Central oxytocin is involved in restoring impaired gastric motility following chronic repeated stress in mice

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Short title: Central oxytocin and chronic stress

Abbreviations: corticotropin releasing factor (CRF), irritable bowel syndrome (IBS) functional dyspepsia (FD), hypothalamus-pituitary-adrenal (HPA) axis, motility index (MI), paraventricular nucleus (PVN), Intracerebroventricular (ICV)

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Abstract

Accumulation of continuous life stress (chronic stress) often causes gastric symptoms. The development of gastric symptoms may depend on how humans adapt to the stressful events in their daily lives. Although acute stress delays gastric emptying and alters upper GI motility in rodents, the effects of chronic stress on gastric motility and its adaptation mechanism remains unclear. Central oxytocin has been shown to have anti-stress effects. We studied whether central oxytocin is involved in mediating the adaptation mechanism following chronic repeated stress. Mice were loaded with acute and chronic stress (repeated stress for 5 consecutive days) and solid gastric emptying and postprandial gastric motility were compared between acute and chronic repeated stress. Expression of oxytocin and corticotropin releasing factor (CRF) mRNA in the hypothalamus was studied following acute and chronic repeated stress. Delayed gastric emptying during acute stress (43.1 ± 7.8 %, n=6, P<0.05) was completely restored to normal levels (72.1 ± 2.4%, n=6) following chronic repeated stress. Impaired gastric motility induced by acute stress was also restored following chronic repeated stress. Intracerebroventricular (icv)-injection of oxytocin (0.1 and 0.5 µg) restored the impaired gastric emptying and motility induced by acute stress. The restored gastric emptying and motility following chronic repeated stress was antagonized by icv-injection of oxytocin antagonists. Oxytocin mRNA expression in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus was significantly increased following chronic repeated stress. In contrast, increased corticotropin releasing factor (CRF) mRNA expression in the SON and PVN in response to acute stress was significantly reduced following chronic repeated stress. Our study suggests the novel finding that the up-regulation of central oxytocin
expression is involved in mediating the adaptation mechanism following chronic repeated stress in mice.

**Key words:** acute stress, chronic stress, corticotropin releasing factor (CRF), hypothalamus-pituitary-adrenal (HPA) axis.
**Introduction:**

Stress is widely believed to play a major role in developing functional GI disorders. Patients with serious stress frequently complain of GI symptoms and these symptoms are, at least in part, likely due to GI motility disorders. Accumulation of continuous life stress (chronic stress) often causes gastric symptoms. The development of dyspeptic symptoms may also depend on how humans adapt to the stressful events in their daily lives. Although some can adapt to chronic stress, the adaptation mechanism against chronic stress remains unclear.

Animal studies demonstrated that gastric emptying of solid and liquid meal was delayed by acute stress in dogs (17), rats (31, 45) and mice (16). Corticotropin releasing factor (CRF) is known to act in the brain to influence the GI tract. Acute restraint stress inhibits solid gastric emptying via central CRF and peripheral autonomic neural pathways in rats (31, 44). Gastric emptying and acid secretion are attenuated when CRF is exogenously applied to the central nervous system (45). Restraint stress is known to increase CRF mRNA in the amygdala and paraventricular nucleus (PVN), resulting in altered GI motor activities (14).

Many animal studies have been conducted to investigate the effects of acute stress on GI motility. However, relatively few studies have been done on chronic repeated stress. Ochi et al. have recently demonstrated that acute stress delays gastric emptying, while repeated stress for 5 days accelerates gastric emptying in rats (36). This suggests that homeostatic adaptation may develop in response to repeated stress.

Oxytocin is a cyclic nona-peptide hormone synthesized in the neurosecretory cells that are located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Besides its well known physiological functions like milk ejection and
induction of labor, oxytocin plays an important role in regulating social behavior and positive social interactions in non-human mammals (32). In humans, intranasal administration of oxytocin was shown to cause a substantial increase in trusting behavior (20).

