peripheral CRH-R1 are thought to be responsible for colorectal distension induced sensitisation. Nevertheless, CRH and CRH-R1 in the brain may play a major role in colorectal distension induced anxiety, ACTH release, visceral hypersensitivity, and changes in colonic motility.

Evidence supporting the concept that peripheral CRH and CRH-R1 play important roles in brain-gut sensitisation is increasing. Several studies have identified immunoreactive CRH[20] and urocortin [21] as well as CRH-R1 and CRH-R2 mRNAs in human colonic mucosa.[21] In addition, reverse transcription-polymerase chain reaction (RT-PCR) has revealed expression of CRH-R1 mRNA in both the myenteric and submucosal plexus in the guinea pig.[22] Application of CRH has been shown to evoke depolarising responses associated with elevated excitability in both myenteric and submucosal neurones.[22] On the other hand, peripheral injection of CRH has been reported to induce discrete effects on colonic secretory and motor function, and permeability.[23] We have previously reported that intravenous administration of a non-selective CRH antagonist (α-helical CRH) blunts the exaggerated motility response in the sigmoid colon to electrical stimulation in IBS patients compared with normal subjects.[24] In the same study, we have shown that administration of α-helical CRH induces a significant increase in barostat bag volume in normal subjects but not in IBS patients, and a significant reduction in the ordinate scale of abdominal pain and anxiety evoked by rectal electrical stimulation in IBS patients. However, plasma ACTH and serum cortisol levels were generally not suppressed following administration of α-helical CRH at 10 μg/kg. Although the precise sites of action of α-helical CRH are unknown, we suggested in our previous study that blunting the colonic motor response is mainly due to blockage of peripheral CRH-R1 and that drug anxiolytic or antinociceptive effects are probably based on inhibition of central CRH-R1 via circumventricular organs, which are relatively unprotected by the blood-brain barrier.[24] These findings and concepts, which put in the context of existing preclinical and clinical data, support the testing of new CRH antagonists, particularly potent CRH-R1 antagonists, in IBS and the view that the CRH-R1 receptor is a promising target for the treatment of IBS.[25]

In this issue of *Gut*, however, Million and colleagues[26] provide a new theory for modifying gut sensitivity via CRH-R2 ***(see page 172)***. Using RT-PCR, they proved the existence of CRH-R2 in the dorsal root ganglia and spinal cord and hypothesised that CRH-R2 activation may influence visceral pain induced by colorectal distension in conscious rats. By assessing the possible sites and mechanisms of action for CRH-R2 activation, they showed that two repeated colorectal distensions produced visceral sensitisation and phosphorylation of extracellular signal related kinase 1/2 (ERK 1/2) and that intravenous administration of human urocortin 2, a selective CRH-R2 agonist, prevented visceral sensitisation and reduced the second response compared with the first one. Million *et al* also demonstrated that administration of human urocortin 2 dampened distension induced phosphorylation of ERK 1/2 and robust inferior splanchnic afferent spike activity and that treatment with astressin$_2$-B, a CRH-R2 receptor antagonist, reversed the inhibitory effects of human urocortin 2 both in vivo and in vitro.[26]

CRH-R2 is highly expressed in the anterior pituitary, hypothalamus, hippocampus, amygdala, lateral septum, and other peripheral tissues, including the spleen, stomach, and gut.[7–9] Compared with CRH-R1, the functional role of CRH-R2 is relatively obscure. However, recent reports put forward the concept that activation of CRH-R2 signalling pathways may be important to reduce anxiety and stress response.[27 28] There are other functional differences between CRH-R1 and CRH-R2. For example, activation of CRH-R1 causes a proinflammatory response whereas stimulation of CRH-R2 provokes anti-inflammatory changes.[29] In addition, the study by Million and colleagues[26] offers evidence of the contrasting roles of CRH-R1 and CRH-R2 in visceral nociception. While CRH-R1 is involved in the pronociceptive effects of visceral pain, CRH-R2 mediates antinociceptive responses. These findings are supported by a recent report from another group.[30]

Several questions arise from these animal experiments. Do endogenous CRH-R2 ligands such as CRH, urocortin 1, urocortin 2, urocortin 3 (stresscopin), and stresscopin related peptides play an inhibitory role in visceral hypersensitivity in IBS patients? If so, are selective CRH-R1 antagonists more effective for visceral hypersensitivity than non-selective CRH antagonists? Moreover, do agents that block CRH-R2 have any adverse effects on the pathophysiology of IBS? Do CRH-R2 agonists have therapeutic value for IBS and/or allied functional gastrointestinal disorders, even though stress induced inhibition of gastric emptying is mainly mediated via CRH-R2? What are the major steps from the synthesis of cAMP by activated CRH-R2 in the dorsal root ganglia and spinal cord to reduced phosphorylation of ERK 1/2 in the laminae I and II? Thus the disclosed nature of CRH-R2 reported in the present issue of *Gut* brings us an exciting paradigm on research and drug development of the CRH neuropeptide family.

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Incretin

To be or not to be—an incretin or enterogastrone?

M Horowitz, M A Nauck

Glucagon-like peptide 1 does not comfortably fulfil the criterion of a gut derived factor responsible for an enhanced meal related insulin response; it appears logical to add the definition of a "physiological incretin hormone"

Incretin hormones are gut derived peptides that augment the insulin releasing action of hyperglycaemia. In his seminal review, based on the 1978 Claude Bernard lecture, delivered at the European Association for the Study of Diabetes Meeting, Werner Creutzfeldt defined the term incretin as "an endocrine transmitter produced by the gastrointestinal tract which is: (a) released by nutrients, especially carbohydrates and (b) stimulates insulin secretion in the presence of glucose if exogenously infused in amounts not exceeding blood levels achieved after food ingestion".[1] At that time, the best characterised incretin candidate was glucose dependent insulinotropic polypeptide (GIP), although there was evidence that GIP was not the only incretin.[1 3] An incretin role for GIP was established, along the lines of Creutzfeldt's definition,[1] by intravenous infusion in healthy subjects, both alone and in combination with glucose, and demonstrating that the insulinotropic property of GIP was dependent on a permissive rise in blood glucose.[2] Subsequent experiments, performed under more physiological conditions, with plasma GIP and glucose concentrations mimicking the postprandial state, confirmed these observations.[4] That relatively uncomplicated infusion experiments had the capacity to predict the physiological role of GIP with regard to its effects on insulin secretion is testimony to the fact that, metabolically speaking, GIP is apparently devoid of additional actions which have the potential to confound such experiments.[5]

The situation with glucagon-like peptide 1 (GLP-1) is far less straightforward. The GLP-1/glucose infusion experiment results in effects similar to those observed with GIP,[6] and GLP-1, accordingly, fulfils the definition of an incretin hormone, as put forward by Creutzfeldt.[1] However, studies which have evaluated the effects of GLP-1 on the metabolic response to a meal, by infusing physiological or pharmacological amounts of GLP-1,[7] or interfering with endogenous GLP-1 action with the well characterised GLP-1 antagonist exendin(9–39),[8–10] have revealed a complex pattern of GLP-1 actions. In particular, as a result of its effect on slowing gastric emptying substantially, exogenous GLP-1 attenuates the postprandial rise in glycaemia, leading to lesser substrate (glucose) mediated insulin secretion and an overall reduction, rather than an increase, in the insulin secretory response to a meal.[7 11 12] In other words, inhibition of gastric emptying by exogenous GLP-1 outweighs its direct insulinotropic effects. This was highlighted in a recent study demonstrating that intravenous erythromycin, as a result of its prokinetic properties, abolishes the deceleration of gastric emptying induced by exogenous GLP-1 in healthy subjects and that this is associated with a marked reduction in its glucose lowering effect.[12] Furthermore, the GLP-1 antagonist exendin(9–39) increases, rather than lowers, the insulin response to a meal.[13] Based on these observations it is clear that GLP-1 does not comfortably fulfil the criterion of a gut derived factor responsible for an enhanced meal related insulin response; furthermore, it appears logical to add the definition of a "physiological incretin hormone" to that provided by Creutzfeldt,[1] and assigning such a role to GLP-1 appears inappropriate based on current data.[11]

In their important study in the current issue of *Gut*, Schirra and colleagues[14] have introduced a new approach to evaluation of the incretin role of GLP-1 in healthy subjects ***(see page 243)***. They used intraduodenal administration

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Corticotropin-Releasing Hormone Receptor 1 Antagonist Blocks Brain–Gut Activation Induced by Colonic Distention in Rats

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Background & Aims: The corticotropin-releasing hormone receptor 1 mediates stress-induced changes in colonic motor activity and emotion. We tested the hypothesis that pretreatment with JTC-017, a specific corticotropin-releasing hormone receptor 1 antagonist, blocks colorectal distention–induced hippocampal noradrenaline release and visceral perception in rats. We also investigated whether pretreatment with JTC-017 blocks acute or chronic colorectal distention–induced adrenocorticotropic hormone release, anxiety, and stress-induced changes in colonic motility. *Methods:* Rats were pretreated intrahippocampally with α-helical corticotropin-releasing hormone (1.25 μg/kg; vehicle), a nonspecific corticotropin-releasing hormone receptor antagonist, or intraperitoneally with JTC-017 (10 mg/kg). Hippocampal noradrenaline release after microdialysis and the frequency of abdominal contractions were measured in response to acute colorectal distention. Plasma adrenocorticotropic hormone levels, anxiety-related behavior, and stress-induced changes in colonic motility were evaluated after acute or chronic colorectal distention followed by exposure to an elevated plus maze. *Results:* Administration of α-helical corticotropin-releasing hormone or JTC-017 significantly attenuated hippocampal noradrenaline release and reduced the frequency of abdominal contractions induced by acute distention. In addition, JTC-017 significantly reduced plasma adrenocorticotropic hormone and anxiety after acute distention. After chronic distention, changes in plasma adrenocorticotropic hormone and anxiety were not significant because of habituation. In contrast, a significant increase in fecal pellet output during the elevated plus maze was observed after chronic distention. This increase in fecal pellet output was blocked by pretreatment with JTC-017. *Conclusions:* Our results suggest that JTC-017, a specific corticotropin-releasing hormone receptor 1 antagonist, attenuates hippocampal noradrenaline release, visceral perception, adrenocorticotropic hormone release, and anxiety after acute colorectal distention in rats. In addition, JTC-017 blocks stress-induced changes in colonic motility after chronic colorectal distention in rats.

