Effects of repeated restraint stress on gastric motility in rats

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Short title: Chronic stress and gastric motility

Abbreviations: corticotropin releasing factor (CRF), functional dyspepsia (FD), growth hormone secretagogue receptor (GHS-R), hypothalamus-pituitary-adrenal (HPA) axis, motility index (MI), migrating motor complex (MMC), paraventricular nucleus (PVN)

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Abstract

In our daily life, individuals encounter with various types of stress. Accumulation of daily life stress (chronic stress) often causes GI symptoms and functional GI diseases. Although some can adapt toward chronic stress, the adaptation mechanism against chronic stress remains unknown. Although acute stress delays gastric emptying and alters upper GI motility, effects of chronic stress on gastric motility still remains unclear. We investigated the effects of acute (single stress) and chronic stress (repeated stress for 5 consecutive days) on solid gastric emptying and interdigestive gastroduodenal contractions in rats. It is well established that acute restraint stress inhibits solid gastric emptying via central corticotropin-releasing factor (CRF). To investigate whether the sensitivity to CRF is altered following chronic stress, CRF was administered intracisternally (ic). Ghrelin is involved to regulate gastric emptying and upper GI motility in rodents. The changes in plasma active ghrelin levels and mRNA expression in the stomach were studied following chronic stress. To evaluate the effects of chronic stress on hypothalamus-pituitary-adrenal (HPA) axis, plasma corticosterone levels were also measured. Delayed gastric emptying observed in acute stress was completely restored following chronic stress. Acute stress abolished gastric phase III-like contractions, without affecting duodenal phase III-like contractions in the interdigestive state. Impaired gastric phase III-like contractions were also restored following chronic stress. Plasma ghrelin levels and ghrelin mRNA expression were significantly increased after chronic stress. Ic-injection of CRF delayed gastric emptying and impaired gastric motility in rats who received chronic stress. Plasma corticosterone concentrations were no more elevated following chronic stress. The restored gastric emptying following
chronic stress was antagonized by the administration of ghrelin receptor antagonists. The adaptation mechanism may involve upregulation of ghrelin expression and attenuation of HPA axis. In contrast, the sensitivity to central CRF remained unaltered following chronic stress in rats.

**Key words:** adaptation, corticotropin releasing factor (CRF), growth hormone secretagogue receptor (GHS-R), hypothalamus-pituitary-adrenal (HPA) axis, migrating motor complex MMC,
Introduction

Functional gastrointestinal (GI) disorders are common in the general population. The incidences of GI disorders such as functional dyspepsia (FD) and irritable bowel syndrome (IBS) have been reported to be about 25–30% and 10–20% of all outpatients consulting gastroenterologists (40), respectively. Functional GI disorders are multifactorial in which the pathophysiological mechanisms are variably combined in different patients.

Stress is widely believed to play a major role in developing functional GI disorders. Patients with serious stress frequently complain of GI symptoms. Common upper GI symptoms include fullness and bloating after small meals, abdominal distention, nausea, and loss of appetite (39). These symptoms are, at least in part, likely to be due to GI motility disorders.

Previous animal studies demonstrated that solid meal gastric emptying was delayed by acute stress in dogs (16) and rats (32, 38). Acute restraint stress inhibits solid gastric emptying via the central corticotropin-releasing factor (CRF) and peripheral parasympathetic/sympathetic neural pathways in conscious rats (32, 37). CRF, a stress-related neuropeptide, is known to act in the brain to influence the GI tract. Gastric emptying and acid secretion are greatly attenuated when CRF is exogenously applied to the central nervous system (26, 38). Restraint stress is known to increase CRF mRNA in the amygdale and paraventricular nucleus (PVN), resulting in altered GI motor activities (14).
In our society, individuals encounter various types of mental and/or social stress during the daily life. Accumulation of continuous life stress (chronic stress) often causes GI symptoms and functional GI diseases. Although some can adapt to chronic stress, the adaptation mechanism against chronic stress remains unclear.

Many animal studies have been conducted to investigate the effects of acute stress on GI motility. However, relatively few studies have been done on repeated chronic stress. Ochi et al. have recently demonstrated that acute stress delays gastric emptying, while repeated stress for 5 days accelerates gastric emptying in rats (34). This suggests that homeostatic adaptation may develop in response to repeated stress.

