Central Neuropeptide Y Plays an Important Role in Mediating the Adaptation Mechanism Against Chronic Stress in Male Rats

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Exposure to continuous life stress often causes gastrointestinal (GI) symptoms. Studies have shown that neuropeptide Y (NPY) counteracts the biological actions of corticotrophin-releasing factor (CRF) and is involved in the termination of the stress response. However, in chronic repeated restraint stress (CRS) conditions, the actions of NPY on GI motility remain controversial. To evaluate the role of NPY in mediation of the adaptation mechanism and GI motility in CRS conditions, a CRS rat model was set up. Central CRF and NPY expression levels were analyzed, serum corticosterone and NPY concentrations were measured, and GI motor function was evaluated. The NPY Y1 receptor antagonist BIBP-3226 was centrally administered before stress loading, and on days 1 through 5 of repeated stress, the central CRF and the serum corticosterone concentrations were measured. In addition, gastric and colonic motor functions were evaluated. The elevated central CRF expression and corticosterone concentration caused by acute stress began to fall after 3 days of stress loading, whereas central NPY expression and serum NPY began to increase. GI dysmotility also returned to a normal level. Pretreatment with BIBP-3226 abolished the adaptation mechanism and significantly increased CRF expression and the corticosterone concentration, which resulted in delayed gastric emptying and accelerated fecal pellet output. Inhibited gastric motility and enhanced distal colonic motility were also recorded. CRS-produced adaptation, overexpressed central CRF, and GI dysmotility observed in acute restraint stress were restored to normal levels. Central NPY via the Y1 receptor plays an important role in mediating the adaptation mechanism against chronic stress. (Endocrinology 159: 1525–1536, 2018)

Stress is widely believed to play a major role in the pathogenesis of many diseases. Gastrointestinal (GI) dysmotility may develop as a result of the accumulation of continuous or repeated stress in some individuals (1).

Corticotrophin-releasing factor (CRF) in the brain plays a substantial role in the central nervous system mediation of stress-induced GI dysmotility (2, 3) via central CRF receptors and the peripheral autonomic nervous system (4, 5). Chronic repeated restraint stress (CRS) produces adaptation, whereas GI dysmotility observed in acute restraint stress (ARS) was restored to normal levels in rodents (6, 7). However, in chronic complicated stress, no adaptation developed (8, 9). Thus, stress habituation or adaptation is likely to be an important mechanism to maintain optimal physiological and psychological functions in the face of repeated stress.

Neuropeptide Y (NPY) is a 36–amino acid peptide belonging to the pancreatic polypeptide family that is widely expressed in the mammalian nervous system, particularly in the arcuate nucleus of the hypothalamus (ARC), the nuclei of the amygdala, the bed nucleus of the stria terminalis, and the hippocampus (10, 11). NPY is also

Abbreviations: ARC, arcuate nucleus of the hypothalamus; ARS, acute restraint stress; CRF, corticotrophin-releasing factor; CRS, chronic repeated restraint stress; ELISA, enzyme-linked immunosorbent assay; FGID, functional gastrointestinal disorder; FPO, fecal pellet output; GE, gastric emptying; GI, gastrointestinal; HPA, hypothalamic-pituitary-adrenal; ICV, intracerebroventricular; MI, motility index; mRNA, messenger RNA; NE, norepinephrine; NPY, neuropeptide Y; NS, nonstressed; OXT, oxytocin; PCR, polymerase chain reaction; PTSD, posttraumatic stress disorder; PVN, paraventricular nucleus.
synthesized and released from peripheral sympathetic neurons (12, 13). Several studies have shown that NPY is involved in stress-related disorders, such as depression, anxiety, and posttraumatic stress disorder (PTSD) (14, 15).

In particular, reports indicate that NPY is crucial for the stress adaptation process. NPY interacts with the hypothalamic-pituitary-adrenal (HPA) axis, counteracts the biological actions of CRF, and is involved in the termination of the stress response (13). Endogenous NPY has anxiolytic properties (16) and may act as a buffer that promotes behavioral adaptation to stress, including moderating and improving the body’s ability to cope with stress (17). In addition, an important fiber tract containing NPY exists between the ARC and the paraventricular nucleus (PVN), the major source of CRF, allowing crosstalk between these two neuropeptide systems (18).