Two major G protein–coupled receptors for the corticotropin-releasing hormone (CRH) have been identified as CRH receptor (CRHR)1 and CRHR2.[1–3] CRHR1, which is highly expressed in the anterior pituitary, neocortex, hippocampus, amygdala, 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,[4–7] and exaggerated stress-related behavior, especially anxiety.[6,8] In addition, stimulation of this receptor is believed to activate adenylate cyclase, an enzyme that catalyzes the formation of adenosine 3′,5′-cyclic monophosphate (cAMP).[1–3]

We have previously reported increased colonic motility and visceral perception in response to the administration of CRH in patients with irritable bowel syndrome (IBS).[9] In addition, earlier studies have indicated that gastrointestinal dysmotility[9,10] and visceral hypersensitivity[11] 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, generalized anxiety, panic, social phobia, and somatization.[12,13] Various studies have suggested a relationship between stress-induced changes in colonic motility and CRH action in the paraventricular nucleus (PVN) of the hypothalamus.[14,15] Accordingly, it has been shown that intracerebroventricular injection of CRH stimulates gastrointestinal motility in a way similar to that induced by stress[7,16,17] and that intraperitoneal injection of CRH induces defecation and clustered spike bursts longer than the basal spike bursts in rats.[5] CRHR1 antagonists have been shown to

Abbreviations used in this paper: CRH, corticotropin-releasing hormone; CRHR, CRH receptor; EPM, elevated plus maze; HPLC, high-performance liquid chromatography; IBS, irritable bowel syndrome; IC_{50}, median inhibitory concentration; LC, locus ceruleus; PVN, paraventricular nucleus.

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prevent stress-like gastrointestinal motor responses after central or peripheral injection of CRH.[5,7] In addition, it has been reported that CRHR1-deficient mice show an impaired response to stress, as indicated by the absence of increased adrenocorticotropic hormone (ACTH) and corticosterone levels after exposure to stress and less pronounced anxiety-related behavior.[6,8] From these findings, it is reasonable to assume that CRH mediates gastrointestinal and behavioral responses to stress via CRHR1.

Colorectal distention is widely used to assess the response to gastrointestinal stimulation in human and animal experiments. Repetitive sigmoid distention has been shown to induce rectal hyperalgesia in patients with IBS,[18] whereas acute noxious colorectal distention under restraint conditions induces abdominal contractions and significant c-Fos expression in the brainstem, limbic areas, cortical areas, and lumbosacral spinal cord in rats.[19–21] Gastrointestinal stimulation especially has been shown to activate the locus ceruleus (LC), a nearly homogeneous nucleus containing approximately 50% of brain noradrenaline neurons.[22] Along this line, we have previously reported that colorectal distention induces hippocampal noradrenaline release and visceral perception in rats.[23] Other studies have suggested that brain noradrenergic response to colonic distention plays an important role in anxiety-related behavior and central symptoms of IBS.[24] Only a few reports have examined the effects of chronic colorectal distention. One study has shown that colon irritation for 2 weeks results in chronic visceral hypersensitivity.[25]

Although the involvement of CRH in stress-related behavior has been widely studied, no study has investigated the effects of CRHR1 antagonists on hippocampal noradrenaline release and visceral perception after gastrointestinal stimulation. In addition, the effects of acute and chronic gastrointestinal stimulation on emotional behavior, which is important to clarify the pathophysiology of IBS, have not been investigated. In this study, we tested the hypothesis that pretreatment with JTC-017, a specific CRHR1 antagonist, blocks colorectal distention–induced hippocampal noradrenaline release and somatic motor response to visceral distention in rats. We also used α-helical CRH (a nonspecific CRHR antagonist) to block CRHR1. If effects of the nonspecific CRHR antagonist and a specific CRHR1 antagonist are almost identical, then CRHR1 may play a salient role in the process described previously. We also investigated whether pretreatment with JTC-017 blocks acute or chronic colorectal distention–induced ACTH release, anxiety-related behavior, and stress-induced changes in colonic motility.

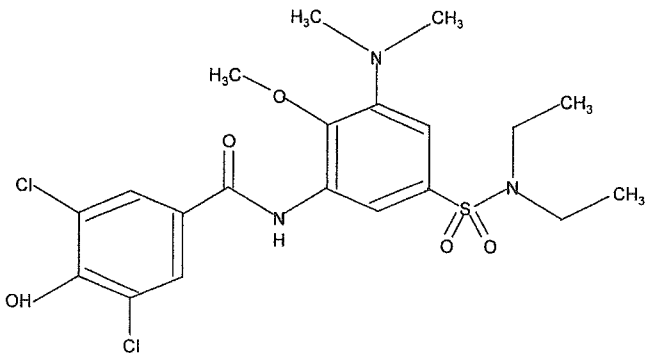

Figure 1. Structure of JTC-017.

Materials and Methods

Animals

Male Wistar rats (n = 73) weighing 180–210 g were provided by Charles River Japan Inc. (Yokohama, Japan). The rats were housed under controlled illumination (12:12-hour light/dark cycle starting at 8:00 AM) and temperature (23°C ± 1°C) with free access to food and water. This study was designed in accordance with the guidelines for animal experiments and was approved by the Ethics Committee of Laboratory Animals of Tohoku University.

Drugs

The drugs used in this study were α-helical CRH, a nonspecific CRHR antagonist, and JTC-017 (3,5-dichloro-*N*-[5-diethylsulfamoyl-3-dimethylamino-2-methoxphenyl]-4-hydroxybenzamide; Figure 1), a specific CRHR1 antagonist developed by Japan Tobacco Inc. α-Helical CRH was dissolved in hydrochloride acidic saline and kept at −40°C, and JTC-017 was dissolved in 10% hydroxypropyl-β-cyclodextrin and kept at 14°C.

Corticotropin-Releasing Hormone Receptor 1 Binding Assays and Cell Biology Assays

Competition binding experiments were performed according to the procedure described by Okuyama et al,[26] with some modifications. Membrane homogenates of rat pituitary glands (150 μg of protein) were incubated for 120 minutes at 22°C with 0.1 nmol/L [^{125}I]Tyr^{0}-CRH in the absence or presence of the competing test compound in 250 μL of assay buffer containing 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $MgCl_2$, 2 mmol/L ethylenediaminetetraacetic acid, and 0.1% bovine serum albumin. After incubation, the samples were rapidly filtered under a vacuum through glass-fiber filters (GF/B; Packard) and rinsed several times with an ice-cold buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.01% Triton X-100 by using a 96-sample cell harvester (Unifilter; Packard). The filters were then dried and counted for radioactivity in a scintillation counter (Topcount; Packard) by using a scintillation cocktail (Microscint 0; Packard). Nonspecific binding was determined in the presence of 1 μmol/L

ovine CRH, which was also used at different concentrations (10^{-10} to 3×10^{-7} mol/L) as a reference compound.

The antagonistic effects of test compounds on CRH1 receptor/G protein coupling were determined by measuring cAMP production in human neuroblastoma cells (SH-SY5Y) according to the procedure described by Schoeffter et al.[27] Cyclic AMP production was measured directly by using an RIA Flashplate detection kit (NEN; reference number SMP004). SH-SY5Y cells (5×10^5 cells per well) and CRH (30 nmol/L) were added directly into the Flashplate in the presence of JTC-017 or water for basal and stimulated controls. After a 30-minute incubation at 30°C, 100 μL of the detection kit buffer containing [^{125}I]cAMP was added, and the mixture was incubated for a further 2 hours at 22°C. The kit buffer causes cell lysis, liberation of cAMP produced, and competition between radiolabeled and nonradiolabeled cAMP for a fixed number of antibodies within each well. Reading of the RIA Flashplate detection kit and measurement of cAMP were performed with a microplate scintillation counter (Topcount; Packard). α-Helical CRH, tested at 8 concentrations ranging from 10^{-9} to 10^{-6} mol/L, was used as inhibition reference compound for determination of median inhibitory concentration (IC_{50}) values.

Measurement of Hippocampal Noradrenaline

Hippocampal noradrenaline was measured 48 hours after in vivo microdialysis, which was performed as previously reported.[23,28] In brief, with the rat under barbiturate anesthesia (50 mg/kg intraperitoneally), a stainless-steel guide cannula was inserted into the right hippocampus of the rat 5.8 mm posterior and 4.8 mm lateral to the bregma with the tip 4.0 mm below the dura. The cannula was secured to the skull with 2 screws and acrylic resin (Unifast; GC-Dental Industrial, Tokyo, Japan). A dialysis probe (full length, 15 mm) was inserted into the hippocampus with the tip 6.0 mm below the dura, and a Ringer solution (NaCl 147 mmol, KCl 4 mmol, and $CaCl_2$ 2.3 mmol) was perfused into the hippocampus at a rate of 2 μL/min by using a microsyringe pump (EP-60; Eicom) connected to the dialysis probe with a plastic tube (internal diameter, 0.1 mm). Noradrenaline was measured in dialysate samples by using high-performance liquid chromatography (HPLC). A total of 40 μL of the neurotransmitter-containing dialysate sample was injected into an HPLC system by using an autoinjector (AS-10; Eicom) every 20 minutes. The HPLC system consisted of a pump (LC-9A; Shimadzu, Kyoto, Japan) equipped with a damper and degasser (DGU-3A; Shimadzu), a catecholamine-specific column (CA-50DS; Eicom), and an electrochemical detector (L-ECD-6A; Shimadzu), which was fitted with a flow cell unit (EC-100; Eicom) with a graphite working electrode set at +550 mV against an Ag/AgCl reference electrode.

The mobile phase in HPLC was a dibasic sodium phosphate buffer 100 mmol/L (pH 6.0) containing sodium octanesulfonic acid 2.5 mmol/L, ethylenediaminetetraacetic acid 0.05 mmol/L, and 7.0% (vol/vol) methanol, and the flow rate was 1.0 mL/min. Rats were killed at the end of the experiment, and the positioning of the dialysis probe in the hippocampus was confirmed.

Colorectal Distention

The rats were first restrained with a restraint instrument to prevent free movements that could interfere with the experimental procedure. A 2-cm polyethylene balloon was then inserted into the colorectum through the anus, and the bag was distended at 80 mm Hg for 20 minutes by an air pump. It is generally accepted that 80 mm Hg of colorectal distention is a noxious stimulus in rats.[29] Comparison between restraint only and colorectal distention under restraint conditions has shown that colorectal distention under restraint conditions increases noradrenaline release more than restraint only and specifically evokes abdominal contractions.[23] For microdialysis, the head of the rat was fixed by using a small-animal stereotaxic instrument (metallic plate), and their limbs were fastened by adhesive tape. This procedure enabled us to continue microdialysis without disruption of the cannula. For comparison between acute and chronic colorectal distention, rats were restrained in plastic tubes for the duration of the distention (they could roll over but not turn head to toe).