We studied and compared the effects of acute and chronic stress on gastric motility in rats. Gastric motility consists of two motor patterns; postprandial contractions and interdigestive contractions. Postprandial contractions mediates gastric emptying while interdigestive contractions plays an important role in mediating mechanical and chemical cleansing of the empty stomach in preparation for the next meal (22, 47).

Migrating motor complex (MMC) of interdigestive contractions consists of phase I (period of motor quiescence), phase II (period of irregular low amplitude contractions), and phase III (period of regular high level contractions. Gastric MMC is also impaired in subsets of FD patients (25). It has been shown that acoustic stress abolished the occurrence of gastric MMC, without affecting intestinal MMC in dogs (15). However, the effects of chronic stress on the interdigestive contractions have not been studied.
Ghrelin, a 28–amino acid peptide, was isolated from the rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (24). Ghrelin is mainly produced in cells lining the fundus of the stomach (19) and has an important role to mediate gastric emptying in rodents (4, 23). In addition to gastric emptying, ghrelin also regulates interdigestive gastric contractions in rodents (5, 13, 41, 51).

Recent studies showed that ghrelin levels are increased following chronic stress in rats (34) and mice (27). We studied whether plasma active ghrelin levels and gastric ghrelin mRNA expression are altered following chronic stress loading in rats.

Our current study showed that delayed gastric emptying observed in acute stress was completely restored following chronic stress in rats. To investigate whether the sensitivity to central CRF is altered following chronic stress, CRF was exogenously administered into the cisterna magna.

Stress responses are mediated by stimulation of the hypothalamus-pituitary-adrenal (HPA) axis. When animals are subjected to stress, CRF is secreted from hypothalamus, which results in the secretion of corticosterone from the adrenal cortex to guard against stress disorders (10). To study the effects of chronic stress on HPA axis, plasma corticosterone levels were evaluated in chronically stressed rats.

**Materials and Methods**

*Restraint stress*

Adult male Sprague-Dawley (SD) rats weighing 260-300 g. were housed in individual cages under conditions of controlled temperature (22-24°C) and illumination (12-hr light
cycle starting at 6:00 AM) for at least 7 days before the experiment. Rats were given *ad libitum* access to food and water. Protocols describing the use of rats were approved by the Animal Care and Use Committee of Clement J. Zablocki Veterans Affairs Medical Center (Milwaukee).

As previously reported (26, 49), rats were placed on a wooden plate with their trunks wrapped in a confining harness consisting of a bandage fabricated for 90 min. The animal was able to move its limbs and head but not its trunk. This restraint stress has been used as a physical stress model in rodents (26, 49). The rats received the same restraint stress for 5 consecutive days. Daily food intake and body weight were monitored.

**Measurement of solid gastric emptying in acute and chronic stress**

After 5 days of chronic restraint stress, rats were fasted for 24 hrs. Pre-weighed pellets (1.6 g) were given as previously reported (20, 21). The rats that did not consume 1.6g of food within 10 min were excluded from the study. Immediately after finishing the feeding, the rats were subjected to the restraint stress. After the restraint stress loading, the rats were sacrificed by pentobarbital (200 mg/kg, IP).

The stomach was surgically isolated and removed. The gastric content was recovered from the stomach, dried, and weighed. Solid gastric emptying was calculated according to the following formula, as previously described (32):

\[
\text{Gastric emptying (\%)} = [1 - (\text{dried weight of food recovered from stomach/weight of food intake})] \times 100.
\]
Another group of rats were subjected to ic-injection of CRF following the chronic stress. After receiving chronic stress for 5 days, rats were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) under a light anesthesia of isoflurane (3%). CRF was injected intra cisternally (ic) by puncture of the occipital membrane with a 10-µl Hamilton microsyringe (Hamilton, Reno, NV). The presence of cerebrospinal fluid in the Hamilton syringe on aspiration before injection confirmed the accuracy of needle placement in the cisterna magna, as previously described (33).

After the recovery from isoflurane, the rats were given 1.6 g of pre-weighed pellets and gastric emptying was measured.

*Monitoring of interdigestive GI motility*

Rats were anesthetized with intraperitoneal injection of pentobarbital (45 mg/kg). Strain gauge transducers were implanted on the antrum and the duodenum for recording gastric and duodenal contractions, as previously described (5, 41). All wires were tunneled subcutaneously to exit at the back of the rat’s neck and protected by a protective jacket (Star Medical, Tokyo, Japan).