The actions of NPY are mediated through at least five G protein–coupled receptors (17, 19). The Y1 and Y2 receptor subtypes are present at significant levels within the central nervous system. The anxiolytic effect of NPY is mediated primarily through the postsynaptic Y1 receptor (13, 16). In contrast to the Y1 receptor, the Y2 receptor has an anxiogenic effect (20, 21). In stress adaptation, there is only a moderate to poor expression of the Y2 receptor in numerous centers and thus may not play a major role in the adaptation mechanism (22).

Previous studies showed that central NPY expression varies depending on the type of stressor and stress-loading duration (23, 24). In addition, the actions of NPY on the regulation of GI motility under stress conditions remain controversial (25–27). In particular, the effect of NPY on GI motility under chronic stress conditions is not well studied.

Therefore, the current study aimed to evaluate whether NPY is involved in the adaptation mechanism in CRS condition, as well as the effects of NPY on the HPA axis’ activity and GI motility. Our work will contribute to the further studies on stress-induced abnormal GI motility and will help to determine the possible physiological adaptation mechanisms following chronic stress.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (laboratory animal center of the China Medical University, Shenyang, China), weighing 260 to 300 g, were housed in individual cages under conditions of controlled temperature (22°C to 24°C) and illumination (12-hour light:12-hour dark cycle starting at 6:00 AM) for at least 7 days before the experiment. Rats were fed with commercial pelleted feed from the Xietong Organism Institute (Nanjing, China) and water ad libitum.

For monitoring of gastric and colonic motility, the rats on a fixed-feeding schedule (food administered 2:00 to 6:00 pm daily) were monitored from 8:00 AM to 4:00 PM for gastric and colonic motility, as previously reported (6, 7).

All experiments were started at 9:00 AM each day. Animal procedures were reviewed and approved by the Animal Care and Use Committee of the China Medical University and conducted according to the guidelines of the laboratory animal ethical standards of the China Medical University.

Fasten restraint stress

For acute fasten restraint stress loading, rats (n = 6 to 8) were placed on a wooden plate with their trunks wrapped in a confining harness for 90 minutes. This stress stress has been used as a physical and psychogenic stress model in rodents (2). For CRS study, the rats were divided into three groups (n = 6 to 8) and received the stress restraint loading for 3, 5, and 7 consecutive days, respectively. For the control groups, the rats were also divided into three groups (n = 6 to 8), and the rats were housed in original individual cages for 90 minutes for 3, 5, and 7 consecutive days, respectively, but had limited access to food and water, as previously reported (7, 8).

Blood collection and hormone assays

The experimental rats were euthanized immediately by pentobarbital sodium (Sigma-Aldrich, St. Louis, MO; 200 mg/kg intraperitoneal injection) after stress loading. At the time of rats’ death, trunk blood was collected immediately via a cardiac puncture, and then the samples were allowed to clot in tubes and centrifuged at 4°C for 10 minutes at 3000 rpm to separate out the serum. The serum fraction was stored at −80°C for further analysis. Corticosterone concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a corticosterone ELISA kit (Assay Design, Ann Arbor, MI). NPY concentrations were measured by an NPY ELISA kit (Phoenix Pharmaceuticals, Belmont, CA). The absorbance was read at 450 nm. All procedures were carried out according to the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction

The rat brain tissue micropunching technique was applied for acquiring hypothalamic tissue samples from specific regions with micropunchers. Briefly, after stress loading, the experimental rats were euthanized and the brains were removed immediately and cut into 450-μm coronal sections. The inner diameter of the punch needle is 1 mm. Punches were collected from the left and right PVN (1.8 mm caudal to bregma; 0.4 mm lateral to midline; 7.6 mm ventral to the brain surface), as previously reported (8). Also the arcuate nucleus was bilaterally collected (an approximate interval of −2.00 to −3.00 mm, relative to bregma), as previously reported (28). All coordinates were based on the rat brain atlas and hypothalamic images of previously reported (29, 30).

Samples were stored at −80°C until use. Total RNA was extracted from the brain tissues using Trizol (Invitrogen, Carlsbad, CA), and trace DNA contamination was removed by DNase digestion (Promega, Madison, WI). Complementary DNA was synthesized from 3 μg total RNA by use of SuperScript III reverse transcription (Invitrogen).