Oxytocin is released from the neurohypophysial terminal into the blood stream and within distinct brain regions in response to stressful or social stimuli. Centrally released oxytocin has anxiolytic effects and anti-stress effects (3). Anxiolytic and anti-stress effects of oxytocin are localized within the central amygdala and the PVN both in females and in males. Oxytocin is released in the PVN in response to various stressors, such as shaker stress (34) and forced swimming stress (52) in rats. The SON consists exclusively of magnocellular neurons. In contrast, the PVN consists of both magnocellular and parvocellular neurons. Forced swimming stress increases oxytocin mRNA expression in the magnocellular neurons, but not the parvocellular neurons, of the PVN in rats (53). Restraint stress induces c-fos expression in oxytocinergic magnocellular neurons in the SON and PVN in rats (28). However, it remains unknown whether restraint stress activates parvocellular oxytocinergic neurons of the PVN.

Oxytocin attenuates CRF mRNA expression in the PVN and the activity of hypothalamus–pituitary–adrenal (HPA) axis in response to stress (51). This suggests that anxiolytic and anti-stress effects of oxytocin are, at least in part, mediated by its inhibitory effect on CRF expression. A Recent study demonstrated that water avoidance stress-induced stimulation of colonic motility is attenuated by central administration of oxytocin in rats (25).

The anxiolytic effects and anti-stress effects of central oxytocin are further supported by the study of oxytocin-deficient mice. Oxytocin-deficient mice display more anxiety-related
behavior in response to a psychogenic stressor and manifest greater stress-induced hyperthermia, compared to wild-type mice (2). Plasma corticosterone levels after shaker stress are higher in oxytocin knockout mice than those in wild-type mice (24). In oxytocin gene-deficient male mice, enhanced CRF mRNA expression is observed in response to restraint stress in the PVN (35).

Gastric motility consists of two motor patterns; postprandial contractions and interdigestive contractions (47). The alterations of gastric motor function in response to food intake may play an important role in stress-related functional GI disorders. We have recently demonstrated that acute restraint stress or central injection of CRF completely abolishes the interdigestive gastric contractions in rats (55). Very few studies have been conducted on chronic stress and its long term effects on GI physiology in mice.

In the current study, we compared the effects of acute and chronic repeated stress on gastric emptying and postprandial gastric motility in conscious mice. Delayed gastric emptying and impaired gastric motility observed during acute stress loading was completely restored following chronic stress in mice. To further investigate the role of oxytocin in stress-induced gastric motor function, oxytocin and oxytocin antagonists were centrally administered. To investigate the changes of central oxytocin and CRF expression following acute stress and chronic stress, oxytocin mRNA expression and CRF mRNA expression in the PVN and SON were measured by real-time PCR. We demonstrated that the central oxytocin may play an important role in regulating the adaptation mechanism following chronic repeated restraint stress in mice.
Materials and Methods

Animals

Male Swiss Webster mice weighing 25–30 g were kept in-group cages under conditions of controlled temperature (22–24°C), humidity and light (12 h light cycle starting at 7:00 am) with free access to laboratory chow and water. All experiments were started at 9:00 am every day. Protocols describing the use of mice were approved by the Institutional Animal Care and Use Committee of Zablocki VA Medical Center at Milwaukee and carried out in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals". All efforts were made to minimize animal suffering and to reduce the number of animal in experiments.

Acute and chronic stress loading

Restraint stress was induced by placing the mice in a 50 ml falcon tube (2.7 cm diameter, 7 cm long) with perforated holes for adequate ventilation. The dimensions of the tube effectively restrained the mice, preventing them from turning around and moving forward or backward. Mice were exposed to restraint stress for 90 minutes. In chronic repeated stress, mice were restrained for 90 minutes for 5 consecutive days.

Measurement of solid gastric emptying

Mice were fasted for 16 hrs with free access to water. Pre-weighed pellets (0.2 g) were given, as previously reported (8). The mice that did not consume 0.2 g of food within 10 min were excluded from the study. Immediately after finishing the feeding, the mice were subjected to the restraint stress (both acute and chronic), as described above. After the restraint stress loading for 90 min, mice were sacrificed by pentobarbital (200 mg/kg, IP).
The stomach was surgically isolated and removed. The gastric content was recovered from the stomach, dried, then weighed. Solid gastric emptying was calculated according to the following formula, as previously described (31).

\[
\text{Gastric emptying (\%) } = [1 - (\text{dried weight of food recovered from stomach/weight of food intake})] \times 100.
\]

**Motility recording**

After an overnight fast, mice were anaesthetized with isoflurane (2 %). Through a midline laparotomy, the stomach was exposed and a miniature strain gauge transducer (5 mm x 4 mm) was implanted on the serosal surface of the gastric antrum, as previously described (54). The wires from transducer were exteriorized through abdominal wall, and tunnelled under the skin to the posterior neck. Wires were contained within a protective jacket. After the surgery, mice were housed individually with access to a standard diet and tap water. Mice were allowed to recover for 1 week before subsequent studies.