We have recently reported that fixed feeding, in which food is available only during restricted hours, causes potent interdigestive contractions (3). Rats on a fixed-feeding schedule (food administered 2:00 – 6:00 PM daily) were monitored from 8:00 AM – 4:00 PM for gastric and duodenal motility. Rats were exposed to restraint stress from 10:00 AM – 12:00 PM. The wires from the transducers were connected to a recording system (Power Lab 8SP, AD Instruments, Colorado Springs, CO). Gastroduodenal interdigestive contractions were monitored before, during and after restraint stress.
The frequency and the maximal amplitude of phase III-like contractions were evaluated. Phase III-like contractions were defined as clustered potent contractions with amplitude of more than 4 g, as previously reported (5, 42).

**Blood collection and hormone assays**

Blood samples were drawn from the sacrificed rats via a cardiac puncture. The blood was collected in tubes containing EDTA and aprotinin (500 kIU/ml), as previously reported (17). After the centrifugation, plasma was aliquoted and for ghrelin, 1.0 N HCl (10% of sample volume) was added. The plasma fraction was stored at -20°C until assayed.

Plasma concentration of corticosterone was measured using a corticosterone RIA kit (ICN Biomedicals Inc., Costa Mesa, CA). Plasma level of active ghrelin was measured by radioimmunoassay using a RIA kit (LINCO Research, St Charles, MO, USA), as previously reported (3, 5). The kit does not have any cross reactivity to des-acyl ghrelin (8). Intra- and inter-assay coefficient variances were <10%, as previously reported (3).

**Ghrelin mRNA detection**

Rats were sacrificed by pentobarbital (200 mg/kg, IP). The body of the stomach was surgically isolated and removed. Total RNA was extracted from the gastric tissues using Trizol (Invitrogen, Carsbad, CA) according to manufacturer’s instructions. Trace DNA contamination was removed by DNase digestion (Promega, Madison, WI). cDNA was synthesized from 3 µg total RNA using Superscript III reverse transcriptase (Invitrogen, Carsbad, CA).

The following primers were designed to amplify rat ghrelin (346bp; accession no.
AB029433), as previously reported (24). Sense primer: 5’-
TTGAGCCCAGAGCACCAGAAA-3’ antisense primer: 5’-
AGTTGCAGAGGAGGCAGAAGCT-3’. For the internal control, the following primers
were designed to amplify a rat β-actin fragment (106 bp; accession no. NM031144), as
previously reported (50). Sense primer: 5’-TTGAGCCCAGAGCACCAGAAA-3’
antisense primer: 5’-GGGTCATCTTTTTACGCTGG-3’.

PCR amplification was carried out with Ex Taq polymerase (Takara Bio, Madison,
WI) according to manufacturer’s instructions. The reaction mixture contained 1 nM of
each primer, 250 nM of each dNTP, 1 U of Ex taq polymerase and reaction buffer, and
the initial template denaturation was programmed for 10 sec at 98 °C with the cycle
profile programmed as follows: 10 sec at 98 °C (denaturation), 30 sec at 55 °C (annealing
and extension) and 60 sec at 72 °C (extension). Forty cycles of the profile were run. PCR
products were visualized by 2% agarose gel electrophoresis.

*Effect of ghrelin receptor antagonists on gastric emptying following chronic restraint*

*stress*

We have previously showed that ghrelin receptor antagonists [(D-lys3)GHRP-6; 1.0
µmol/kg)] almost completely abolished phase III-like contractions induced by
exogenously applied ghrelin (5). To study whether endogenous ghrelin is involved in
mediating the adaptation mechanism to restore gastric emptying, (D-lys3) GHRP6 (1.0
µmol/kg, ip) was administered intraperitoneally 15 min before feeding. Gastric emptying
was measured in chronically restraint rats. Saline-injected rats served as controls.
Substances

A rat/human CRF (Sigma, St. Louis, MO) and (D-lys3)GHRP-6 (Bachem, King of Prussia, PA) were kept in powder form at -70°C. CRF was dissolved in 0.9% saline immediately before use.