The following primers were designed to amplify rat NPY (83 bp; accession no. NM_012614.2), as previously reported.
(31): sense primer 5'-CAGAGGCCGCCAGACG-3' and antisense primer 5'-CAGCCCATCTGTGTTACC-3'. And the following were designed to amplify rat CRF (81 bp; accession no. NM_031019): sense primer 5'-CCAGGGCAGAGCAGTTAGCT-3' and antisense primer 5'-CAAGCCGCAATTTGATTCC-3'. For an internal control, the following were designed to amplify rat β-actin fragment (106 bp; accession no. 118505324): sense primer 5'-GGGATCTTTCTTTACAGAG-3' and antisense primer 5'-GGGTATCTTTTCCGTTG-3', as previously reported (8).

Quantitative polymerase chain reaction (PCR) was performed by using SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China). Amplification reactions were performed using the ABI 7500 Real-Time PCR Instrument (Applied Biosystems, San Mateo, CA). Initial template denaturation was performed for 30 seconds at 95°C. The cycle profiles were programmed as follows: 5 seconds at 95°C (denaturation), 20 seconds at 60°C (annealing), and 15 seconds at 72°C (extension). Forty-five cycles of the profile were run, and the final cooling step was continued for 30 seconds at 40°C. Quantitative measurements of each messenger RNA (mRNA) sample were achieved by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. Amplicon size and specificity were confirmed by melting curve analysis. The relative amount of each mRNA was normalized by the amount of β-actin mRNA, as previously reported (8).

ICV cannulation and administration of NPY receptor antagonist

The rats were anesthetized with isoflurane (2%; RWD Life Science, Shenzhen, China) and placed in a stereotaxic apparatus (RWD Life Science). After the skin and muscles of the head were dissected, a 24-gauge plastic sterile cannula was implanted into the right lateral ventricle (1.5 mm caudal; 2 mm lateral from the bregma; 6 mm ventral from the skull surface), as previously reported (8). The cannula was fixed with cement (Kyowa, Tokyo, Japan) and acrylic resin (Shofu, San Marcos, CA). After cannulation, the rats were allowed to recover for 1 week.

The rats were divided into four groups (n = 6 to 8): non-stressed (NS) rats and ARS (day 1) and 3- and 5-day repeated restraint stress (day 3 and day 5) groups.

NPY Y1 receptor antagonist BIBP-3226 (200 nmol/5 μL in 0.15 M saline; Sigma-Aldrich, Natick, MA) was injected intracerebroventricularly daily 15 minutes before stress loading. Intracerebroventricular (ICV) administration of BIBP-3226 was effective in antagonization of NPY Y1 receptor subtypes without any side effects, as reported before (32–34).

For the control groups (n = 6 to 8), the rats were injected with 5 μL saline intracerebroventricularly and then housed in original individual cages for 90 minutes for 1, 3, and 5 consecutive days, respectively, as mentioned previously.

At the end of the experiment, the implantation site of ICV cannula was confirmed by the presence of Evans blue (5%, 1 μL; Sigma-Aldrich, St. Louis, MO) after injection via the cannula, as previously reported (8).

Measurement of solid GE

Rats were fasted for 24 hours prior to the measurement of gastric emptying (GE). The standard rodent pellets (1.6 g) were provided, and the rats that did not consume 1.6 g of food within 10 minutes were excluded from the study. For the control group, the rats, after finishing the feeding, were back to their original cages for 90 minutes, but with limited access to food and water. Then the rats were euthanized by pentobarbital sodium. For the stress group, immediately after finishing the feeding, the rats were subjected to restraint stress for 90 minutes. Then the experimental rats were euthanized as mentioned previously. The stomach was surgically isolated and removed. The gastric contents were recovered from the stomach, dried, and weighed. Solid GE was calculated according to the following formula, as previously described (7, 8):

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

Measurement of fecal pellet output

Rats were exposed to restraint stress treatment of 90 minutes, as mentioned previously, and the fecal pellet output (FPO) numbers were counted after stress loading as measure of colonic transit function. For the control group, the rats after finishing the feeding were back to their original cages for 90 minutes, and the expelled fecal pellets were also counted.

Monitoring of gastric and colonic motility

Rats were anesthetized with isoflurane (2%). Strain gauge transducers were implanted on the antrum for recording gastric contractions and distal colon for colonic contractions. 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). The abdominal wall was closed, and rats were allowed to recover for 7 days. Rats on a fixed-feeding schedule (food administered 2:00 to 6:00 PM daily) were monitored from 8:00 AM to 4:00 PM for gastric and colonic motility, as previously reported (6, 35).