Wires from the transducer were connected to a recording system (Power-Lab model 8SP; ADI instruments, Colorado Springs, CO). Although the animals were wired and connected to the recording system, the animals could freely move during recording, as previously reported (54). Postprandial gastric contractions were measured in conscious, freely moving mice except during the period of restraint stress loading. Gastric motility was monitored before, during and after the restraint stress. After recording the basal gastric motility for 2 hrs, mice were exposed to restraint stress for 90 min. After releasing the restraint stress, gastric motility was recorded for 2 more hrs.
**Intracerebroventricular (icv)-injection**

For icv-injection, mice were placed in a stereotaxic instrument under the isoflurane anesthesia, 1 week prior to the gastric emptying and gastric motility studies. A hole was made in each skull using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24 gauge cannula in 3 mm length was implanted into the right lateral ventricle for icv-injection. The injector was the same length as the implanted cannula. The cannula was fixed to the skull with dental cement and capped with silicon.

To investigate whether central oxytocin is involved in mediating gastric motility and emptying, oxytocin (0.05, 0.1 and 0.5 µg in 2 µl saline) was injected (icv) 30 min prior to stress loading. It has been shown that oxytocin (0.1-1.0 µg; icv) dose dependently reduced the anxiety responses in mice (42).

To investigate whether endogenous oxytocin is involved to restore gastric motility and emptying following chronic stress, oxytocin receptor antagonists, tocinoic acid (2 µg) and [d(CH₂)₅¹,Tyr(Me)²,Orn⁸]-Oxytocin (100 ng) were injected icv 30 min prior to stress loading. Saline (2 µl, icv)-injected mice served as controls.

Tocinoic acid (20 µg) has been shown to prevent the effect of oxytocin-induced social behavior in male rats (46). [d(CH₂)₅¹,Tyr(Me)²,Orn⁸]-Oxytocin (1-100 ng) has been shown to inhibit the action of oxytocin in sexual physiological stimuli in rats (4, 26).

We used separate mice for each study group. After finishing the experiment, mice were sacrificed and the implantation site of icv-cannula was confirmed by the visualization after injection of a dye via the cannula, as previously reported (7, 19).
**Quantitative RT-PCR**

Immediately after finishing acute or chronic stress loading, mice were sacrificed by pentobarbital (200 mg/kg, IP). Brain tissues were collected between -0.56 mm and -0.96 mm from bregma, medially 0.8 mm above the ventral tissue edge around the dorsal end of the 3rd ventricle to obtain the PVN, and bilaterally from the optic tract in order to acquire the SON (10, 39). Total RNA was extracted from the brain 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 mouse CRF (81 bp; accession no. NM_205769), as previously reported (15). Sense primer: 5’-CCCAGGCAGACGAGTGGTGTG-3’. Antisense primer: 5’-CAAGCCGGAACATTTCTTTCATGTC-3’.

The following primers were designed to amplify mouse oxytocin (82 bp; accession no. NM_011025), as previously reported (49). Sense primer: 5’-CCTACAGCGGATCTCAGACTGA-3’. Antisense primer: 5’-TCAGAGCCAGTAAGCCAAGCA-3’. For the internal control, the following primers were designed to amplify a mice β-actin fragment (106 bp; accession no. EF095208). Sense primer: 5’-TGGCACCACACCTTCTACAATGAG-3’. Antisense primer: 5’-GGGTCACTCTTTTCACGTTG-3’.

Quantitative RT-PCR was performed using SYBR Premix Ex Taq (TakaraBIO, Madison, USA) according to manufacturer’s instructions. Amplification reactions were performed using a LightCycler 480 (Roche Diagnostics). Initial template denaturation
was performed for 30 sec at 95°C. The cycle profiles were programmed as follows: 5 sec at 95°C (denaturation), 20 sec at 60°C (annealing) and 15 sec at 72°C (extension). Forty-five cycles of the profile were run, and the final cooling step was continued for 30 sec at 40°C. Quantitative measurement of each mRNA was achieved by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. The relative amount of each mRNA was normalized by the amount of β-actin mRNA. Amplicon size and specificity were confirmed by melting curve analysis and 2% agarose gel electrophoresis.

