz *et al.*[24] reported the same for sham distention of the stomach in patients with functional dyspepsia. However, these reports have not investigated gastrointestinal motility function. Only the animal study with rat model revealed that conditioned fear after repeated foot shock as US caused a significant increase in colonic spike burst frequency but failed to affect jejunal motility.[7] The colonic motility changes that have been identified in the present study are considered to be as one of secondary phenomena that would occur during the anticipation of pain. Anticipation affects the autonomic nervous function. The observation that anticipation of painful/aversive stimulus resulted in changes in heart rate was confirmed by several studies in both human and animal models.[25–27] Tests with drugs blocking the sympathetic or parasympathetic fibres revealed that

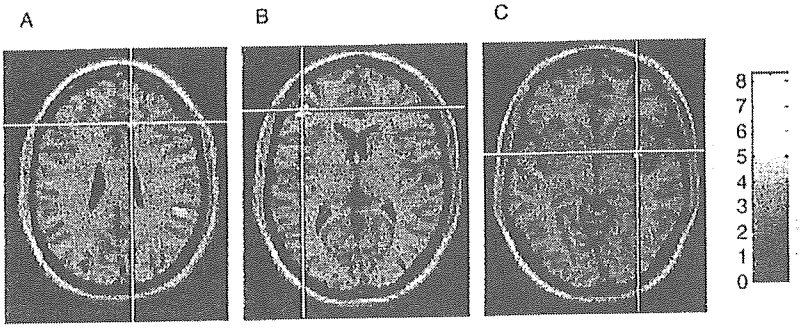

Figure 5 Conditioning effects on regional cerebral blood flow (rCBF). Parametric maps of regional cerebral blood flow increase during the postconditioning CS-alone trial compared with the preconditioning CS-alone trial is superimposed on Talairach–Tournoux stereotactic space. Sagittal, coronal and axial views are centred at 12, 34, 30, in the right anterior cingulate cortex (left; A), at −42, 40 and 10, in the left prefrontal cortex (middle; B) and at 36, 2, −4, in the right insula (right; C), respectively. In the view of A, activation of the right parietal cortex also can be seen. Significant changes are marked with a split-grey scale ($P \leq 0.001$, uncorrected).

Table 3 Areas of rCBF significantly increased in the CS with full intensity US trial compared to the preconditioning CS-alone trial ($P \leq 0.001$, uncorrected)

Area (Broadmann area)	Hemi-sphere	Talairach co-ordinate			Z-score
		x	Y	z	
Primary sensory cortex (1)	Left	−34	−28	56	3.8
Primary sensory cortex (2)	Left	−48	−22	36	3.4
	Right	52	−22	30	3.4
Orbitofrontal cortex (11)	Left	−34	36	−16	4.1
Precentral gyrus (6)	Left	−38	2	26	3.9
Posterior cingurate cortex (31)	Left	−18	−52	20	3.6
Middle temporal gyrus (22)	Left	−42	−42	6	3.8
Occipital cortex (18)	Left	−20	−82	24	4.0
Pons	Left	−8	−40	−40	4.2
	Right	16	−24	−34	3.8

rCBF, regional cerebral blood flow.

Table 4 Areas of rCBF significantly increased in the CS with weak intensity US trial compared to the preconditioning CS-alone trial ($P \leq 0.001$, uncorrected)

Area (Broadmann area)	Hemi-sphere	Talairach co-ordinate			Z-score
		x	Y	z	
Primary somatosensory cortex (2)	Left	−34	−32	40	3.4
Prefrontal cortex (9)	Left	−40	8	30	3.8
Prefrontal cortex (10)	Left	−36	40	20	3.8
Orbitofrontal cortex (11)	Left	−34	34	−16	3.6
Anterior cingulate cortex (24)	Left	−4	10	36	4.6
Insura (13)	Left	−42	−16	4	3.7
Parietal cortex (40)	Left	−50	−30	46	3.9
Superior frontal gyrus (6)	Left	−6	6	58	3.7
Middle frontal gyrus (6)	Left	−10	−6	50	3.7
Precentral gyrus (6)	Left	−56	0	20	3.7
	Right	54	2	14	3.5
Primary motor cortex (4)	Left	−34	−24	60	3.4
Putamen	Left	−28	−22	−4	4.5
Pons	Left	2	−20	−20	4.2

rCBF, regional cerebral blood flow.

the conditioned group, showing anticipatory fear, actually had a large sympathetic increase that was partly masked by a simultaneous parasympathetic increase.[28,29] Considering the previous studies on autonomic nervous activity during anticipation, increase in colorectal motility observed following a series of painful stimuli to the hand may result as dominant parasympathetic arousal. Further pharmacological intervention should be needed to confirm this hypothesis.