The wires from the transducers were connected to a recording system (Power Laboratory 8SP; AD Instruments, Colorado Springs, CO). Gastric and colonic contractions were monitored before, during, and after restraint stress. Quantification of gastric motility and colonic 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 Laboratory; AD Instruments), as previously reported (6, 35).

Experimental design

Experiment 1

In the CRS rat model, central CRF (in PVN) and NPY (in ARC and PVN) mRNA expression were analyzed, serum corticosterone and NPY concentrations were measured, and GI motor function was evaluated by GE and FPO.

Experiment 2

In the CRS rat model, NPY Y1 receptor antagonist BIBP-3226 was administered intracerebroventricularly (daily, before stress loading), the central CRF expression and the serum corticosterone concentrations were measured, and gastric and colonic
motor functions were also evaluated by GE, FPO, and a motility recording system.

Statistical analysis

Analysis was performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL). Results were shown as mean ± standard error of the mean. Statistical analyses were performed using a two-way classification analysis of variance. Experiment 1 was a 2 x 5 factorial design, and statistical significance was considered with a Bonferroni-adjusted α level of P < 0.01. Experiment 2 was a 2 x 4 factorial design, and statistical significance was considered with a Bonferroni-adjusted α level of P < 0.0125.

Results

Experiment 1

Effects of chronic stress on central CRF and NPY mRNA expression

CRF mRNA expression in the PVN showed no significant difference between the NS rats and the control group (n = 6). However, CRF mRNA expression increased significantly in response to acute stress (day 1, n = 6, P < 0.01) and then began to decrease from day 3. On days 5 and 7, there was no significant difference observed between the restraint group and the control group (n = 6; Fig. 1A). NPY mRNA expression in the ARC showed no significant increase in response to acute stress (day 1). On day 3, NPY mRNA expression increased significantly (n = 6, P < 0.01); however, on days 5 and 7, there was no significant difference observed between the restraint group and the control group (Fig. 1B). NPY mRNA expression in the PVN showed significant increase on day 3 (n = 6, P < 0.01); however, at days 5 and 7, there was no significant difference observed between the restraint group and the control group (n = 6; Fig. 1C).

Effects of chronic stress on serum corticosterone and NPY levels

Serum corticosterone levels in NS rats were 20 ± 3 μg/dL (n = 6). Post-stress serum corticosterone levels significantly increased to 82 ± 5 μg/dL (n = 6, P < 0.01) at day 1 in restrained rats and then began to fall after day 3 (67 ± 6 μg/dL, n = 6, P < 0.01). At day 5 (25 ± 5 μg/dL, n = 6) and day 7 (20 ± 3 μg/dL, n = 6), there was no significant difference observed between the restraint group and the control group (Fig. 2A). The serum NPY level in the NS rats was 1.7 ± 0.1 ng/mL (n = 6). The serum NPY level did not increase significantly in response to acute stress (day 1, 1.7 ± 0.1 ng/mL, n = 6). However, the NPY levels at day 3 increased significantly to 2.2 ± 0.2 ng/mL (n = 6, P < 0.01). At day 5 (2.1 ± 0.1 ng/mL, n = 6) and day 7 (1.8 ± 0.2 ng/mL, n = 6), there was no significant difference observed between the restraint group and the control group (Fig. 2B).

Figure 1. Effects of chronic stress on central CRF and NPY mRNA expression. (A) The CRF mRNA expression in the PVN of the hypothalamus on days 1, 3, 5, and 7 of repeated restraint stress. CRF on days 1 and 3 were significantly increased in response to repeated restraint stress. (B) The NPY mRNA expression in the ARC. NPY on day 3 was significantly increased in response to repeated restraint stress. (C) The NPY mRNA expression in the PVN of the hypothalamus. NPY on day 3 significantly increased in response to repeated restraint stress. The mRNA expression was standardized with the ratio of internal control of β-actin. (n = 6, *P < 0.01 compared with controls.)