**Statistical analysis**

As previously reported (54), quantification of gastric motility was studied by calculating motility index (MI). MI was equivalent to the area under the curve of the motility recording from baseline. MI was calculated using a computer-assisted system (Power Lab; AD Instruments, AD Instruments, Colorado Springs, CO).

Comparison between group values was performed by one way ANOVA for gastric emptying and motility studies. Two-way ANOVA was performed for experiments comparing acute and chronic restraint stress with and without drug dosing. The students t-test was used to determine the significance among groups. Bonferroni post test was done to compare data replicates in a group. Pearson correlation analysis was performed for oxytocin mRNA expression and gastric emptying rate. P-value < 0.05 was considered to be statistically significant. Results were shown as mean ± standard error of the mean (SEM).
Chemicals

Oxytocin and tocinoic acid was purchased from (Sigma Aldrich, St. Louis, MO). [d(CH2)51,Tyr(Me)2,Orn8]-Oxytocin was purchased from (Bachem, Torrance, CA).

Results

Effect of acute and chronic repeated restraint stress on gastric emptying

In non-restraint mice, solid gastric emptying was 76.7 ± 4.6% (n=6), 90 min after feeding 0.2 g mice chow. Gastric emptying was significantly delayed in mice that received restraint stress at the day 1 (43.1 ± 7.8 %, n=6, P<0.05) and day 3 (52.1 ± 1.7%, n=6, P<0.01). Delayed gastric emptying was completely restored to normal levels (72.1 ± 2.4%, n=6) after 5 consecutive days of restraint stress (Fig 1).

Effect of acute and chronic repeated restraint stress on postprandial gastric motility

Postprandial gastric motility was measured in mice before, during and after restraint stress loading. Before stress loading, regular gastric contractions were observed. Immediately after the loading of restraint stress, gastric contractions were significantly attenuated on day 1 (Fig 2a). Motility index was significantly reduced during the stress loading to 69.0 ± 2.9% of basal (n=5, P< 0.05). Regular gastric contractions were soon recovered to basal levels after finishing stress loading (Fig 2a). After 3 consecutive days of restraint stress, the impaired gastric contractions during stress loading were partially restored. On the day 5, restraint stress had no longer an inhibitory effect on gastric contractions (Fig 2a).
The reduced MI in response to restraint stress was gradually restored at the day 2 and day 4. On day 5, MI was no longer attenuated during stress loading (Fig 2b).

**Effect of oxytocin on gastric emptying during acute stress loading**

Icv-injection of oxytocin (0.05 µg) did not have a significant effect on delayed solid gastric emptying induced by acute stress (47.9 ± 0.7%, n=6). In contrast, icv-injection of oxytocin (0.1 and 0.5 µg) significantly antagonized the delayed gastric emptying induced by acute stress (54.4 ± 1.3 % by 0.1 µg and 67.5 ± 1.2 % by 0.5 µg, n=6, P<0.05), compared to that of saline (icv)-injected mice (47.6 ± 3.6%, n=6 (Fig.3). A two-way ANOVA showed a significant difference in gastric emptying between control and acute stress group (F 1,19= 25.68; p < 0.001).

**Effect of oxytocin on gastric motility during acute stress loading**

We studied whether icv-injection of oxytocin itself affects basal gastric motility before stress loading. MI was slightly yet significantly increased by 7.9 ± 1.8 % (n=5, P<0.05) of basal contractions by icv-injection of oxytocin (0.5 µg). The stimulatory effect of oxytocin was observed immediately after the injection and lasted for 15 to 20 min.

Icv-injection of oxytocin (0.5 µg) completely restored impaired gastric contractions induced by acute restraint stress (Fig.4a). MI was significantly reduced during stress loading to 57.1 ± 2.4 % (n=5, P<0.05) of basal in saline-injected mice. However, MI was not significantly altered during stress loading of basal in oxytocin-treated mice (Fig. 4b).
**Effect of oxytocin antagonist on gastric emptying in response to acute and chronic repeated restraint stress**