Measurement of Abdominal Contractions

The frequency of contractions of the abdominal wall was considered as a reliable marker of visceral perception and was videotaped during the colorectal distention period (20 minutes). Contractions were counted visually and were clearly separated from normal abdominal wall respiratory movements.

Measurement of Plasma Adrenocorticotropic Hormone

Immediately after rats were decapitated, blood was collected in chilled polyethylene tubes containing 200 μL (200 U) of heparin and separated, and the plasma was stored at −40°C until assay. Plasma ACTH levels were measured by radioimmunoassay.

Evaluation of Anxiety-Related Behavior and Stress-Induced Changes in Colonic Motility

Anxiety-related behavior and stress-induced changes in colonic motility were evaluated when rats were exposed to an elevated plus maze (EPM). In this type of maze, loss of normal behavior in the open arms reflects increased anxiety; compounds well known to cause anxiety in human (eg, yohimbine, pentylenetetrazole, caffeine, and amphetamine) have been shown to significantly reduce the percentage of entries into and time spent on the open arms in rats.[30,31] In addition, an increase in the number of entries into the open arms of the EPM and in the time spent on these arms is considered to be inversely related to anxiety.[31] However, the number of fecal pellets produced in the EPM is considered to be a simple marker of stress-induced changes in colonic motility.[32] Rats were placed facing an enclosed arm in the center of the EPM.

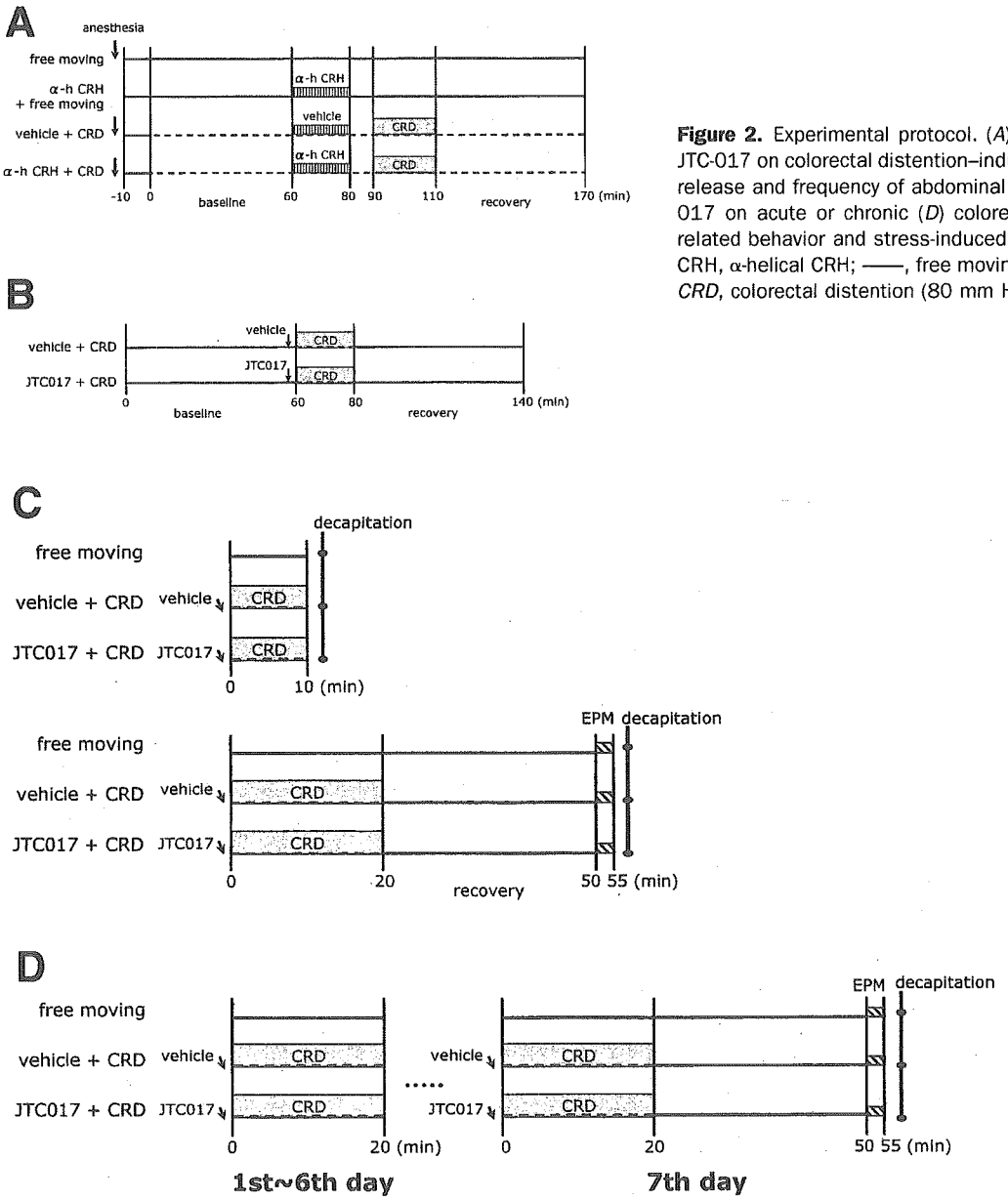

Figure 2. Experimental protocol. (*A*) Effects of α-helical CRH or (*B*) JTC-017 on colorectal distention–induced hippocampal noradrenaline release and frequency of abdominal contractions. (*C*) Effects of JTC-017 on acute or chronic (*D*) colorectal distention–induced anxiety-related behavior and stress-induced changes in colonic motility. α-h CRH, α-helical CRH; ——, free moving; – – –, restraint; gray box with *CRD*, colorectal distention (80 mm Hg); striped box, EPM.

This 5-minute test provided a computer-automated measure of arm exploration. Testing was conducted in a quiet and dedicated room. All experiments used a between-individuals design in which separate groups of rats were tested on the EPM only once.

Experimental Protocol

Determination of the effects of α-helical CRH on colorectal distention–induced hippocampal noradrenaline release and abdominal contractions. Rats were divided into 4 groups (6 per group) and exposed to 1 of the following 4 experiments: free movement, treatment with α-helical CRH followed by free movement, treatment with vehicle followed by colorectal distention, or treatment with α-helical CRH followed by colorectal distention (Figure 2*A*). After in vivo microdialysis, α-helical CRH (1.25 μg/kg) or vehicle was microinjected into the right hippocampus of each rat 30 minutes before colorectal distention or free movement. Hippocampal noradrenaline release and abdominal contractions were measured as described previously. Baseline measurements started 2 hours after perfusion was initiated, ie, when the transmitter level reached a plateau, and were maintained for 60 minutes. Rats were freely moving during the baseline measurement and recovery periods.

Determination of the effects of JTC-017 on colorectal distention–induced hippocampal noradrenaline release and abdominal contractions. Rats were divided into 2 groups (5 per group) and intraperitoneally treated with

either vehicle or JTC-017 at a dose of 10 mg/kg (Figure 2*B*). Two minutes after drug or vehicle administration, the rats were subjected to colorectal distention as described previously (Figure 2*B*). Hippocampal noradrenaline release and abdominal contractions were measured as indicated previously. Baseline measurements started 2 hours after perfusion was initiated and were maintained for 60 minutes. Rats were freely moving during the baseline measurement and recovery periods.

Determination of the effects of JTC-017 on acute and chronic colorectal distention–induced ACTH release, anxiety-related behavior, and stress-induced changes in colonic motility. Nine groups of rats (5–8 per group) were used in these experiments. During the experiments for acute colorectal distention, rats were treated with vehicle or JTC-017 (10 mg/kg intraperitoneally) 2 minutes before colorectal distention or left free (control rats; Figure 2*C*). The effects of JTC-017 on acute colorectal distention–induced ACTH release, anxiety-related behavior, and stress-induced changes in colonic motility were determined by measuring plasma ACTH levels after an EPM test followed by decapitation. In the rats that underwent colorectal distention, the colorectum was distended for 10 or 20 minutes. In the 10-minute colorectal distention experiments, blood was sampled immediately after distention; however, in the 20-minute colorectal distention experiments, rats were allowed a 30-minute recovery period before being subjected to the EPM test. Blood was sampled by decapitation immediately after the EPM session.

During the chronic colorectal distention experiments, rats were treated with vehicle or JTC-017 (10 mg/kg intraperitoneally) 2 minutes before colorectal distention or left free (control rats; Figure 2*D*). For colorectal distention, the colorectum was distended for 20 min/day for 7 days (Figure 2*D*). On the Seventh day, anxiety-related behavior and stress-induced changes in colonic motility were evaluated by EPM after the last colorectal distention session and a recovery period of 30 minutes. Blood was sampled by decapitation immediately after the EPM session.

Statistical Analysis

All data are expressed as the mean ± SE. Results of CRHR1 binding assays and cell biology assays are expressed as percentages of control values and as percentage variation of control values obtained in the presence of JTC-017. IC_{50} values, median effective concentration values, and Hill coefficients were determined by nonlinear regression analysis of the concentration–response curves. These parameters were obtained by the Hill equation curve fitting. Inhibition constants (K_i) were calculated from the Cheng–Prusoff equation. The mean value of 3 baseline samples was set as 100% noradrenaline level. Each datum was calculated as the percentage of noradrenaline level, as previously reported.[23,28] Statistical significance was evaluated by using analysis of variance (1 and 2 way; repeated measures), the post hoc test, and the Wilcoxon rank sum test. A probability level of ≤.05 was considered to be statistically significant.

Results

CRHR1 Binding Assay and the Antagonistic Effects of Test Compounds on CRHR1

The affinity of JTC-017 for human CRHR1 was much lower than that of α-helical CRH (K_i = 43 nmol/L and IC_{50} = 105 nmol/L vs K_i = 5.2 nmol/L and IC_{50} = 13 nmol/L). Cyclic AMP production in human neuroblastoma cells indicated that JTC-017 antagonizes CRHR1 with an IC_{50} value of 3.6 nmol/L, whereas α-helical CRH antagonizes CRHR1 with an IC_{50} value of 0.3 nmol/L. Preliminary studies have indicated that JTC-017 does not bind to CRHR2 because its IC_{50} for this receptor is more than 100 mmol/L at 100 nmol/L ^{125}I-sauvagine (data not shown).