Effects of chronic stress on GE and FPO

In the NS rats, solid GE was 52 ± 4% (n = 6). GE was delayed significantly in rats that received restraint stress for 90 minutes at day 1 (27 ± 3%, n = 6, P < 0.01). Delayed GE was restored to the normal level at day 5 (55 ± 3%, n = 6) and day 7 (59 ± 3%, n = 6; Fig. 3A). In the NS rats, the FPO was 2.8 ± 0.6 (number/90 min, n = 6). The FPO increased significantly in rats that received restraint stress for 90 minutes at day 1 (9.8 ± 0.8, n = 6, P < 0.01) and day 3 (6.8 ± 0.7, n = 6, P < 0.01). The FPO was restored to normal levels at day 5 (3.3 ± 0.5, n = 6) and day 7 (3.5 ± 0.4, n = 6; Fig. 3B).
Experiment 2

Effects of an NPY Y1 receptor antagonist on central CRF mRNA expression and corticosterone concentration in response to CRS

In the NS group, ICV-administered NPY Y1 receptor antagonist BIBP-3226 (200 nmol/5 µL) did not change the CRF mRNA expression significantly (n = 6). BIBP-3226 also had no effect on CRF mRNA expression in the acute stress loading group (day 1). Meanwhile on days 3 and 5 in the repeated restraint stress group, BIBP-3226 ICV administration (daily, before stress loading) significantly increased the CRF mRNA expression (n = 6, P < 0.0125, 5 µL saline injected intracerebroventricularly as a control; Fig. 4A). ICV injection of BIBP-3226 had no effect on the GE and FPO in the NS group or in the repeated restraint stress loading group on days 1 and 3. Meanwhile, on day 5 in the restraint stress group, ICV-administered BIBP-3226 significantly increased the serum corticosterone concentration (from 26 ± 5 µg/dL to 77 ± 5 µg/dL, n = 6, P < 0.0125, 5 µL saline injected intracerebroventricularly as a control; Fig. 4B).

Effects of the NPY Y1 receptor antagonist on gastric and colonic motility recording in response to CRS

In the NS group, ICV injection of BIBP-3226 had no effect on the amplitude and frequency of gastric phase III–like contractions, or on the amplitude of distal colonic contraction (data not shown). ARS (day 1) abolished gastric phase III–like contractions in the saline group (5 µL saline injected intracerebroventricularly as a control) and abolished the contractions in the BIBP-3226 group (ICV injection of the NPY1 receptor antagonist BIBP-3226, 200 nmol/5 µL before stress loading; Fig. 6A). ARS (day 1) enhanced the amplitude of distal colonic contraction in the saline group, and in the BIBP-3226 group (Fig. 6B). Gastric phase III–like contractions were restored partially at day 3 in the repeated restraint saline group, whereas in the BIBP-3226-administered groups, the contractions were still abolished (Fig. 6C).
The amplitude of the distal colonic contractions was partially attenuated after 3 days of repeated restraint stress, whereas in the BIBP-3226 groups, the contractions remained enhanced (Fig. 6D). Gastric phase III–like contractions were completely restored after 5 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, gastric phase III–like contractions were still abolished (Fig. 6E). The amplitude of the distal colonic contractions was completely restored after 5 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, the amplitude of distal colonic contractions remained enhanced (Fig. 6F). Each recording experiment was individually repeated at least three times and similar results were obtained (5 μL saline injected intracerebroventricularly as a control).

**Effects of the NPY Y1 receptor antagonist on gastric and colonic MI changes in response to CRS**

In the NS group, ICV-administered NPY Y1 receptor antagonist BIBP-3226 did not significantly alter the gastric MI change (n = 6; Fig. 7A), as well as in the acute stress loading group (day 1) and on day 3 in the restraint stress group. However, the restored gastric MI change on day 5 in the repeated restraint stress groups was significantly attenuated (from 97% ± 11% to 47% ± 9%), compared with that of the ICV saline-injected stress group (5 μL saline injected intracerebroventricularly as a control, n = 6, #P < 0.0125 compared with control).

**Discussion**

Functional gastrointestinal disorders (FGIDs), such as functional dyspepsia and irritable bowel syndrome, are common pathologies of the gut. The pathogeneses of functional dyspepsia and irritable bowel syndrome are highly associated with stress in humans (1, 36). GI dysmotility might develop as a result of the accumulation of continuous or repeated stress in some individuals, whereas others are able to adapt to a stressful environment without developing GI symptoms.
Restraint stress has been used frequently as a physical and psychogenic stress model in rodents. ARS stimulates CRF release in the PVN of the hypothalamus, which plays a dominant role in influencing motor function of the GI tract. CRF delays GE via central CRF2 receptors and sympathetic pathways (4). However, the accelerated colon transit induced by ARS is mediated via central CRF1 receptors and parasympathetic pathways in rats (5).