Icv-injection of oxytocin antagonists, tocinoic acid (2 µg) and (d(CH$_2$)$_3$¹,Tyr(Me)$^2$,Orn$^8$)-Oxytocin (100 ng) did not have a significant effect on gastric emptying in non-restraint mice (Fig.5). Oxytocin antagonists also did not alter the stress-induced delay in gastric emptying in response to acute stress loading (Fig.5). A two-way ANOVA indicated a significant difference in gastric emptying between acute and chronic stress group (F $^2$,$^28$= 8.95; p < 0.01). In chronically stressed mice, icv-injection of oxytocin antagonists, tocinoic acid (2 µg) and [d(CH$_2$)$_3$¹,Tyr(Me)$^2$,Orn$^8$]-Oxytocin (100 ng) significantly delayed solid gastric emptying to 50 ± 1.7% (n=6, P<0.01) and 37.2 ± 1.6% (n= 6, P<0.01), respectively, compared to that of saline injected (icv) mice (67.8 ± 3.8%, n=6) (Fig. 5).

**Effect of oxytocin antagonist on gastric motility in response to acute and chronic repeated restraint stress**

At day 5, MI was no longer attenuated during stress loading. In contrast, MI was significantly reduced to 51.6 ± 1.9 % of basal (n=5, P<0.05) following the administration of an oxytocin antagonist, [d(CH$_2$)$_3$¹,Tyr(Me)$^2$,Orn$^8$]-Oxytocin (100 ng) (Fig.6).

**Effects of acute and chronic stress on CRF and oxytocin mRNA expression in the PVN and SON**

CRF mRNA expression was significantly increased in the PVN and SON in response to acute restraint stress, compared to that of controls. The increased CRF mRNA observed
during acute stress was significantly reduced in the PVN following chronic stress. There was no significant increase of CRF mRNA expression observed in the SON following chronic stress loading (Fig. 7A).

Oxytocin mRNA expression was not significantly increased in the PVN in response to acute stress. In the SON, a small but significant increase of oxytocin mRNA expression was observed in response to acute stress. Chronic stress significantly increased oxytocin mRNA expression in the PVN as well as SON, compared to that of controls and acutely stressed mice (Fig. 7B).

**Correlation between oxytocin mRNA expression and gastric emptying**

There was a significant positive correlation observed between oxytocin mRNA expression and gastric emptying in the PVN and SON in control (Fig. 8a), acute stress (Fig. 8b) and chronic repeated stress groups (Fig. 8c).

**Discussion**

We have previously shown that acute stress delays solid gastric emptying via central CRF and peripheral sympathetic pathways in rats (31). Others have suggested that the inhibitory effect of acute stress on gastric emptying is mediated via the impaired activity of parasympathetic pathway in rats (44).

In contrast to acute stress, recent study suggests that chronic stress seems to have an inverse effect on gastric emptying in rats (36). Our recent study demonstrated that delayed gastric emptying induced by acute restraint stress is no longer observed following 5-day consecutive repeated stress in rats (55). Our current study also demonstrates that delayed gastric emptying
observed in acute stress was fully restored following stress loading for 5 consecutive days in mice.

Oxytocin and its receptors are also found all throughout the GI tract in humans (29) and rats (48). Oxytocin exerts its activity on GI tract through both central and peripheral mechanisms. In healthy fasting humans, intravenous infusion of oxytocin (0.33 U/min) increases gastric emptying of semi solid meals (40). In contrast, intraperitoneal administration of oxytocin (0.8 mg/kg) inhibits gastric emptying of a non nutrient liquid meal in rats (22). Others have shown that administration of oxytocin (4 pmoles) into the dorsal motor nucleus of the vagus (DMV) inhibits gastric motility in anesthetized rats (43). Intravenous injection of oxytocin (0.5 to 2.0 U/kg) abolishes the peristaltic contractions of GI tract in anesthetized dogs (27). On the other hand, intravenous injection of oxytocin (0.1-0.8 µg/kg) was shown to increase the gastric motility in rabbits (21). Oxytocin (10^-9 – 10^-6 M) dose-dependently increased the contraction of the muscle strips of gastric body and antrum in rats. Tetrodotoxin and atropine do not influence the effect of oxytocin, suggesting the direct action on smooth muscle cells (41).