Effects of α-Helical Corticotropin-Releasing Hormone on Colorectal Distention–Induced Hippocampal Noradrenaline Release and Abdominal Contractions

Basal noradrenaline levels did not significantly differ among the rats with free movement (110% ± 6%), treated with α-helical CRH followed by free movement (111% ± 20%), vehicle followed by colorectal distention (118% ± 12%), and α-helical CRH followed by colorectal distention (87% ± 15%). However, at the end of colorectal distention, noradrenaline levels in the rats treated with α-helical CRH followed by colorectal distention were significantly lower than those in the rats treated with vehicle followed by colorectal distention (77% ± 11% vs 400% ± 65%; $P < .01$) but did not significantly differ from those in the rats with free movement (98% ± 29%) or those in the rats treated with α-helical CRH followed by free movement (133% ± 31%; Figure 3*A*). In addition, at the end of the recovery period, noradrenaline levels in the rats treated with α-helical CRH followed by colorectal distention were significantly lower than those in the rats treated with vehicle followed by colorectal distention (99% ± 9% vs 541% ± 108%; $P < .01$) but did not significantly differ from those in the rats with free movement (69% ± 12%) or those in the rats treated with α-helical CRH followed by free movement (100% ± 32%). However, abdominal contractions in the rats treated with α-helical CRH followed by colorectal distention were significantly less frequent than those in the rats treated with vehicle followed by colorectal distention (25 ± 2 vs 57 ± 7; $P < .01$; Figure 3*B*). Evaluation of statistical significance using analysis of variance revealed significant main effects (drug conditions: $F = 17.2$, $P < .001$; period: $F = 12.0$, $P < .001$) and significant interaction ($F = 7.0$; $P < .001$).

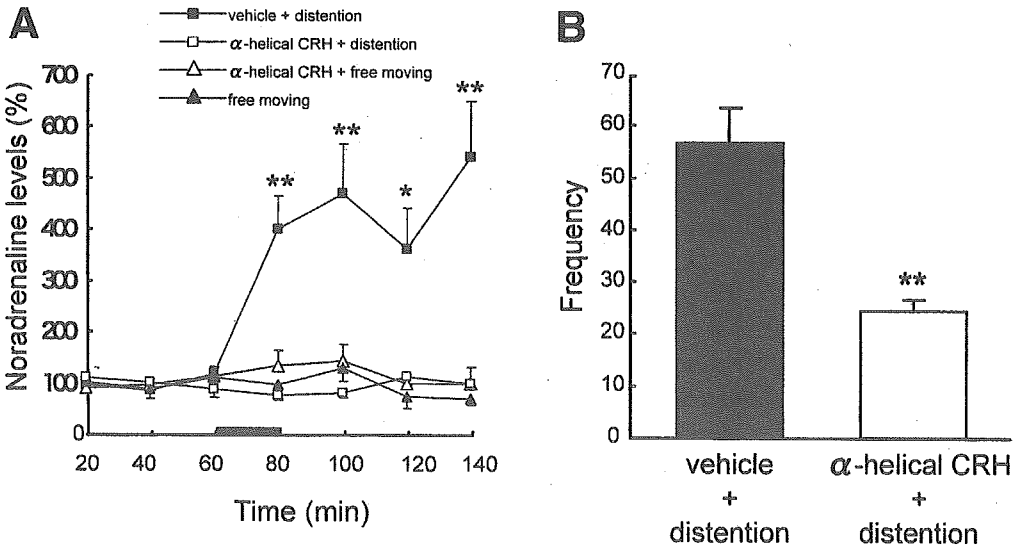

Figure 3. Effects of α-helical CRH on colorectal distention–induced hippocampal noradrenaline release and frequency of abdominal contractions. (*A*) Colorectal distention–induced hippocampal noradrenaline release and (*B*) abdominal contractions. Data are expressed as the mean ± SE (n = 6). $*P < .05$; $**P < .01$ (vehicle + distention vs α-helical CRH + distention, α-helical CRH + free moving, or vehicle + free moving).

Effects of JTC-017 on Colorectal Distention–Induced Hippocampal Noradrenaline Release and Abdominal Contractions

Analysis of variance on hippocampal noradrenaline revealed significant main effects (drug conditions: F = 12.0, $P < .01$; period: F = 10.2, $P < .001$) and significant interaction (F = 9.5; $P < .001$). Basal noradrenaline levels did not significantly differ between the rats treated with JTC-017 followed by colorectal distention (100% ± 12%) and those treated with vehicle followed by colorectal distention (110% ± 10%). However, at the end of colorectal distention, noradrenaline levels in the rats treated with JTC-017 followed by colorectal distention (130% ± 17%) were significantly lower than those in the rats treated with vehicle followed by colorectal distention (407% ± 112%; $P < .05$; Figure 4*A*). In addition, at the end of the recovery period, noradrenaline levels in the rats treated with JTC-017 followed by colorectal distention were significantly lower than those in the rats treated with vehicle followed by colorectal distention (106% ± 20% vs 474% ± 113%; $P < .05$). However, abdominal contractions in the rats treated with JTC-017 followed by colorectal distention were significantly less frequent than those in the rats treated with vehicle followed by colorectal distention (29 ± 10 vs 54 ± 15; $P < .05$; Figure 4*B*).

Effects of JTC-017 on Acute and Chronic Colorectal Distention–Induced Adrenocorticotropic Hormone Release

Plasma ACTH levels in the rats treated with vehicle followed by a 10-minute colorectal distention were significantly higher than those in the control rats

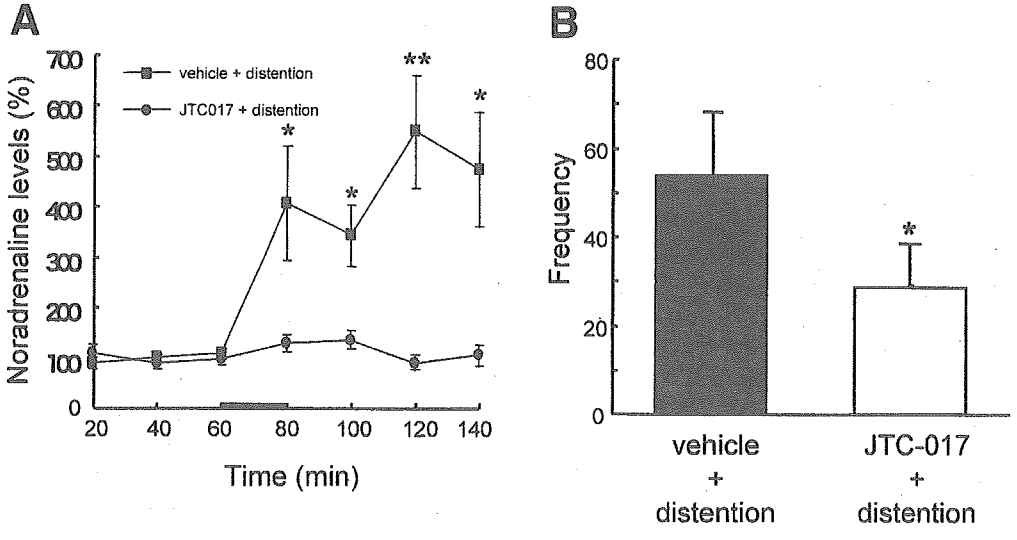

Figure 4. Effect of JTC-017 on colorectal distention–induced hippocampal noradrenaline release and frequency of abdominal contractions. (*A*) Colorectal distention–induced hippocampal noradrenaline release and (*B*) abdominal contractions. Data are expressed as the mean ± SE (5 rats per group). $*P < .05$; $**P < .01$ (vehicle + distention vs JTC-017 + distention).

Table 1. Effects of Specific CRHR1 Antagonist on Acute or Chronic Colorectal Distention–Induced Plasma ACTH

	Plasma ACTH (pg/mL)		
	Acute		Chronic
	10 min distention	20 min distention + 30 min recovery	20 min distention + 30 min recovery
control	38.7 ± 12.5	48.8 ± 14.7	39.4 ± 13.9
vehicle + distention	427.4 ± 40.7[ab]	182.7 ± 34.2[b]	57.0 ± 9.9[e]
JTC017 + distention	312.5 ± 33.3[b]	140.3 ± 17.6[c]	47.8 ± 13.2[d]

NOTE Data are expressed as the mean ± SE (n = 5–8 rats/group).
[a]$P < .05$ (vs JTC017 + distention).
[b]$P < .01$ (vs control).
[c]$P < .05$ (vs control).
[d]$P < .01$ (vs acute 20 min distention + 30 min recovery).
[e]$P < .05$ (vs acute 20 min distention + 30 min recovery).

without colorectal distention ($P < .01$; Table 1). In addition, plasma ACTH levels in the rats treated with JTC-017 followed by a 10-minute colorectal distention were significantly higher than those in the control rats ($P < .01$) but markedly lower than those in the rats treated with vehicle followed by a 10-minute colorectal distention ($P < .05$). In the rats that underwent acute colorectal distention for 20 minutes and their controls, plasma ACTH levels varied in a manner similar to that in the rats that underwent 10-minute colorectal distention.

Conversely, plasma ACTH levels in the rats treated with JTC-017 followed by chronic distention 20 min/day for 7 days or in those treated with vehicle followed by chronic distention 20 min/day for 7 days did not differ from those in the control rats (Table 1). A comparison between the acute and chronic effects of JTC-017 on acute colorectal distention–induced ACTH release showed that plasma ACTH levels in the rats treated with vehicle followed by chronic distention 20 min/day for 7 days were significantly lower than those in the rats treated with vehicle followed by acute 20-minute colorectal distention ($P < .05$). In addition, plasma ACTH levels in the rats treated with JTC-017 followed by chronic distention 20 min/day for 7 days were significantly lower than those in the rats treated with JTC-017 followed by acute 20-minute colorectal distention ($P < .01$; Table 1).

Effects of JTC-017 on Acute and Chronic Colorectal Distention–Induced Anxiety-Related Behavior and Stress-Induced Changes in Colonic Motility

During EPM experiments, the percentage of time spent in EPM open arms in the rats treated with vehicle followed by 20-minute acute colorectal distention (11% ± 2%) was significantly lower than that in the control rats (24% ± 3%; $P < .05$; Figure 5*A*). However, the percentage of time spent in EPM open arms in the rats treated with JTC-017 followed by 20-minute acute colorectal distention (27% ± 6%) was significantly higher than that in the rats treated with vehicle followed by 20-minute acute colorectal distention ($P < .05$) but was not markedly different from that in the control rats. Conversely, the number of fecal pellets, which reflects changes in colonic motility, during EPM did not significantly differ among the 3 groups of rats (Figure 5*B*).

During chronic distention experiments, the percentage of time spent in EPM open arms did not significantly differ among the control rats (25% ± 4%), the rats treated with vehicle followed by chronic colorectal distention (16% ± 8%), and rats treated with JTC-017 followed by chronic colorectal distention (20% ± 9%; Figure 5*C*). In contrast, the number of fecal pellets in the rats treated with vehicle followed by chronic colorectal distention increased significantly as compared with the control rats ($P < .05$; Figure 5*D*). In addition, the number of fecal pellets in the rats treated with JTC-017 followed by chronic colorectal distention was significantly lower than that in the rats treated with vehicle followed by chronic colorectal distention ($P < .05$) but was not significantly different from that in the control rats (Figure 5*D*).