Statistical analysis

Quantification of gastric motility was studied by calculating motility index (MI). MI was equivalent with the area under the curve of the motility recording. MI was calculated using a computer-assisted system (Power Lab; AD Instruments, AD Instruments, Colorado Springs, CO), as previously reported (3, 5).

Frequency of gastric and duodenal phase III-like contractions was also calculated by counting the number of phase III-like contractions every 30 min. All results were expressed as means ± SE. A multiple-group comparison was performed by ANOVA followed by Scheffe’s test. A $P$ value <0.05 was considered statistically significant.

Results

Effect of chronic restraint stress on body weight and food intake

Body weight increase (Fig. 1A) and daily food intake (Fig. 1B) were not significantly altered during repeated stress loading for 5 days, compared to those of controls.

Effect of chronic stress on gastric emptying

In non-restraint rats, solid gastric emptying was 52.4 ± 3.1% ($n = 9$) 90 min after the feeding of 1.6 g rat chow. Gastric emptying was significantly delayed in rats that received
restraint stress for 90 min at the day 1 (25.6 ± 3.9%, n=6, P<0.01) and day 3 (34.8 ± 2.8%, n=6, P<0.01). Delayed gastric emptying was completely restored to the normal levels (53.3 ± 3.6%, n=6) after 5 consecutive days of restraint stress (Fig. 2A).

Effect of ic-injection of CRF on gastric emptying

As previously reported (32), ic-injection of saline did not significantly affect solid gastric emptying. IC-injection of CRF (1.0 µg) significantly delayed solid gastric emptying to 28.8 ± 4.4% (n = 6, P<0.01) in non-restraint rats (Fig. 2B).

In chronically restraint rats, ic-injection of CRF also significantly delayed gastric emptying to 40.9 ± 6.1% (n = 6, P<0.05), compared to that of saline-injected rats (Fig. 2B).

Effects of chronic stress on plasma ghrelin and gastric ghrelin mRNA expression

Plasma active ghrelin levels in control rats were 21.8 ± 1.9 pg/ml (n = 6) at the day 1. No significant difference of the plasma ghrelin levels was observed between restraint rats and control rats at the day 1. However, active ghrelin levels were significantly increased to 52.5 ± 5.3 pg/ml (n=6, P<0.01) at the day 3 in restraint rats. The ghrelin levels continued to increase at the day 5, (65.6 ± 5.4 pg/ml, n=6, P<0.01) (Fig. 3A) in restraint rats.

No significant difference of ghrelin mRNA expression was observed between restraint rats and control rats at the day 1 (Fig. 3B and 3C). In contrast, ghrelin mRNA expression was significantly increased at the day 5 in restraint rats, compared to that of control rats (Fig. 3B and 3C).
Effects of acute and chronic stress on interdigestive gastroduodenal motility

Gastroduodenal phase III-like contractions were recorded in fixed-fed rats before during and after restraint stress loading (for 90 min) for 5 consecutive days. On the day 1, regular gastric phase III-like contractions were observed before the restraint stress loading. Appearance of phase III-like contractions was completely abolished during restraint stress loading (Fig. 4). Regular phase III-like contractions were observed immediately after finishing the restraint stress.

After 3 consecutive days of restraint stress, however, appearance of gastric phase III-like contractions was partially restored. On the day 5, appearance of phase III-like contractions was completely restored. Duodenal phase III-like contractions remained unaffected by restraint stress in the entire duration of the study (Fig. 4).

Reduced frequency of gastric phase III-like contractions in response to restrain stress was gradually increased at the day 2 and day 4 (Fig. 5A). At the day 5, there was no more reduced frequency observed in restraint rats. The frequency of duodenal phase III-like contractions was not affected in response to restraint stress for 5 days (Fig. 5B).

Effects of ic-injection of CRF on interdigestive gastroduodenal motility

Ic-injection of CRF (1.0 µg) completely abolished gastric phase III-like contractions (Fig. 6A) in non-restraint rats. The inhibitory effect of CRF persisted for approximately 150 min. In contrast, duodenal phase III-like contractions were not affected by CRF injection in non-restraint rats (Fig. 6A).
CRF was also administered (ic) to chronically restraint rats at the day 5. Restored gastric phase III-like contractions were observed in the chronically restraint rats. Gastric phase III-like contractions, but not duodenal phase III-like contractions, were completely abolished immediately after the injection of CRF in chronically restraint rats (Fig. 6B).