It remains unclear whether central administration of oxytocin affects solid gastric emptying and postprandial gastric contractions in rodents. Flanagan et al. have previously shown that centrally administered oxytocin (10 µg; icv) inhibits gastric motility under normal conditions in conscious rats (13). Our current study shows that oxytocin (0.5 µg, icv) fails to alter gastric emptying in non-stressed mice, while gastric motility was slightly increased by icv-injection of oxytocin. Enhanced gastric motility induced by central administration of oxytocin may not influence solid gastric emptying in conscious mice under normal conditions. Thus, it is likely that the effects of oxytocin on the GI tract may vary among species under normal conditions.
Next, we studied the central effects of oxytocin on gastric emptying and gastric motility in response to acute and chronic repeated stress in mice. We demonstrated that central administration of oxytocin antagonized the inhibitory effect of acute restraint stress on gastric emptying and motility. As the central administration of oxytocin did not cause any effects on gastric emptying in non-stressed conditions, the normalization of gastric emptying caused by icv-oxytocin observed in acute stress loading may be associated with the stress responses.

To further investigate the role of oxytocin in the adaptation mechanism following chronic repeated stress, two different oxytocin antagonists (Tocinoic acid and \([d(CH_2)_2^{1}\text{Tyr(Me)}^2,\text{Orn}^8]\)-Oxytocin) were administered. Either of the oxytocin antagonists failed to evoke any significant effects on gastric emptying in non-restrained mice. In contrast, oxytocin antagonists reversed the restored gastric emptying and gastric motility following chronic restraint stress in mice. This suggests that endogenous oxytocin may play an important role in the adaptation mechanism following chronic stress.

Previous studies have shown that central administration of oxytocin antagonists into the PVN resulted in a significant increase in basal release of ACTH in male and female rats (33). This suggests that endogenous oxytocin in the PVN mediates its anti-stress responses through the inhibition of HPA axis.

When animals are subjected to acute stress, CRF is secreted from the hypothalamus, resulting in the secretion of corticosterone from the adrenal cortex to guard against stress disorders. Central administration of CRF significantly elevates oxytocin secretion both in
male and female rats (9). On the other hand, central oxytocin attenuates CRF mRNA expression in the PVN in response to restraint stress (50). This suggests that oxytocin may have a direct or indirect influence on CRF production and/or secretion from the PVN through a negative feedback mechanism (37).

We studied the changes of CRF and oxytocin mRNA expression in the PVN and SON following acute and chronic stress in mice. CRF mRNA expression in PVN and SON was significantly increased following acute restraint stress, which was reduced to control levels at day 5 of chronic repeated stress. In contrast, oxytocin mRNA expression was significantly increased following chronic repeated stress. Further analysis showed that there was a significant positive correlation between oxytocin mRNA expression and gastric emptying e. To our knowledge, this is the first demonstration of up-regulated oxytocin expression at the hypothalamus following chronic repeated stress in mice.

Plasma corticosterone levels are significantly reduced following chronic repeated stress in mice (38) and rats (55), suggesting the attenuation of HPA axis following chronic stress. Oxytocin attenuates stress-induced activity of HPA axis and thus modulates the regulation of stress response (50), it is very likely that chronic repeated stress may up-regulate oxytocin expression at the PVN and SON, which in turn attenuates CRF expression and HPA activity. Further studies are needed to elucidate the CRF-oxytocin interactions in the adaptation mechanism following chronic repeated stress.

The mechanism of up-regulation of oxytocin expression following chronic repeated stress remains to be determined. Ghrelin plays an important role in mediating gastric emptying (6)
and interdigestive gastric contractions (5, 54) in rodents. It has been shown that the expression of gastric ghrelin is up-regulated following chronic stress in rats (36, 55) and mice (23). Plasma ghrelin levels are increased by beta-adrenergic agonists (18) and sympathetic nerve stimulation (30) in rats. We have previously showed that restraint stress stimulates the sympathetic pathway in rats (31). Thus, the up-regulation of ghrelin expression might be explained by activated sympathetic nerves following chronic stress (23). Ghrelin released from the stomach has been shown to activate various neuropeptides and neuronal circuits at the CNS (1, 11). We cannot exclude the possibility that increased gastric ghrelin expression may directly or indirectly stimulate oxytocin expression during the adaptation process following chronic stress.

Another possibility for the mechanism of up-regulated oxytocin expression following chronic repeated stress is the lack of feed back system. Glucocorticoids secreted in response to stress activate the HPA axis and cause negative feedback onto the hypothalamus suppressing oxytocin secretion (12). Reduced plasma cortisol levels following chronic repeated stress may result in up-regulation of oxytocin expression.

Although mice 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. A better understanding of the mechanism of stress-induced GI tract dysfunction and how adaptation to stress takes place may lead to better treatments for these disorders in humans.