In contrast to ARS, repeated experiences with the same stressor produce habituation or diminution of behavioral responses (23). In the current study, we developed a CRS model and found that the upregulated HPA axis (central CRF expression and serum corticosterone level) caused by ARS was attenuated on days 5 and 7 of consecutive stress loading. ARS-induced GI dysmotility, such as delayed GE and accelerated FPO, gradually returned to normal levels. These results are consistent with previous studies (6, 7).

CRF is the main mediator of the response to stress. ARS stimulates CRF in the amygdala and PVN of the hypothalamus, resulting in the activation of the HPA axis (2, 37).

In contrast, CRS significantly lowered the number of CRF-positive neurons and CRF mRNA expression in the PVN (8, 38). Plasma adrenocorticotropic hormone and corticosterone levels are also significantly reduced following chronic repeated stress (8, 39), suggesting that the attenuation of the HPA axis following CRS is caused by reduced CRF expression in the hypothalamus. In this respect, the results of the current study are consistent with previous studies (6, 7). Furthermore, intracisternal injection of CRF still had an inhibitory effect on GE in chronically stressed rats, even though chronic stress showed no further inhibitory effects on GE. This suggested that the inhibitory pathway in response to central CRF is not altered following chronic stress (7).

CRF exerts its action through the activation of the CRF1 and CRF2 receptors, not only in the central nervous system, but also in peripheral tissues (4, 5). To date, there has been no report of whether and how central CRF signaling interacts with peripheral CRF signaling.

Figure 6. Effects of ICV injection of an NPY Y1 receptor antagonist on gastric and colonic motility in response to CRS. (A) The gastric phase III-like contractions in the saline and BIBP-3226 groups during ARS (day 1) loading. (B) The distal colonic contraction in the saline and BIBP-3226 groups during ARS (day 1) loading. (C) Gastric phase III-like contractions were restored partially after 3 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, gastric phase III-like contractions were still abolished. (D) The amplitude of the distal colonic contractions was partially attenuated after 3 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, the contractions were still enhanced. (E) Gastric phase III-like contractions were completely restored after 5 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, the contractions were still abolished. (F) The amplitude of distal colonic contractions was completely restored after 5 days in the repeated restraint stress group, whereas in the BIBP-3226 groups, the contractions were still enhanced. (5 μL saline injected intracerebroventricularly as a control; BIBP-3226: ICV injection of NPY1 receptor antagonist BIBP-3226 200 nmol/5 μL, daily, before stress loading.)
However, there is evidence that peripheral CRF acts on myenteric neurons, which determine the activity of colonic functions during stress (40). However, the precise mechanism of altered gastric motility induced by peripheral CRF or stress has not been clarified. Further studies are needed to elucidate these issues.

Oxytocin (OXT) is a cyclic nonapeptide hormone synthesized in the hypothalamus. In addition to its well-known physiological functions in lactation and induction of labor, studies also suggest that central OXT has an anxiolytic effect and attenuates the HPA axis in response to stress, and OXT has even been suggested as a likely candidate to treat patients with PTSD (41–43). Our previous studies also found that under CRS conditions, accompanied by downregulated HPA axis activity, hypothalamic OXT is upregulated, suggesting that it plays an important role in mediating the stress adaptation mechanism (8, 9). However, the hypothalamus is not the only source of CRF, which is widely distributed within the nervous system (44, 45), whereas OXT neurons and OXY receptors are less widespread in the central nervous system (46). OXT has no significant effect on the HPA axis activity in the amygdala region under complicated stress conditions (47). As mentioned previously, hypothalamic OXT might only react to moderate or slight degrees of chronic stress. Thus, other key factors should be involved in the adaptation mechanism to chronic stress.

NPY is one of the most widely expressed neuropeptides in the central nervous system, and its broad distribution suggests its involvement in numerous physiological processes, such as regulation of feeding, energy homeostasis, daily rhythms, neuronal excitability, and related seizures (12, 13). Several studies also indicated that NPY is involved in stress-related disorders, such as depression, anxiety (14, 48), and PTSD (22). Endogenous NPY has anxiolytic properties (16, 49, 50). NPY might interact with the HPA axis, thereby counteracting the biological actions of CRF, and might be involved in the termination of the stress response (13).