**Effects of chronic stress on plasma corticosterone levels**
Plasma corticosterone levels in non-restraint rats were 70.2 ± 12.0 ng/ml (n=6). Plasma corticosterone levels significantly increased to 1315.3 ± 220.4 ng/ml (n = 6, P<0.01) at the day 1 in restraint rats. Plasma corticosterone levels began to fall after the day 3. At the day 5, there was no significant difference observed between the restraint group and the control group (Fig. 7).

**Effect of ghrelin receptor antagonists on gastric emptying of chronic restraint rats**
As described above, gastric emptying was completely restored to the normal levels after 5 consecutive days of restraint stress. In chronically restraint rats, ip-injection of ghrelin receptor antagonists, (D-lys3) GHRP-6 (1.0 µmol/kg), significantly delayed gastric emptying to 36.1 ± 2.5% (n = 6, P<0.01), compared to that of saline-injected rats (53.4 ± 5.6%, n=6).

**Discussion**
It is well known that acute stress inhibits solid gastric emptying in humans (36, 43), dogs (16, 48) and rats (32, 45). We have previously shown that acute stress delays gastric emptying via central CRF and peripheral sympathetic pathways in rats (32).
In contrast, recent study suggests that chronic stress seems to have an inverse effect on gastric emptying. Ochi et al. (34) reported that under chronic stress gastric emptying is restored to normal levels in rats. Our current study demonstrated that delayed gastric emptying observed in acute stress was fully restored following consecutive stress loading for 5 days.

Ghrelin is has an important role to mediate gastric emptying in rodents. Exogenously applied ghrelin accelerates gastric emptying via stimulation of antro-pyloric coordinations in rats (3). Ghrelin antagonists delay gastric emptying in mice (23). We have recently demonstrated that gastric emptying is accelerated in the early phase of diabetes (2) and fixed feeding regimen (3). The increased ghrelin release mediates the accelerated gastric emptying in these rats (3).

In addition to gastric emptying, ghrelin also regulates interdigestive gastric contractions in rodents. We have previously demonstrated that plasma ghrelin levels are associated with gastric phase III-like contractions in rats (5). Ghrelin antagonists abolished spontaneous phase III-like contractions of the stomach in rats (5, 13, 41) and mice (51).

Circulating ghrelin can be found in two forms: active (acylated) ghrelin and inactive (not acylated) ghrelin. Recent study showed that active ghrelin levels are increased following chronic stress in rats (34) and mice (27).

Our current study demonstrated that plasma active ghrelin levels and gastric ghrelin mRNA expression are significantly increased following chronic stress. Consistent with
our study, plasma active ghrelin levels are significantly elevated after 11-39 days of chronic social defeat stress in mice (27). These findings suggest that ghrelin may play a role in the adaptation process following chronic stress. We also showed that the restored gastric emptying following chronic stress was antagonized by the administration of ghrelin receptor antagonists. The restored gastric emptying following chronic stress may be mediated, at least in part, via upregulated ghrelin signalling pathways.

The mechanism of upregulated ghrelin expression following chronic stress remains to be investigated. Ghrelin secretion from the stomach is regulated by both the cholinergic and adrenergic arms of the autonomic nervous system. Plasma ghrelin levels are increased by muscarinic agonists and beta-adrenergic agonists in rats (18). Sympathetic nerve stimulation increases gastric ghrelin secretion in rats (30). We have previously showed that restraint stress stimulates sympathetic pathway in rats (32). We cannot exclude the possibility that repeated stress may act to upregulate ghrelin expression and secretion via the stimulation of ghrelin cells by catecholamine following activation of the sympathetic nerves.

It is widely accepted that delayed gastric emptying induced by acute stress is mediated via central CRF and that central administration of CRF delays solid gastric emptying (28, 32). Our current study demonstrated that ic-injection of CRF still has an inhibitory effect on gastric emptying in chronically stressed rats even though chronic stress has no more inhibitory effects on gastric emptying. This suggests that the inhibitory pathway in response to central CRF is not altered following chronic stress.
Numerous CRF-immunoreactive neurons were observed in the PVN in acutely stressed rats. In contrast, repeated stress for 11 consecutive days significantly lowered the number of CRF neurons in the PVN (7). Others showed the down-regulation of CRF mRNA expression at the hypothalamus and pituitary in chronically stressed rats (9). We showed that plasma corticosterone levels are significantly reduced following chronic stress, suggesting the attenuation of HPA axis. It is conceivable that chronic stress downregulates CRF pathway at the PVN, resulting in reduced activity of HPA axis. It remains unknown whether increased ghrelin expression of the stomach may inhibit CRF expression at the PVN during the adaptation process following repeated stress.