On the other hand, the anticipatory rectosigmoid motor responses could represent nonspecific arousal or anxiety. It has been reported that psychological stress induces significant changes in gastrointestinal motility, which were associated with alterations in autonomic nervous activity.[30,31] However, nonspecific arousal or anxiety cannot explain main effects of the conditioned phasic contractions of the rectosigmoid colon observed in this study for two reasons: (i) the overall anxiety score which is considered to be very global did not show a significant difference between before and after the series of painful stimuli. (ii) The increases in PVEs were limited to the poststimulus interval and were not seen during the baseline interval in the postconditioning CS-alone trial although this baseline interval was preceded by a series of painful stimuli.

Considering the conditioning effect in the brain, our findings of the brain imaging (Table 2 and Fig. 5) were in accordance with previous studies showing cerebral activation in the frontal and parietal cortices following Pavlovian conditioning.[12–14] Activation of the prefrontal cortex was seen during somatic stimulus, and has been implicated in cognitive appraisal of the stimulus.[32] In addition, significant cortical activation in the anterior cingulate cortex (ACC), which is believed to play a role in mediating the affective qualities of the pain experience[33,34] and expectation of pain,[35] and in the insula which serves as limbic integration cortex[36] was also seen as anticipatory responses in this study. Therefore, our results support that activation of the cognitive- and affective-related brain regions may contribute to the learned anticipatory responses and that this learned process was confirmed after the conditional trials in this experimental model. However, this model has been set up to study the anticipatory colonic motor response and brain activation patterns that have been identified only reflected that. The direct relationships between the brain activation and the gastrointestinal response during anticipation have not been clarified with this model.

When comparing brain-imaging data between the postconditioning buzzer with high- or low-mA stimulus and the preconditioning buzzer-alone trials, increased rCBF not only in the left primary somatosensory cortex but also in the prefrontal cortex was observed (Tables 3 and 4). These findings were in concordance with the previous reports on the central processing of painful stimulus using with brain-imaging studies,[32,35,37] Thus, in the present study, these comparisons revealed that the transcutaneus electrical stimulation to the right hand induced painful sensation. Furthermore, activation of the cognitive- and affective-related brain regions was observed in common, suggesting that cerebral responses involved in painful/fearful anticipation might be different from the nociceptive process.[38]

There are some limitations in this study. First of all, participants of the study were limited to small group of healthy volunteers from a local university. Lee *et al.* have investigated differences in gastrointestinal symptom severity in males *vs* females and variations with menstrual cycles.[39] Female subjects might show different patterns in the brain and/or colonic motility function for the conditioned responses. Secondly, additional examinations such as an anorectal morphological study were not explored. However, existence of severe megarectum[40] and/or the other anorectal disorders,[41] which might affect rectal wall motor function, were unlikely because no subjects had been reported any problems of bowel movement and any abnormal physical findings. Finally, the reliable visceral sensory tests (e.g. the ascending method of limits and/or the random sequence)[19] were not used because of the limited time in the PET scanning room. Despite of these limitations, we believe that this study could be worth to report the conditioned phenomena in this model as a first step to understand anticipatory colonic motility responses to somatosensory painful stimulus in humans. The available data on conditioned gastrointestinal responses are very limited and deserve further studies.

In summary, the Pavlovian conditioning study is significant because of positive findings that the conditioned phenomenon in this model is a first step to understand the anticipatory colonic motility responses. Significant increases in colonic phasic contractions and significant increases in cerebral blood flow in the cognitive- and affective-related cortical regions were observed in this study. This conditioning paradigm could be a model to investigate anticipatory responses in gastrointestinal motility and brain function, which may contribute to development of functional gastrointestinal disorders. We concluded that the rectosigmoid motility could become conditioned by pairing a painful somatosensory stimulus with a neutral stimulus in humans.

ACKNOWLEDGMENTS

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as the letter states, this treatment does resolve both OSA and GER. It is not appropriate, however, to state that '...sleep disorder treatment will effectively treat both sleep and GER'. This applies only to the condition of OSA.

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Development of irritable bowel syndrome may be associated with a parental history of bowel problems

SIRS, We read with special interest the review article by Kang[1] on the cross-cultural epidemiological comparisons for irritable bowel syndrome (IBS). The author pointed out that there was a gender difference in the prevalence of IBS, health care utilization and stool frequency among different ethnic and/or cultural populations. Unfortunately, data from Japan were not cited on the review. We have reported the epidemiological data for IBS using Rome II diagnostic criteria[2] from 417 Japanese subjects in the community.[3] In this study, 14.2% (15.5% of females and 12.9% of males) were diagnosed as IBS and there was no difference in gender ratio. Only 22.0% of subjects with IBS had met a doctor for IBS-related symptoms.[2] Our results for Japanese supported the statement of the review article in which female predominance was not seen using Rome I or II criteria in eastern population.[1]