NPY expression in the brain under stress conditions has been well studied; however, the magnitude and the direction of stress-induced NPY alterations heavily depend on the type and duration of the stress. For example, NPY gene expression in the amygdala and ARC is increased after foot shock stress (51, 52), but was unaltered by mild stress loading comprising acute water avoidance stress and acute air puff stress (24, 25). However, in studies of restraint stress loading, which is a moderate degree of stimulus, the NPY gene expression was still debatable: some studies showed increased expression (53, 54), and unaltered or decreased expression has also been reported (23, 55). In studies of chronic stress, central NPY expression is usually upregulated in response to repeated stress loading (54, 56). Thus, as mentioned previously, NPY may usually react to acute stress rather than moderate or chronic stress.

Within the brain, the amygdala (together with the lateral septum, periaqueductal gray matter, locus coeruleus, etc.) gets projection of the NPY pathways originating in the ARC of the hypothalamus (57), where the highest levels of NPY mRNA are found (58). Moreover, an important fiber tract containing NPY exists.
between the ARC and the PVN, allowing crosstalk between these two neuropeptide systems (18). Thus, it was reasonable to clarify NPY expression in the ARC of the hypothalamus under stress conditions.

In line with previous findings, the current study observed that hypothalamic NPY mRNA expression (both in the ARC and the PVN) did not change after day 1 of stress loading but significantly increased on day 3 of consecutive stress loading; however, by days 5 and 7, there were no more significant increases. This indicated that, initially, hypothalamic NPY might react to repeated restraint stress loading, and then subsequently, amygdala-expressed NPY is upregulated, as previously reported (54, 55). Thus further investigation of the expression of NPY in the extrahypothalamic area in chronic stress conditions is required.

The results of the current study support those of a previous report, in which stressors initiated rapid CRF release within the CNS and in which NPY release is thought to follow during a later phase to mediate an adequate termination of the acute response. Even after repeated stress exposure, NPY is upregulated as a coping mechanism (13). It was proposed that upregulation of NPY expression might contribute to successful behavioral adaptation to stress.

The circulating plasma NPY levels are in the low range under resting conditions; however, in many stress conditions, NPY levels were related to the intensity and duration of stress. For example, mild and brief stress tests failed to increase the plasma NPY level; however, it increased in an intensity-dependent manner during foot shock stress and cold restraint stress, but not during immobilization (59). In the peripheral nervous system, NPY is found in three main pools: sympathetic nerves, the adrenal medulla, and platelets. The release of NPY is dependent on the intensity and the pattern of sympathetic nerve activation (59). Norepinephrine (NE) is widely considered to be the primary sympathetic neurotransmitter, whereas NPY is a cotransmitter in the sympathetic nervous system (60). Physiologically, NPY is released during stress, along with NE, but the proportions of these two transmitters vary depending on the type of stress. NE is secreted during the mildest acute stress; however, the release of NPY requires a more prolonged and/or intense stimulation of sympathetic nerves (61). In line with these findings, the fasten restraint stress in our present study is a moderate stress stimulus, and we found that serum NPY levels did not increase significantly in response to ARS but were significantly increased at day 3 of CRS, before leveling off on days 5 and 7. This is in agreement with previous studies (59, 61). The current study was focused on the role of central NPY in mediating the adaptation mechanism against chronic stress; therefore, we did not study the effect of the Y1 receptor in peripheral administration. Further investigation is needed to determine the role of peripheral NPY in chronic stress conditions.

The significance of NPY in stress adaptation seems to be receptor dependent. NPY shows strong affinity for the Y1, Y2, and Y5 receptors. Whereas the Y1 and Y2 receptors are widely distributed throughout the brain (17, 57), the Y5 receptor occurs in several limbic brain areas, but is less abundant than the Y1 or Y2 receptor (19).

Many studies have demonstrated that the anxiolytic behavioral effect of NPY is mediated primarily through postsynaptic Y1 receptors (13, 57). The Y2 receptor in the brain shows a similar distribution to the Y1 receptor. However, in numerous centers involved in stress adaptation, the Y2 receptor shows only low or moderate expression. This indicated that the Y2 receptor might not play a major role in the chronic stress adaptation mechanism (14, 19).