Discussion

This is the first study that shows the effects of acute and chronic colorectal distention on ACTH release, anxiety-related behavior, and colonic motility. Our results indicate that both nonspecific and specific CRHR1 antagonists attenuate colorectal distention–induced hippocampal noradrenaline release and abdominal contrac-

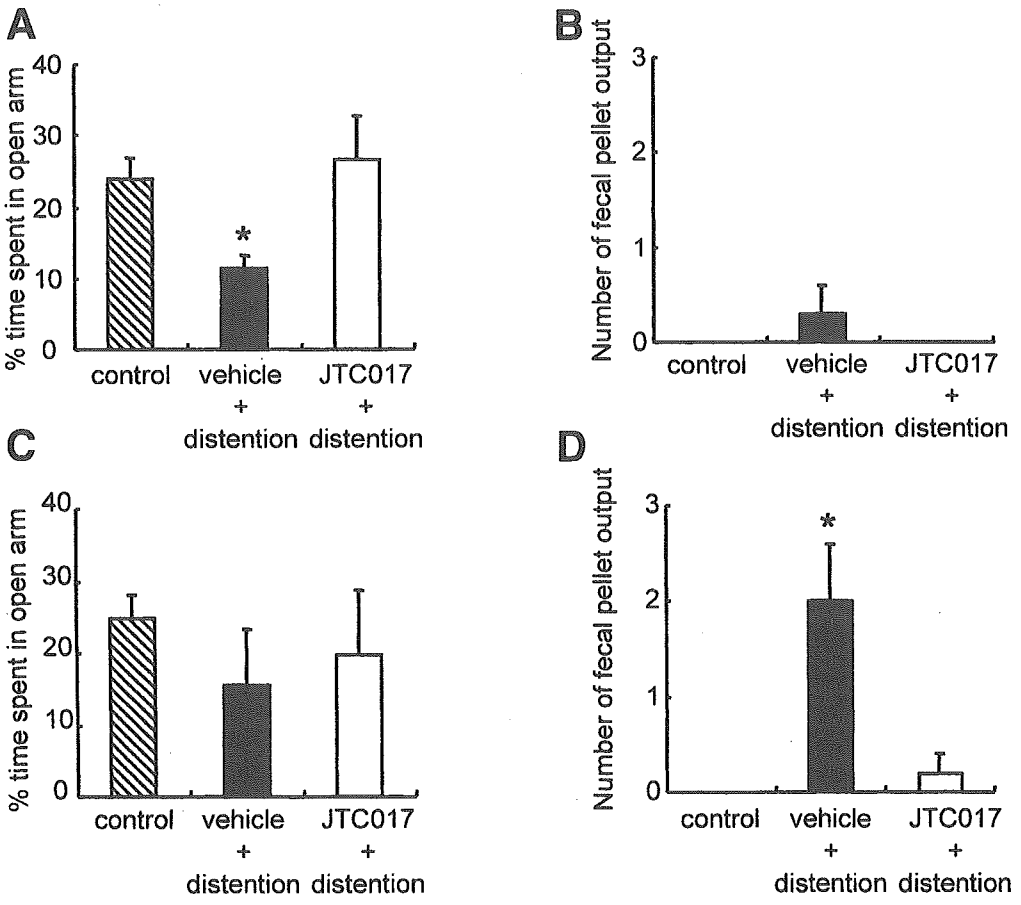

Figure 5. Effect of JTC-017 on acute or chronic colorectal distention–induced anxiety-related behavior and stress-induced changes in colonic motility. (*A*) Acute colorectal distention–induced percentage of time spent in the open arms of the EPM and (*B*) fecal pellet output. (*C*) Chronic colorectal distention–induced percentage of time spent in the open arms of the EPM and (*D*) fecal pellet output. Data are expressed as the mean ± SE (5–6 rats per group). $*P < .05$ (vehicle + distention vs JTC-017 + distention and control).

tions. In addition, treatment with JTC-017, a specific CRHR1 antagonist, attenuates acute colorectal distention–induced hippocampal noradrenaline release, somatic motor responses to visceral distention, ACTH release, and anxiety in rats. Moreover, JTC-017 blocks stress-induced colonic motility increases after chronic colorectal distention.

CRH secretion by PVN neurons has been shown to be under positive-feedback regulation by central noradrenaline pathways (including those originating from LC), thereby forming a bidirectional positive-feedback loop between the CRH and noradrenaline systems.[33] Moreover, microinfusion of CRH into the LC is known to induce long-lasting stimulation of colonic transit and bowel discharge.[34] However, microinfusion of CRH into the PVN increases both phasic and tonic motor activity in the proximal colon.[35] Gastrointestinal stimulation has been shown to increase the firing rate of LC,[36] noradrenaline release in the hippocampus,[23] and c-Fos expression in the LC and PVN.[20] One previous report indicated that CRHR1 immunoreactivity was detected at the LC.[37] In addition, the administration of CRHR1 antagonists has been shown to block the stimulatory effects of exogenous corticotropin-releasing factor on LC neuronal firing and the startle response amplitude.[38,39] This study indicates that colorectal distention–induced hippocampal noradrenaline release and abdominal contractions are reduced by a CRHR1 antagonist. These findings provide direct evidence that CRHR1 and noradrenaline participate in the gut-to-brain pathway.

Previous studies have indicated that even unilateral microinjection of CRH into the LC/subceruleus nucleus stimulates the propulsive motor functions of the lower intestinal tract in rats.[34] In this study, α-helical CRH was injected into the hippocampus via a microdialysis probe with minimum distress to rats according to a previously reported method.[40] From our results, it is plausible to assume that intrahippocampally injected α-helical CRH inhibits the firing of nerve terminals of noradrenaline neurons. Moreover, it has been reported that a unilateral increase in γ-aminobutyric acid in the cerebral cortex results in a clear and consistent bilateral analgesia.[41] Therefore, one possibility for the analgesic effect of α-helical CRH injected unilaterally into the hippocampus is increased γ-aminobutyric acid in the brain.

The results of this study provide evidence that CRHR1 and noradrenaline participate in activation of

the hypothalamo-pituitary-adrenal axis and anxiety-induced colonic stimulation and that specific CRHR1 antagonists, including JTC-017, attenuate acute colorectal distention–induced hippocampal noradrenaline release, plasma ACTH release, and anxiety-related behavior. It has been shown that CRHR1 antagonists decrease stress-induced ACTH release and anxiety-related behavior.[6,8] In addition, several reports have indicated that CRHRs located in, or close to, the LC are involved in the response to stress-induced stimulation.[42] Moreover, acute immobilization stress causes strong and transient expression of messenger RNA encoding the CRHR1 in the rat PVN.[43] Although previous studies have indicated that under acute stress, the LC activates both the hypothalamo-pituitary-adrenal axis and the behavioral response to stress via a multisynaptic mechanism involving noradrenergic activation of PVN-excitatory neural pathways,[44] the role of CRHR1 or noradrenaline in this mechanism is not clear.

Another important result of this study is that chronic colorectal distention slightly decreased ACTH release but had no remarkable effect on anxiety-related behavior and that treatment with the specific CRHR1 antagonist JTC-017 did not influence these responses. Previous studies have suggested that chronic stress induces habituation to stress-induced ACTH release and anxiety-related behavior.[45–47] This habituation was also shown to occur after exposure to various chronic intermittent stressors, such as handling,[45] novelty,[46] and restraint.[47] Because habituation of catecholaminergic-containing neurons of the brain stem may participate in the attenuation of neuroendocrine CRH neuron activity, the CRHR1 receptor may thus be down-regulated in the rat PVN after repeated exposure to the same neurogenic stressor.

As for changes in colonic motility, the number of fecal pellets, which represents a simple marker of stress-induced changes in colonic motility, increased by chronic colorectal distention–induced stress in rats. However, treatment with JTC-017 blocked this increase. An earlier study showed that the fecal pellet output decreases in chronic immobilization–induced stress in rats and that this phenomenon parallels the down-regulation of CRHR1 expression within CRH-containing cells of the PVN.[43] In this study, despite the possibility of habituation of the PVN-LC complex (which is suggested by decreased ACTH release and anxiety), the number of fecal pellets increased by stress after chronic colorectal distention. These findings may indicate that stress-induced changes in colonic motility are specifically sensitized and conditioned by repetitive colorectal distention at the central and/or peripheral levels.

Previous reports have indicated that acute noxious colorectal distention induces abdominal contractions, microscopic inflammation in the colon, and c-Fos expression in the central nervous system.[19–21,48] These findings indicate that acute gastrointestinal stimulation may lead to peripheral and central sensitization. Besides, chronic colon irritation in neonates results in chronic visceral hypersensitivity.[25] To clarify the effects of chronic colorectal stimulation on peripheral and central sensitization, we would like to examine colonic mucosa and sensitized colonic afferents in the future.

The results of CRHR1 binding assays and cell biology assays indicated that JTC-017 affinity for the CRHR1 was approximately 10-fold lower than that of α-helical CRH. However, the dose of JTC-017 used in this study (10 mg/kg) was much higher than that of α-helical CRH and may have compensated for the lower affinity for the CRHR1. Preliminary pharmacokinetic experiments in mice have indicated that peripherally administered JTC-017 (10 mg/kg) crosses the blood–brain barrier with a plasma and brain concentration 15 minutes after injection of 7.84 and 1.18 μg/mL, respectively (unpublished data). Previous studies have also indicated that intraperitoneal administration of JTC-017 (10 mg/kg) decreases stress-induced anorexia and foot shock–induced conditioned fear and that peripherally administered JTC-017 reasonably blocks peripheral CRHR1 (unpublished data). Therefore, intraperitoneally administered JTC-017 blocks the CRHR1 receptor both in the central nervous system and in peripheral organs. Because in this study, intrahippocampal administration of α-helical CRH and intraperitoneal administration of JTC-017 produced very similar results, it is suggested that CRHR1 in the brain plays a major role in colorectal distention–induced behavior. Finally, because clinically CRHR1 antagonists have to be administered peripherally, our selection of a peripheral route of administration for JTC-017 has clear clinical relevance.

In conclusion, our results suggest that JTC-017, a specific CRHR1 antagonist, attenuates colorectal distention–induced hippocampal noradrenaline release, somatic motor responses to visceral distention, ACTH release, and anxiety-related behavior after acute colorectal distention in rats. In addition, JTC-017 blocks stress-induced changes in colonic motility after chronic colorectal distention in rats. Controlling noradrenaline levels and CRH in the brain may, at least in part, contribute to the treatment of IBS patients who have anxiety disorders and visceral hypersensitivity.