In addition to the gastric emptying, we also studied whether acute and chronic stress affects interdigestive GI contractions in conscious rats. GI motility during the interdigestive state was monitored via strain gauge transducers placed on the antrum and duodenum in rats. It is widely accepted that the strain gauge transducer method is reliable and accurate to record GI motor function in freely moving conscious animals, including dogs (46), rats (31) and mice (51).

Acute acoustic stress inhibits phase III contractions of the antrum in dogs. In contrast, duodenal phase III-like contractions remains unaltered by acute acoustic stress in dogs (15). Intracebroventricular (ICV)-injection of CRF abolished gastric phase III, but not duodenal phase III, in dogs (15). Similar results were observed in our rat study. Spontaneous gastric phase III-like contractions, but not duodenal phase III-like contractions, were abolished during acute restraint stress loading.
The difference in motor response to stress between the stomach and duodenum can be attributed to the mechanism of regulation. Gastric motor function is regulated via vagal efferents (1), while duodenal motor function is regulated via intrinsic neurons (12). Our recent study demonstrated that spontaneous phase III-like contractions of the stomach are mainly regulated by ghrelin and its own receptors, whereas spontaneous phase III-like contractions of the small intestine are regulated by 5-HT and 5HT_4 receptors in rats (41). Released ghrelin from the gastric mucosa initiates gastric phase III-like contractions via vago-vagal reflex. In contrast, released 5-HT from enterochromaffin (EC) cells of the duodenal mucosa induces intestinal phase III-like contractions via 5-HT_4 receptors and intrinsic neurons in rats (41).

Acute restraint stress has been shown to inhibit vagal efferent pathway (38) and/or stimulates sympathetic pathway (26, 32) in rats. As intestinal phase III-like contractions are independent of extrinsic neurons, acute stress may not cause any inhibition on duodenal phase III-like contractions in rats.

In our current study, gastric phase III-like contractions reappeared after the day 3 of consecutive restraint stress loading. On the day 5, gastric phase III-like contractions were completely restored to normal levels. This suggests that interdigestive gastric motility is adapted to repeated stress in rats. Increased ghrelin release may be responsible for the restored gastric phase III-like contractions following chronic stress. Similar to gastric emptying study, ic-injection of CRF attenuated restored gastric phase III-like contractions following chronic stress.
Chronically stressed individuals often develop deleterious weight gain, abdominal obesity, type II diabetes and increased cardiovascular morbidity (11). Although ghrelin plays an important role in appetite and weight regulation, it remains to be investigated whether endogenous ghrelin is involved in the eating response to stress in humans. Recent study suggests that the post-stress induced urge for uncontrolled eating is not related to the elevated ghrelin levels in humans (35).

Exogenously administered ghrelin stimulates feeding activity in rodents (6). In our study, plasma ghrelin level and ghrelin mRNA expression were significantly increased following 5 days of restraint stress loading. In contrast, there was no significant changes observed in daily food intake and body weight between chronically restraint rats and control rats. Further study is needed whether the prolonged period of chronic stress loading (more than 5 days) affects food intake and body weight.

GI motility disorders such as delayed gastric emptying has been suggested to be associated with the symptoms of dyspepsia (39). In contrast, gastric emptying in FD patients is not necessarily delayed, and 28% of such patients exhibits a rapid gastric emptying (29). Recent study also showed three patterns of gastric emptying (normal, delayed and accelerated gastric emptying) in FD patients (44).

It is conceivable that symptoms of dyspepsia and gastric motility disorders depend on the degree of strength, duration and accumulation of various types of mental and social stress. In addition, dyspeptic symptoms and motility disorders could depend on how we adapt to the stressful events in our daily life.
In conclusion, acute restraint stress significantly delayed solid gastric emptying and impaired interdigestive gastric contractions in rats. In contrast, there were no delayed solid gastric emptying and impaired interdigestive gastric contractions observed following chronic stress loading for 5 days. The adaptation mechanism may involve upregulated ghrelin expression of the stomach. As plasma corticosterone levels were significantly reduced following chronic stress, attenuation of HPA axis is also involved in the adaptation process. The inhibitory pathway of gastric motility in response to central CRF is not altered following chronic stress. It is conceivable that chronic stress downregulates CRF pathway at the PVN, resulting in reduced activity of HPA axis.