In the CRS model used in the current study, to detect whether central NPY is involved in the adaptation mechanism, the rats were treated centrally (intracerebroventricularly) with the NPY receptor Y1 antagonist BIBP-3226 before stress loading. The nonpeptide NPY Y1 receptor antagonist BIBP-3226 acts both in vitro and in vivo and has no affinity for the Y2, Y4, and Y5 receptors (13). ICV administration of BIBP-3226 effectively antagonized the NPY Y1 receptor subtype without any side effects, as recently reported (32–34). However, a previous study also found BIBP-3226 might produce nonreceptor-mediated effects, such as inhibiting NPY-induced food intake in a nonspecific manner (62), and BIBP-3226 also has affinity for neuropeptide FF receptors, which is a part of a neurotransmitter system acting as a modulator of endogenous opioid functions (63). Thus, the nonreceptor-mediated effects of BIBP-3226 in chronic stress conditions should be investigated in a future study.

However, in the current study, we found that under CRS conditions, BIBP-3226 significantly increased CRF mRNA expression and the serum corticosterone concentration. BIBP-3226 also attenuated gastric motility and accelerated colonic transit. These results indicated that BIBP-3226 abolished the habituation of behavioral responses in the CRS model; therefore, central NPY might play an important role in mediating the habituation mechanism against chronic stress via the Y1 receptor. Besides the Y1 receptor, NPY also shows strong affinity for the Y5 receptor. The Y5 receptor may be involved in antistress effects (58), and in some cases, the Y5 receptor, rather than the Y1 receptor, appears to mediate NPY's anxiolytic action (21). Thus, further investigation of the role of the Y5 receptor in chronic stress conditions is required.
Previous studies on the effects of NPY on GI motility indicated that NPY inhibits GI motility under acute stress conditions (25, 26). However, another study showed NPY is not required for an inhibition of GE during stress conditions (27). Thus, the actions of NPY on the regulation of GI motility under stress conditions remain controversial, especially under chronic stress conditions. The current study found that in the CRS models, the rats underwent habituation, during which GI dysmotility caused by acute stress returns to normal levels. Meanwhile, treatment with the NPY Y1 receptor antagonist BIBP-3226 prior to stress loading significantly attenuated the restored GI motility, resulting in impaired gastric motility and accelerated colon transit, again indicating that central NPY might play an important role in mediating the habituation mechanism against the chronic stress via the Y1 receptor.

As we mentioned previously, many studies have documented that NPY usually reacts to acute stress more than moderate or chronic stress and that NPY is crucial for the stress adaptation process; NPY interacts with the HPA axis, counteracts the biological actions of CRF, and is involved in the termination of the stress response. The current study, therefore, sought to evaluate, in a chronic repeated stress condition, whether NPY is involved in the adaptation mechanism, as well as the effects of NPY on the activity of the HPA axis and on GI motility. In a rat model, we compared nonstress conditions with ARS and CRS and found NPY via Y1 receptor plays an important role in the response to CRS conditions.

Furthermore, both NPY and OXT are chronic modulatory factors that have anxiolytic effects and are related with the adaptation mechanism. Our present study showed that hypothalamic NPY mRNA expression was upregulated on day 3 of stress in rats, but returned to baseline on days 5 and 7. In our previous study, we showed that OXT mRNA expression was significantly increased on days 5 and 7 of stress loading (8). The highly elevated CRF mRNA expression following ARS was reduced to control levels at day 7 of CRS. It is very likely that CRS upregulates NPY mRNA expression in the PVN of the hypothalamus first. The fasten restraint stress used in our present study is a moderate level of stress loading, and when adaptation developed, NPY was not further elevated. Subsequently, OXT expression in the PVN of the hypothalamus was highly elevated, which, in turn, attenuated CRF expression and HPA activity. Thus, the relationship and interaction between NPY and OXT, and their effects on CRF/HPA axis in chronic stress conditions, should be the subject of further studies.

In conclusion, CRS produced adaptation, and the overexpressed central CRF and GI dysmotility observed in ARS was restored to normal levels. In addition to central OXT, central NPY via the Y1 receptor also plays an important role in mediating the adaptation mechanism against chronic stress. Our study provides a better understanding of the mechanism of stress-induced FGIDs. Determining how this adaptation to stress takes place might lead to better treatments for FGIDs in humans.

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