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Temporal variation of acute stress responses in sympathetic nervous and immune systems

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Abstract

Sympathetic nervous activity plays a prominent role in acute stress responses in the immune system, enhancement of innate immunity and suppression of specific immunity. The present study was conducted to examine the temporal characteristics of such immune responses to acute stress and to determine their association with sympathetic activity in detail. For this purpose, 15 female undergraduates engaged in a continuous mental arithmetic task for 14 min, and we collected their blood samples for immune indices (CD3+ T cells, CD4+ T cells, NK cells) each 3 min during the task and saliva samples before and after the task. Our results showed that the proportion of Natural Killer cells (NK cells) increased even 2 min after initiation of the task, whereas proportions of CD3+ and CD4+ lymphocytes decreased 8 min after initiation of the task. Moreover, we found significant correlations between cardiovascular activity and the variations of immune indices.

Keywords: Stress; Sympathetic nervous; Mental arithmetic; Psychoneuroimmunology

1. *Introduction*

A variety of experimental protocols for the induction of psychological acute stress in laboratory settings (Bachen et al., 1992; Herbert et al., 1994; Breznitz et al., 1998; Gerra et al., 2001; Atanackovic et al., 2002; Scarpa and Luscher, 2002; Willemsen et al., 2002; Kunz-Ebrecht et al., 2003; Lutgendorf et al., 2004) have indicated that the function of innate immunity represented by Natural Killer cell (NK cell) number was enhanced while the function of specific immunity including B cells and CD4+ helper T cells was suppressed in acute stress situations, especially in active coping situations (Delahanty et al., 1996; Mills et al., 1996; Pike et al., 1997; Burleson et al., 1998; Marsland et al., 2001; Willemsen et al., 2002; Isowa et al., 2004). Furthermore, the volume of secretory immunogloulin A (s-IgA), which is a dominant antibody in mucosal immunity, increases in such acute stress situations (Willemsen et al., 1998; Ring et al., 1999, 2000; Bosch et al., 2001; Winzer et al., 1999).

An important cause of immune modulation by stress is the activity of the hypothalamus–pituitary–adrenal cortex (HPA) axis, which secretes cortisol from the adrenal cortex, resulting in immunosuppression (Rupprecht et al., 1990; Garvy and Fraker, 1991; Northrop et al., 1992; Slade and Hepburn, 1983). However, at least in acute stress situations, immunomodulation should be affected more by autonomic nervous system activity than by HPA axis activity because secretion of cortisol begins about 15 min after the initiation of stressful tasks and reaches a peak about 20 min later (Kuhn, 1989). In spite of the delay in the rise of cortisol secretion, even brief stress tasks for 6 or 4 min influenced the immune cells in peripheral blood (Willemsen et al., 2002). Considering those findings, the autonomic nervous system should contribute to stress responses of immune cells at least in acute stress situations. The fact that adrenalin and noradrenalin secreted during excitation of the sympathetic nervous system can influence the surface receptors of immune cells in blood supports this idea (Benschop et al., 1994, 1996). However, little is known about the characteristics of temporal changes in immune functions during acute stress.

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In previous studies, variations in immune indices were typically evaluated before and after tasks, and the duration of tasks varied among studies. Tasks used for this purpose included performing mental arithmetic for 8 min, experiencing 4 min of a cold pressor (immersion of the forearm in cold water) and performing public speaking for 6 min (Farag et al., 2002; Willemsen et al., 2002). The fact that activation of cardiovascular activities can occur within a second of exposure to stress tasks (Kuhn, 1989) suggests that variation in immune functions can be evoked at the early stage of such stress situations. Unfortunately, the simple pre-post design adopted in previous studies prevented study authors from making a detailed examination of temporal variations in immune functions during acute stress. The aim of the present study, therefore, was to examine at which stage the indices of the immune system change during an acute stress situation and to determine the influence of sympathetic nervous activity in these changes. For this purpose, we also focused on a peripheral manifestation of sympathetic nervous system activity as revealed by heart rate variability (HRV) because it is necessary to confirm whether our stress task admittedly elicits the activation of sympathetic nervous system. HRV is determined by the balance of parasympathetic and sympathetic nervous system activity regulating the heart, and the ratio of the spectrally derived low-frequency to high-frequency band power (LF/HF ratio) is considered to be a reliable index of sympathetic activation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).

In the present study, therefore, we measured proportions of subsets of blood lymphocytes (CD3+ T cell, CD3+CD4+ helper T cell, CD3–CD16+CD56+ NK cell) and concentration of salivary secretory immunogloblin A as indices of natural, specific and mucosal immune functions every 3 min during a mental arithmetic task that lasted 14 min. Additionally, to examine the autonomic–endocrine–immune association, we evaluated the concentration of salivary cortisol as an endocrine index before and after the task and measured the heart rate and blood pressure and HRV as autonomic indices continuously during the task. But because our research interest was paid to the sympathetic nervous activity and immune changes to the acute stress, with consideration of a participant's burden, blood sampling at short intervals was performed only during the task and taken only for the immune indices.

2. Methods

2.1. Subjects

Fifteen female undergraduates in Mie Prefectual College of Nursing (age range 20–26 years; mean = 21.67; S.D. = 1.88) participated in the present study. The mean body-mass index of the subjects was 20.88 kg/m^2 (S.D. = 2.48). Since the stress of the cannula insertion itself was taken into consideration, only the students of nursing who are usually familiar with the cannula insertion participated. None was suffering from any chronic or oral illness, and none was taking medication known to influence immunity. In addition, no subjects were using oral contraceptives. Although there were several reports that differences in the balance of the sex hormone changing with the menstrual cycle influenced on an immune system (Pehlivanoglu et al., 2001), significant difference in immune measures about a menstrual cycle was not observed in this research (data not shown).

All subjects signed informed consents to participate in the study, which was approved by the Ethics Committee of Mie Prefectural College of Nursing.

2.2. Immunological measures

Blood samples for immunological testing were collected in heparinized tubes. The numbers of total white blood cells (WBC), lymphocytes, monocytes and granulocytes per sample were determined by standard means. Percentages of lymphocyte subsets were determined by flow cytometry (FACS Calibur, Becton-Dickinson). A whole-blood lysis method was used to stain the cells with the following pairs of Fluorescein isothiocyanate (FITC)/Phycoerythrin (PE)-conjugated monoclonal antibodies (DAKO, Inc.). The isotype-matched antibodies used were Mouse IgG1, CD3+/CD4+ indicating helper T cells and CD3−/CD16+/CD56+ indicating NK cells.

To determine the volume of secreted saliva and the concentration of s-IgA, we collected samples of unstimulated saliva using cotton swabs (Salivettes, Sarstedt Ltd.). A cotton swab was placed underneath the tongue of each participant for 3 min. After that, the cotton swab was removed and saliva was extracted from the cotton by centrifugation at 3.5×10^3 rpm for 10 min. Saliva was stored frozen in capped test tubes at −20 °C until assay. We determined the s-IgA concentration in saliva (in micrograms per milliliter) by an enzyme-linked immunoabsorbent assay (using IgA test; MBL, Inc.). The thawed saliva aliquots (10 μl) were diluted 40 times. Saliva samples were reacted with polystyrene beads that labeled the antihuman secretary component. After incubation at 37 °C for 1 h, the beads were washed twice and reacted with peroxidase standard antihuman IgA (rabbit IgG/Fab') (secondary reaction). After incubation at room temperature for 1 h, the beads were washed three times, and then enzyme metrical fluid (orthophenylenediamine + 4 mM H_2O_2) (third reaction) was added. After incubation at room temperature for 30 min, the reaction was stopped by the addition of H_2SO_4. The reaction product was quantified spectrophotometrically at 492 nm with a microplate reader (Bio-Rad, Inc., Model 550). The s-IgA secretion rate (in micrograms per minute) was calculated as the product of s-IgA concentration and saliva flow rate.

2.3. Endocrine measures

Samples of saliva for measurement of the concentration of salivary cortisol were obtained by the same method as

for s-IgA and stored at −20 °C until assay. The concentration of cortisol in the saliva (in micrograms per milliliter) was determined by an enzyme-linked immunoabsorbent assay. Ninety-six-well Costar™ microplates pre-coated with anticortisol rabbit antibody were prepared. A saliva sample of 50 μl was added to each well in triplicate, and then an enzyme conjugate of 50 μl was added to each well and samples were incubated at room temperature for 1 h. After incubation, each well was washed three times. A substrate of 50 μl was added to each well and incubated at room temperature for 30 min. After that, 1N HCL 50 μl was added to each well to stop the enzyme reaction. To measure the concentration of cortisol the plate was read at 450 nm with a microplate reader (Bio-Rad, Inc., Model 550).

2.4. Cardiovascular measures

Cardiodynamic activity was recorded using electrocardiography (ECG) and non-invasive finger blood pressure (FINAP) measurements. ECG was recorded using a MP 100 system (Biopac Systems, Inc.). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded through a finger cuff of a Portapres Model 2 (TNO Biomedical Instrumentation, Inc.) attached to the third finger of the non-dominant hand of each subject. Each indicator was recorded continuously during the task and rest periods. The ECG data were subsequently analyzed to yield HRV. The data were first subject to ocular inspection and only completely artifact-free data were used for estimation of the R–R intervals. The R–R interval data were resampled at 4 Hz to obtain equidistant time series values. A power spectrum density was then obtained through a fast Fourier transformation of the tachogram. In connection with the fast Fourier transformation, the data were detrended linearly and filtered through a rectangular window. The integral of the power spectrum was studied in two major frequency bands: the high-frequency band (0.15–0.5 Hz) and the low-frequency band (0.05–0.15 Hz). The former related to respiratory sinus arrhythmia and is exclusively attributable to parasympathetic influence reflecting vagal activity and in the latter case, it mirrors the baroreceptor feedback loop that controls blood pressure and appears to reflect both sympathetic and parasympathetic activity. Consequently, the HF component and the relative contributions of LF and HF power (LF/HF) which reflect sympathovagal balance, were considered in the present paper. Analyses of ECGs and FINAP waveforms were performed using AcqKnowledge software for the MP 100.

2.5. Psychological measures

The subjects were asked to evaluate subjectively the intensity of the stress on analog-visual scales (0–100%). In addition, they completed a Japanese version (Katsuharu and Tadanobu, 1982) of the State-Trait Anxiety Inventory (STAI) (Speilberger et al., 1970). The STAI is composed to measure state anxiety and consists of 40 items.