**Perspectives and significance**

Humans encounter various types of stressors in our daily life. Accumulation of continuous life stress (chronic stress) often causes gastric symptoms. Symptoms of dyspepsia may depend on the degree, duration and accumulation of stress. Dyspeptic symptoms may also depend on how we adapt to the stressful events in our daily life. Although some can adapt to chronic stress, the adaptation mechanism against chronic stress remains unclear.

Our current study reveals that the delayed gastric emptying observed in acute stress loading was completely restored following repeated chronic stress in rats. The adaptation mechanism involves upregulation of gastric ghrelin secretion and attenuation of HPA axis. Our study could contribute to the better understanding of the mechanism of stress-induced functional GI disorders.
Individuals encounter various types of physical, mental and social stress in daily life. Although animals can adapt to a single, repeated stressful event, it is necessary to establish an animal model of chronic loading of different types of stress (chronic complicated stress) and to study whether adaptation develops following chronic complicated stress loading.
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Figure Legends

Fig. 1. Effect of chronic stress on body weight (A) and daily food intake (B). Chronic restraint stress did not significantly altered body weight and food intake (n = 6).

Fig. 2. Effect of chronic stress on solid gastric emptying (A) and ic-injection of corticotrophin-releasing factor (CRF; 1 µg/rat) on solid gastric emptying following chronic stress (B). Gastric emptying was significantly delayed in restraint rats at the day 1 and day 3. Delayed gastric emptying was completely restored to the normal levels after 5 consecutive days of restraint stress (A). IC-injection of CRF significantly reduced solid gastric emptying in non-restraint rats. In chronically stressed rats, IC-injection of CRF also significantly delayed gastric emptying, compared with controls (n = 6, **P < 0.01, * P < 0.05 compared with saline-injected rats).

Fig. 3. Effect of chronic stress on plasma active ghrelin levels (A), gastric ghrelin mRNA expression (B) and change of ghrelin mRNA expression relative radioactivity (C). Plasma active ghrelin levels showed no significant increase at the day 1, compared to that of controls. However, plasma ghrelin levels were significantly increased at day 3 and day 5 (n=6, **P < 0.01 compared with controls) (A). Gastric ghrelin mRNA expression showed no significant difference at the day 1 of restraint rats, compared to that of controls. At the day 5 of chronically restraint rats, ghrelin mRNA expressions were significantly increased (n=6, **P < 0.01
compared with controls). The mRNA expression was standardized with the intensity of β-actin (B and C).

Fig. 4. Effects of acute and chronic stress on interdigestive gastroduodenal motility. Acute restraint stress completely abolished gastric phase III-like contractions (at the day 1). Gastric phase III-like contractions partially restored at the day 3, and completely restored at the day 5. In contrast, duodenal phase III-like contractions remained unaffected in response to restraint stress throughout the study.

Fig. 5. Effects of acute and chronic stress on the frequency of phase III-like contractions in the stomach (A) and duodenum (B). The number of gastric phase III-like contractions, which was reduced in response to acute stress gradually restored following repeated stress lading (at the day 2 and day 4). On the day 5, appearance of gastric phase III-like contractions was completely restored. Duodenal phase III-like contractions were unaffected by acute or chronic stress (n = 6, **P < 0.01 compared with basal).

Fig. 6. Effects of ic-injection of CRF (1.0 µg) on interdigestive gastroduodenal motility following acute stress (A) and chronic stress (B). Ic-injection of CRF completely abolished gastric phase III-like contractions, while duodenal phase III-like contractions were not affected by CRF (A). In the chronically restraint rats, restored gastric phase III-like contractions were completely abolished by CRF (B).
Fig. 7. Effects of acute and chronic stress on plasma corticosterone levels. Acute restraint stress significantly increased the plasma corticosterone levels (at the day 1). The plasma corticosterone levels began to fall at the day 3. At the day 5, there was no more significant increase observed between the restraint group and control group (n = 6. **P < 0.01 compared with controls).