We were also concerned about another factor affecting prevalence of IBS among the countries. A total of 417 subjects from the community sample in Sendai, Japan, and 56 patients with IBS in the gastrointestine (GI) clinic were asked whether their parents had a history of chronic or recurring abdominal pain/discomfort with abnormal bowel habit (parental history). Significantly more IBS patients (33.9%) and IBS nonconsulters (26.1%) had a parental history, compared to control subjects who were not diagnosed as IBS in the community (12.6%). The parental history was an independent risk factor for IBS diagnosis after adjusting for trait anxiety, depression and perceived stress. These results supported the results from the twin study in the USA[4] and Australia.[5] Furthermore, IBS patients with parental history had significantly higher scores of major symptoms for IBS (7-point scales); abdominal pain, abdominal discomfort, changes in bowel movement with abdominal pain/discomfort and softer stools with abdominal pain/discomfort than those without the history. Our results suggest that the parental history of bowel problems may affect the severity of IBS symptoms. Thus, this aggregation of IBS within families – namely, genetics and social learning through modelling the illness behaviour of parents – may be one of the risk factors for development and reinforcement of IBS symptoms. In fact, it has been reported that children of mothers with IBS have more somatic as well as GI symptoms, disability days and clinical visits.[6] The recent genetic studies on the serotonin transporter (SERT) gene-linked polymorphic region (5-HTTLPR) revealed that IBS is not equivalent to SERT dysfunction but SERT dysfunction may contribute to behavioural and functional gut disorders.[7] In the further research on epidemiological studies for IBS, such genetic and environmental (e.g. diet, belief and social habit) factors should be taken into account.

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Visceral sensitivity

Can modulating corticotropin releasing hormone receptors alter visceral sensitivity?

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Activation of corticotropin releasing hormone (CRH) receptor 2 (CRH-R2) reduces visceral sensitivity induced by colorectal distension in conscious rats. This finding is relevant to the increased interest in the potential use of therapeutic agents that act on CRH receptors in the treatment of irritable bowel syndrome

Clarifying the adverse effects of stress on bodily function is a crucial paradigm for medical research. Evidence that psychosocial stress aggravates digestive diseases has been accumulating and stress induced exacerbation of symptoms in patients with functional gastrointestinal disorders is well recognised.[1] Corticotropin releasing hormone (CRH), a 41 amino acids peptide produced mainly in the paraventricular nucleus of the hypothalamus, is considered to be a major mediator of the stress response.[2] Indeed, stress is known to induce release of hypothalamic CRH, resulting in pituitary secretion of adrenocorticotropic hormone (ACTH). In addition, stress related activation of CRH receptors has been reported to alter gastrointestinal functions.[3] Moreover, physical or psychological stress is known to delay gastric emptying,[4] accelerate colonic transit,[5] and evoke colonic motility[6] in rats.

Two major G protein coupled receptors for the CRH have been identified, CRH receptor 1 (CRH-R1) and receptor 2 (CRH-R2).[7–9] CRH-R1, which is highly expressed in the anterior pituitary, neocortex, hypothalamus, hippocampus, amygdala, locus coeruleus, and cerebellum, has been reported to mediate stress induced physiological changes, including stimulation of the hypothalamo-pituitary-adrenal axis, elevation of plasma levels of catecholamines, increased colonic motility,[10] and exaggerated stress related behaviour, especially anxiety.[11 12] In addition, stimulation of this receptor is believed to activate adenylate cyclase, an enzyme that catalyses the formation of cyclic AMP (cAMP).[7–9]

We have previously reported increased colonic motility and visceral perception in response to administration of CRH in patients with irritable bowel syndrome (IBS).[13] In addition, earlier studies have indicated that gastrointestinal dysmotility[14] and visceral hypersensitivity[15] are major events in the pathophysiology of IBS. Moreover, patients with IBS have been reported to suffer from a variety of chronic or acute psychiatric conditions, including depression, generalised anxiety, panic, social phobia, and somatisation.[16] Various studies have suggested a relationship between stress induced changes in colonic motility and CRH action in the paraventricular nucleus of the hypothalamus.[17] Accordingly, it has been shown that intracerebroventricular injection of CRH stimulates gastrointestinal motility in a way similar to that induced by stress[18] and that intraperitoneal injection of CRH induces defecation and clustered spike bursts longer than basal spike bursts in rats.[10]

CRH-R1 antagonists have been shown to prevent stress-like gastrointestinal motor responses following central or peripheral injection of CRH.[10] In addition, it has been reported that CRH-R1 deficient mice show impaired response to stress, as indicated by absence of increased ACTH and corticosterone levels following exposure to stress, as well as less pronounced anxiety related behaviour.[11 12] From these findings, it is reasonable to assume that CRH mediates gastrointestinal and behavioural responses to stress via CRH-R1. Actually, in a recent study,[19] we have shown that administration of an α-helical CRH or CRH-R1 antagonist attenuates hippocampal noradrenaline release and reduces the frequency of abdominal contractions induced by acute colorectal distension in rats. We have also shown that the CRH-R1 antagonist used in that study[19] reduced plasma ACTH and anxiety after acute colorectal distension but not after chronic colorectal distension, probably due to habituation. Another important finding of our previous study[19] is that pretreatment with the CRH-R1 antagonist blocked chronic colorectal distension induced increase in rats faecal pellet output. Because the CRH-R1 antagonist used in our previous study[19] is an agent that crosses the blood-brain barrier, both central CRH-R1 and