2.6. Stress task

The subjects performed mental arithmetic for 14 min. The subjects were told to add the currently displayed number (from 2 to 9) to the next one displayed on the PC monitor, and to answer only one digit of the current answer by pressing a key (from 0 to 9). Each number was displayed for 500 ms and followed by a 1300 ms interval of no number display. The task included 460 numbers. During the task, once participants responded by key after each set, O or x was shown on the monitor as feedback indicating whether the participant's previous answer was correct or wrong.

2.7. Procedure

The subjects had been instructed to eat a light breakfast on the morning of the experiment; caffeine-containing beverages were not allowed. Also, the subjects had been instructed to paste a monoanesthetic seal (PENLES; Wyeth Lederle, Inc.) at the location of the cannula insertion in their arms about 1 h before the experimental sessions to reduce pain. Subjects suffering from an infectious illness within 2 weeks of the experiment were rescheduled.

The experimental sessions were composed of a mental arithmetic task and two rest periods. The subjects were tested individually in a temperature- and humidity-controlled room. After each subject entered the experiment room, a cannula was inserted into the forearm vein of her non-dominant arm. Next, electrodes for electrocardiographic measurements and a finger cuff for blood pressure recording were attached. For the next 15 min, the subjects filled in psychological questionnaires. Then, instruction was given for the mental arithmetic task and the subjects were allowed to practice the mental arithmetic task for 1 min. During the instruction period, the subjects were told that four blood samples would be taken during the arithmetic task but they were to perform the task continuously and not stop concentrating on it.

After a rest period of 10 min, the first blood sample (for assays of immunological parameters) and the first saliva sample (for assays of s-IgA and cortisol) were taken as a baseline sample, and the subjects were asked to fill in the questionnaire. After that the subjects performed the mental arithmetic task for 14 min. While subjects performed the task, the four blood samples were taken at 2, 5, 8 and 11 min.

Immediately after the task, the sixth blood and the second saliva sample were taken and each subject filled in the questionnaire again. After a second rest period of 15 min, the seventh blood and the third saliva sample were taken and the questionnaires were filled in again. Autonomic indices (ECG and BP) were measured continuously throughout the experimental session. After the end of the procedure, the electrodes, blood pressure cuff and cannula were removed,

and the subjects were fully debriefed and thanked. Each subject was paid 800 Japanese yen for their participation.

2.8. Statistical analysis

Prior to statistical analysis, mean values of HR, SBP and DBP data were calculated for the last 2 min of the pre-experimental baseline period, periods during the stress task (1–2, 4–5, 7–8, 10–11 and 13–14 min), and the last 2 min of each rest period after the task. The cardiovascular data were analyzed using repeated-measures analyses of variance (ANOVAs) with a within-subjects factor of periods (baseline, $stress_{2\ min}$, $stress_{5\ min}$, $stress_{8\ min}$, $stress_{11\ min}$, $stress_{14\ min}$, rest). Immune data excluding s-IgA were analyzed using repeated-measures ANOVAs with a within-subjects factor of period (baseline, $stress_{2\ min}$, $stress_{5\ min}$, $stress_{8\ min}$, $stress_{11\ min}$, $stress_{14\ min}$ rest). Cortisol, s-IgA, HF component of HRV, LF/HF ratio and psychological data were analyzed using repeated-measures ANOVAs with a within-subjects factor of period (baseline, stress, rest). The Greenhouse–Geisser epsilon correction factor, ε (Jennings and Wood, 1976), was used where appropriate. In cases where significant main effects were found in the ANOVAs, post hoc analyses using LSD tests ($p < .05$) were conducted to examine which combinations of data points differed significantly. Pearson correlation coefficients were computed among changed scores (scores at the stress period–scores at the baseline) of these indexes to examine the relationship between immune, cardiovascular and endocrine reactivity.

3. Results

3.1. Immunological measures

The data including WBCs, lymphocytes, monocytes and granulocytes at the baseline, stress (2, 5, 8, 11, 14 min) and rest periods are summarized in Table 1. Fig. 1 illustrates changes in the percentages of CD3+ T cells, CD4+ T cells and NK cells.

The main effects of period, reflecting temporal variation of indices during the acute stress task, were significant for granulocytes, $F(6, 84) = 3.61, p < .01$; CD3+ T cells, $F(2.63, 36.81) = 4.97, p < .01$; CD4+ T cells, $F(3.06, 42.83) = 5.12, p < .01$; NK cells, $F(2.59, 36.28) = 8.97, p < .01$. Post hoc comparisons indicated that CD3+ T cells and CD4+ T cells decreased, and granulocytes and NK cells increased during the acute stress task. Furthermore, CD3+ T cells decreased significantly at 5 min and CD4+ T cells showed significant decreases at 8 min after the start of the task and remained at a level lower than that at baseline during the task. The increments of granulocytes and NK cells were found to be significant at 2 min from the start of the task and were maintained throughout the acute stress task. However, there were no significant main effects between baseline and rest in NK cells. Therefore, it can be deduced that, as predicted, the acute stress task influenced immune indices, which returned to normal a short time after the task. Meanwhile, ANOVAs yielded no significant main effects of task for WBCs, $F(1.75, 24.56) = 2.53$, ns, lymphocytes, $F(3.46, 48.48) = 1.53$, ns or monocytes, $F(3.14, 43.99) = 1.41$, ns.

3.2. Salivary measures

The changes in salivary flow, s-IgA concentrations and concentrations of cortisol at three points (baseline, stress, rest) are shown in Table 2, which also shows that an ANOVA showed tendency of main effects of period about s-IgA $F(2, 28) = 2.74$, $p < .10$. This indicates that s-IgA tended to increase in response to an acute stress task. There were no significant main effects of period in connection with the other two indices.

3.3. Cardiovascular measures

Changes in cardiovascular indices are illustrated in Fig. 2. ANOVAs yielded a significant main effect of period for HR, $F(3.83, 53.60) = 18.05$, $p < .01$; SBP, $F(2.07, 28.92) = 14.19, p < .01$; DBP, $F(2.55, 35.62) = 25.67, p < .01$. These results mean that all of these cardiovascular indices showed higher levels during execution of the stress task than at baseline or during the rest periods. Moreover, there were significantly maintained increments of these indices during all the time of the task. While the increments of HR and DBP returned to the baseline level after the acute stress task, SBP did not return and remained higher than baseline. On the other hand, the HF component of HRV was significantly reduced during task period compared with the baseline and rest periods $F(2, 28) = 13.47, p < .01$. And the LF/HF ratio showed significant increase in the task period, $F(1.43, 20.01) = 6.50$, $p < .05$, but no difference was observed between baseline and rest periods. The decrease in HF

Table 1
Means (S.D.s) of immunological measures and results of ANOVAs

	Baseline	Task (2 min)	Task (5 min)	Task (8 min)	Task (11 min)	Task (14 min)	Rest	Main effect
WBC ($\times 10^3$, μl)	52.07 (17.04)	54.60 (17.82)	54.67 (17.78)	54.13 (18.30)	54.53 (18.00)	54.80 (18.04)	54.00 (16.00)	ns
Lymphocyte ($\times 10^3$, μl)	15.62 (4.87)	15.40 (5.26)	15.47 (5.65)	15.87 (5.45)	16.02 (5.77)	15.84 (4.98)	15.84 (4.98)	ns
Monocyte ($\times 10^3$, μl)	3.20 (1.35)	3.48 (1.52)	3.55 (1.50)	3.53 (1.49)	3.49 (1.40)	3.37 (1.33)	3.45 (1.20)	ns
Granulocyte ($\times 10^3$, μl)	32.04 (12.98)	34.40 (13.66)[a]	34.37 (13.01)[a]	33.57 (14.03)[a]	33.78 (13.71)[a]	34.40 (14.26)[a]	34.64 (13.11)[a]	Period**

Main effects as results of ANOVAs.
[a] Significant different from baseline at this time point.
** $p < .01$

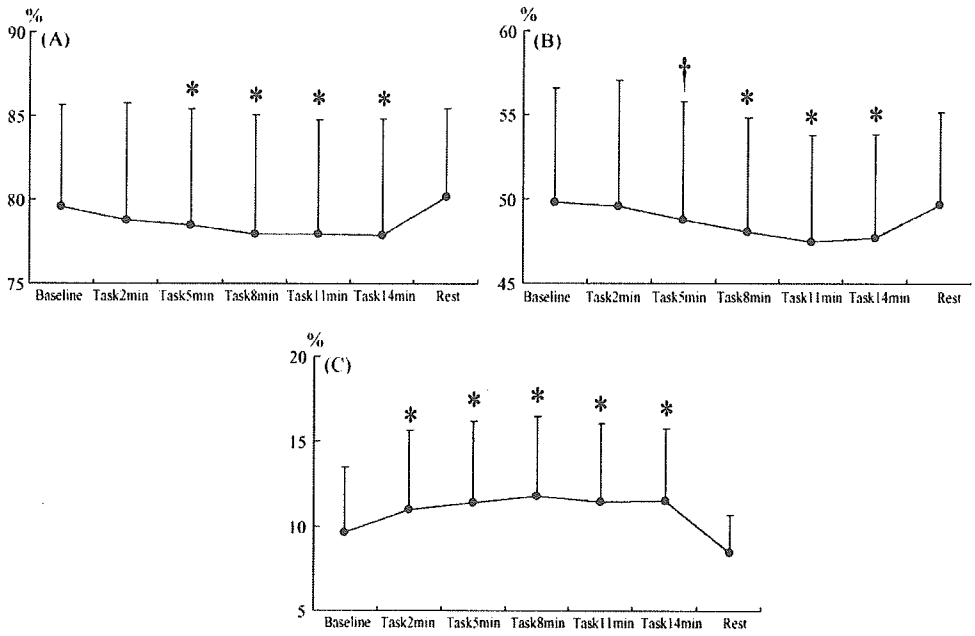

Fig. 1. Changes in percentages of CD3+ T cells (A), CD4+ T cells (B) and CD16+CD56+ NK cells (C) at each measurement point. Error bars indicate standard deviations. Symbol (*) denotes a significant ($p < .01$) difference of each index in comparison to baseline. Symbol (†) denotes a tendency toward a significant difference ($p < .1$) of each index in comparison to the baseline.

Table 2
Means (S.D.s) of salivary measures and results of ANOVAs

	Baseline	Task (14 min)	Rest	Main effect
Saliva flow rate (μl/3 min)	709.93 (713.16)	871.00 (823.59)	756.13 (776.02)	ns
s-IgA concentration (μg/ml)	61.58 (13.31)	67.58 (21.16)	58.12 (11.41)	†
Cortisol (ng/ml)	9.83 (6.69)	9.83 (5.76)	10.70 (6.44)	ns

Main effects as results of ANOVAs.
† $p < .1$.

component and the increase in LF/HF ratio could suggest that our mental arithmetic task induced the dominance state of sympathetic nervous system. The data derived from HRV are presented in Table 3.