**Perspectives and Significance**

Functional GI disorders are frequently associated with continuous life stress (chronic stress). In our modern society, most individuals encounter both mental and social stress
on daily basis. GI symptoms may develop as a result of accumulation of daily stress in some individuals, however others will adapt to the stressful environment without developing GI symptoms. Our current study reveals that delayed gastric emptying observed in acute stress loading was completely restored following repeated chronic stress in mice. The adaptation mechanism involves up-regulation of oxytocin expression and down-regulation of CRF expression. Our study contributes to the better understanding of the mechanism of stress-induced functional GI disorders.
References


Figure Legends:

**Fig. 1** Effect of acute and chronic restraint stress on solid gastric emptying. Restraint stress significantly delayed the gastric emptying at the day 1 and the day 3. In contrast, the delayed gastric emptying was completely restored following chronic stress at the day 5 ($n=6$, *P*<0.05, **P**<0.01).

**Fig. 2** Effect of acute and chronic repeated restraint stress on postprandial gastric motility (a) and motility index (b). Acute restraint stress significantly attenuated gastric contractions during the restraint stress loading. The reduced gastric motility in response to acute stress was gradually restored following the repeated stress loading (at the day 2 and day 4). On the day 5, restraint stress had no more inhibitory effects on gastric contractions (b) ($n=5$, **P**<0.01).

**Fig. 3** Effect of icv- injection of oxytocin (0.5 µg) on gastric emptying during acute restraint stress. Icv-injection of oxytocin completely restored delayed gastric emptying induced by acute stress ($n=6$, *P*<0.05). A two-way ANOVA showed a significant difference in gastric emptying between control and acute stress group ($F_{1,19}=25.68$; $p<0.001$).

**Fig 4** Effect of icv-injection of oxytocin (0.5 µg) on gastric motility (a) and motility index (b) during acute stress loading. Icv-injection of oxytocin completely restored the attenuated gastric motility during acute stress loading (a). The calculated
motility index showed that the reduction of gastric contractions induced by acute stress was no more observed in mice treated with oxytocin (n=5, ** P<0.01).

**Fig. 5** Effect of icv- injection of oxytocin antagonists on gastric emptying during chronic repeated restraint stress loading. In chronically restraint mice, icv- injection of oxytocin antagonists, tocinoic acid (2 µg) and [d(CH₂)₅¹,Tyr(Me)²,Orn⁸]-Oxytocin (100 ng) significantly antagonized the restored gastric emptying (n=6, ** P<0.01), compared to that of the saline injected mice. In contrast, icv- injection of oxytocin antagonists did not alter delayed gastric emptying induced by acute restraint stress. Two-way ANOVA indicated a significant difference in gastric emptying between acute and chronic stress group (F ₂,₉₈= 8.95; p < 0.01).

**Fig. 6** Effect of icv-injection of an oxytocin antagonist, [d(CH₂)₅¹,Tyr(Me)²,Orn⁸]-Oxytocin (100 ng) on gastric motility following chronic repeated restraint stress. At the day 1, restraint stress significantly attenuated gastric motility, which was partially restored at the day 3 and completely restored at the day 5. The administration of an oxytocin antagonist significantly antagonized the restored gastric motility following chronic stress loading. MI was reduced to 51.6 ± 1.9% (n=5, P<0.05) of basal by an oxytocin antagonist.

**Fig. 7** Effect of acute and chronic repeated restraint stress on CRF mRNA expression (A) and oxytocin mRNA expression (B) in the PVN and SON. CRF mRNA expression showed a significant increase in response to acute stress. The increment of CRF
mRNA was much less at the PVN following chronic stress, compared to that of acutely stressed mice. Oxytocin mRNA expression at the PVN and SON showed a more pronounced increase following chronic stress, compared to that of acutely stressed rats. The mRNA expression was standardized with the ratio of internal control, β actin (n=6, *P < 0.05, **P < 0.01 compared with controls).

**Fig. 8** Correlation between oxytocin mRNA expression and gastric emptying in the PVN and SON in control (a), acute stress (b) and chronic repeated stress (c) in mice. There was a significant positive correlation observed between oxytocin mRNA expression and gastric emptying (n=6, p<0.05).
Figure a: Graph showing motility index over different days with and without restraint stress.

Figure b: Bar graph comparing motility index before, during, and after stress on different days.