3.4. Psychological measures

The psychological data at the baseline, stress and rest periods are presented in Table 4. In the table, perception of stress is shown on a visual analog scale. From the results of ANOVAs, significant differences were observed in perception of stress $F(1.72, 24.08) = 8.77$, $p < .01$ and state of anxiety $F(1.50, 21.01) = 7.16$, $p < .01$. Post hoc comparisons revealed that perception of stress and state of anxiety were higher in the stress period than baseline and rest periods. These results could show the task used in this study functioned as acute stress task for participants.

Table 3
Means (S.D.s) of HF component of HRV, LF/HF ratio and results of ANOVAs

	Baseline	Stress	Rest	Main effect
HF (%)	53.74 (23.39)	37.43 (20.53)	53.46 (19.90)	Period**
LF/HF ratio	1.37 (1.38)	2.76 (2.67)	1.32 (1.52)	Period*

Main effects as results of ANOVAs.
* $p < .05$.
** $p < .01$.

3.5. Associations between immune and cardiovascular measures

Correlations between changes in immune and cardiovascular parameters were computed for the whole sample (see Table 5). The results indicated that, for the mental arithmetic task, systolic and diastolic blood pressure in measures of

Table 4
Means (S.D.s) of psychological measures and results of ANOVAs

	Baseline	Stress	Rest	Main effect
Perception of stress	29.80 (20.02)	43.07 (13.77)	25.13 (13.96)	Period**
STAI State anxiety	42.00 (6.67)	44.70 (5.80)	38.93 (4.56)	Period**

Main effects as results of ANOVAs.
** $p < .01$.

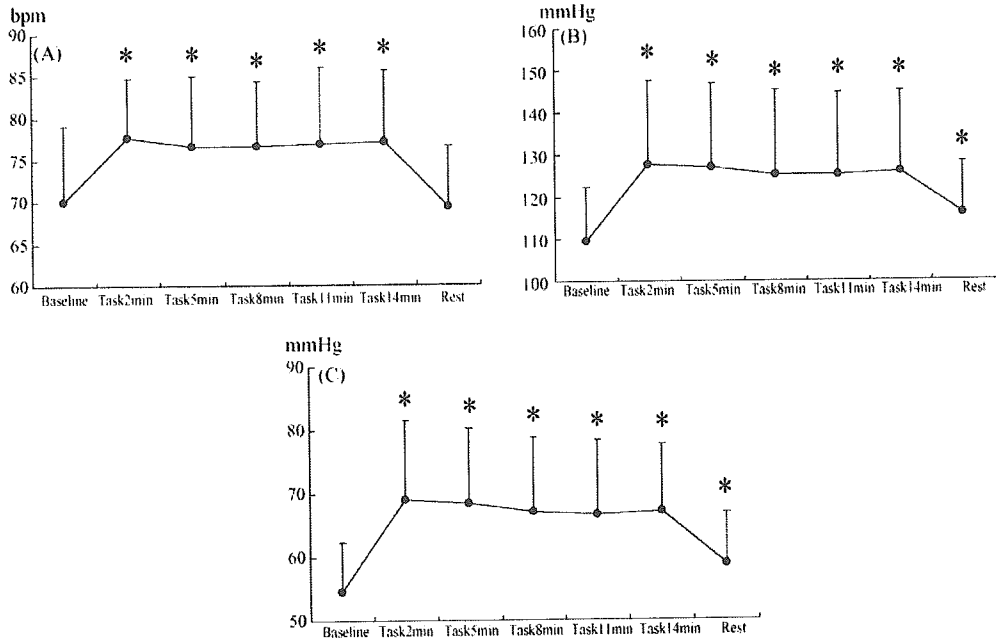

Fig. 2. Changes of HR (A), SBP (B) and DBP (C) at seven measurement points. Error bars indicate standard deviations. Symbol (*) denotes a significant ($p < .01$) difference of each index in comparison to the baseline.

Table 5
Correlations between changes in immune and cardiovascular measures

	HR	SBP	DBP	HF	LF/HF ratio
WBC	.588**				
Lymphocyte					
Monocyte					
Granulocyte	.728**				
CD3+		−.865**	−.739**		
CD4+		−.622*			−.535*
NK cell		.861**	.732**		
s-IgA					
Cortisol					

* $p < .05$.
** $p < .01$.

cardiovascular activity correlated negatively with change in the percentage of CD3+ T cells and CD4+ T cells but positively with change in the percentage of NK cells. And LF/HF ratio in HRV data showed significant negative correlation only with CD4+ T cells. Interestingly, NK cells and CD3+ T cells correlated with the cardiovascular measurements immediately after the task was started. On the other hand, the significant correlations in CD4+ T cells were observed at 8 min after the beginning of the task (data not shown). Additionally, significant positive correlation between heart rate and granulocytes or WBCs was also indicated.

4. Discussion

The present study replicated and confirmed previous findings of an increased proportion of NK cells and a reduction of that of CD3+CD4+ helper T cells, suggesting enhancement of innate immunity and suppression of specific immunity in an acute stress challenge (Delahanty et al., 1996; Pike et al., 1997; Burleson et al., 1998; Willemsen et al., 2002; Isowa et al., 2004). The main contribution of the present study is the finding, for the first time, that such typical immune responses to acute stress can happen within 2 min from initiation of the task, especially concerning NK cells. This finding suggests that variations of immune functions can happen in a very early stage of acute stress situations.

Another important point in the present study is our finding of a time lag in reactivity to acute stress of NK cells, CD3+ T cells and CD4+ T cells. We observed a significant increase of NK cells immediately after initiation of the task (2 min), whereas reductions of CD3+ T cells and CD3+CD4+ helper T cells reached significant levels 5 or 8 min after initiation of the task. In previous studies (Peters et al., 1999; Pehlivanoglu et al., 2001), changes of immune functions have been evaluated by indices of proportions of subsets of lymphocytes. Because such proportion measures are substantially relative, it is not clear whether increases of NK cells and decreases of T cells (especially helper T cells) are independent processes. By examining temporal variations of both immune measures, we were able in the present study to offer evidence that enhancement of innate immunity and suppression of specific immunity in acute stress should be dissociable processes. Those present findings are consistent with a previous finding that absolute numbers, not relative proportions, of NK cells and T cells were independently influenced by acute stress (Schmid-Ott et al., 2001; Adler et al., 2002; Mills et al., 2003; Steptoe et al., 2004).

Observed highly significant correlations between cardiovascular and immune indices and lack of a significant change of salivary cortisol suggest that sympathetic activity rather than the HPA activity mediates the temporal variations in immune functions during acute stress. The increments of heart rate and systolic and diastolic blood pressure observed in the present study are part of a typical pattern of cardiovascular activities. This type of response has been observed in previous studies using acute stress tasks including mental arithmetic tasks (Willemsen et al., 1998, 2002; Ring et al., 2000; Isowa et al., 2004), public speaking tasks and Stroop tasks (Owen and Steptoe, 2003; Redwine et al., 2003). The common pattern of this response in these studies is characterized by dominant sympathetic nervous system, especially beta-adrenergic activity compared to alpha-adrenergic activity (Ring et al., 2000; Isowa et al., 2004). Our HRV data might confirm evidently that the task used in this study has elicited the pre-dominance state of sympathetic nervous system. During this kind of stress response, adrenaline or noradrenalin is secreted from sympathetic glands and activates NK cells located on the vascular endothelium via beta-adrenergic receptors on their surface, resulting in an increase in the number of NK cells in peripheral blood (Benschop et al., 1994, 1996). Elevated blood pressure and increased bloodstream elicited by sympathetic activity might drop NK cells into the peripheral blood physically (Benschop et al., 1993). Since the increment of blood pressure influences immune cells directly from this kind of mechanism, it might be considered that the correlations with immunological measures were more strongly observed in blood pressure than in HRV. Moreover, the observed result showed that the increment of granulocytes and significant correlation with heart rate was also related to beta-adrenergic activity. Granulocytes, especially neutrophils, have a number of receptor types expressed on their surface, in particular beta-adrenergic ones (Dhabhar et al., 1996). The present result is consistent with a previous finding that activation of beta-adrenergic receptors induces a functional rise of neutrophils (Ellard et al., 2001). Thus, the observed correlation between WBC and heart rate could be also interpreted by the increment of neutrophils, which is a type of granulocyte via beta-adrenergic receptor activation. But this speculation needs more empirical research.

The dissociation of temporal activity between NK cells and T cells reported above can also be interpreted as resulting from a difference of their sensitivity to noradrenalin and adrenalin. Both NK cells and CD3+ T cells and CD3+CD4+ helper T cells express beta2-adrenergic receptors on their surface and therefore can be influenced by noradrenalin and adrenalin. However, NK cells express more beta2-adrenergic receptors than do CD3+CD4+ helper T cells (Landmann et al., 1984; Van Tits et al., 1990; Wahle et al., 2001). Since there are relatively few distributions of beta-adrenergic receptors in T cells, their sensitivity to adrenaline and noradrenaline might be lower than that of NK cells. Therefore, the influence of these substances could not take place easily in T cells (Schedlowski et al., 1996; Kohm and Sanders, 2001). That might be a reason why the reduction of proportions of CD3+ T cells and CD4+ helper T cells appeared in the later stage of the acute stress task. However, these speculations need further examination.

Several limitations in this study must be recognized. First, the number of participants ($N = 15$) was relatively small. As indicated in the results, however, the subjects experienced robust temporal variations in immune parameters and correlations between autonomic and immune indices. A second limitation was that we examined only female subjects, whereas the previous study (Willemsen et al., 2002) reported sex differences in some immune responses to acute stress. Thus, the generalizability of the present findings must be further tested using a larger sample composed of both sexes. A third limitation was that, contrary to previous studies (Willemsen et al., 2000; Bosch et al., 2001), the concentration of salivary s-IgA did not increase significantly after the acute stress task, although we observed a tendency for the concentration to increase. This unexpected result might has been caused by the small number of participants in our study. On the other hand, although we found strong association between immune and sympathetic nervous system, this study was a correlational design. We speculated the mechanism indirectly from cardiovascular responses but did not measure catecholamine itself. Further study measuring catecholamine or using adrenergic receptor blocker is needed for more precise inspection. Moreover, blood sampling at short intervals were performed only in the task period. There is, however, not only an acute stress response but researches which suggest the importance of the recovery period (Steptoe et al., 2003; Gold et al., 2004). It is thought that performing precise evaluation in recovery period is necessary for understanding the mechanism of an immune system and other systems. Further studies to overcome these limitations are awaited.

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