Central apelin mediates stress-induced gastrointestinal motor dysfunction in rats

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Central apelin mediates stress-induced gastrointestinal motor dysfunction in rats. Am J Physiol Gastrointest Liver Physiol 310: G249–G261, 2016. First published December 17, 2015; doi:10.1152/ajpgi.00145.2015.—Apelin, an endogenous ligand for APJ receptor, has been reported to be upregulated in paraventricular nucleus (PVN) following stress. Central apelin is known to stimulate release of corticotropin-releasing factor (CRF) via APJ receptor. We tested the hypothesis that stress-induced gastrointestinal (GI) dysfunction is mediated by central apelin. We also assessed the effect of exogenous apelin on GI motility under nonstressed (NS) conditions in conscious rats. Prior to solid gastric emptying (GE) and colon transit (CT) measurements, APJ receptor antagonist F13A was centrally administered under NS conditions and following acute stress (AS), chronic homotypic stress (CHS), and chronic heterotypic stress (CHeS). Plasma corticosterone was assayed. Strain gage transducers were implanted on serosal surfaces of antrum and distal colon to record postprandial motility. Stress exposure induced coexpression of c-Fos and apelin in hypothalamic PVN. Enhanced hypothalamic apelin and CRF levels in microdialysates were detected following AS and CHeS, which were negatively and positively correlated with GE and CT, respectively. Central F13A administration abolished delayed GE and accelerated CT induced by AS and CHeS. Central apelin-13 administration increased the plasma corticosterone and inhibited GE and CT by attenuating antral and colonic contractions. The inhibitory effect elicited by apelin-13 was abolished by central pretreatment of CRF antagonist F13A in antrum, but not in distal colon. Central endogenous apelin mediates stress-induced changes in gastric and colonic motor functions through APJ receptor. The inhibitory effects of central exogenous apelin-13 on GI motility appear to be partly CRF dependent. Apelin-13 inhibits colon motor functions through a CRF-independent pathway.

Apelin; stress; corticotropin releasing factor; gastrointestinal motility; microdialysis

The functional gastrointestinal disorders (FGID) are a heterogeneous group of chronic conditions that are considered to have no structural or biochemical abnormalities that account for the symptoms (14, 51, 52, 69). It is well known that stressful and traumatic life events trigger exacerbations of symptoms in FGID patients (14, 77). Most individuals experience multiple types of stress by encountering mental, physical, and social stressors. Exposure to chronic stress can lead prolonged activation of the stress response, impaired brain-gut axis, and autonomic dysfunction, which are considered as the relevant pathways modulating FGID symptoms (6, 30, 63, 64). Interestingly, gastrointestinal (GI) motor disorders may develop in some individuals; however, others are able to adapt to a stressful condition without developing GI symptoms. Corticotropin-releasing factor (CRF) is known to act in the brain to influence the GI motor functions via autonomic neural pathways (6, 36, 63, 64). It has been shown that CRF is upregulated upon stress in hypothalamic paraventricular nucleus (PVN), which in turn accelerates colon transit (CT), while inhibiting gastric emptying (GE) via CRF receptor type-1 (CRF1) and type-2 (CRF2), respectively (46, 50). In rodents, increased hypothalamic CRF mRNA expression induced by acute stress (AS) was found to be downregulated following chronic homotypic stress (CHS) for 5 consecutive days, whereas the increased CRF mRNA expression did not significantly alter upon chronic heterotypic stress (CHeS) loading (4, 10, 84). Likewise, impaired GE and accelerated CT were completely restored following chronic homotypic stress for 5 consecutive days, but not upon 7-day chronic complicated stress loading (1–5, 9, 83, 85). Although latter findings suggest that hypothalamic CRF-producing neurons are involved in maladaptation, the mechanism of the adaptation is not fully understood.

Apelin, the endogenous ligand for the G protein-coupled APJ receptor, was initially isolated from bovine stomach in 1998 (71). Apelin gene encodes the preproapelin molecule, which contains 77 amino acids with a signal peptide in the NH2-terminal region. Several forms of apelin such as apelin-36, apelin-26, apelin-19, apelin-17, apelin-13, and apelin-12 are processed after the cleavage of the signal peptide (29, 71). Among the preproapelin end products, pyroglutamyl form of apelin-13 (pGlu-apelin-13) has been shown to be the most abundant form with a greater affinity for APJ in rat (13, 40). Expression of apelin and APJ receptor mRNA has been shown in a variety of tissues including the heart, lung, alimentary tissues, placenta, ovary, adipose, thyroid gland, mammary gland, and costal cartilage (20, 23, 74, 75).

Besides the peripheral tissues, the apelinergic system is also widely expressed in brain, suggesting that apelin may be involved in regulation of certain central nervous system (CNS) functions (29, 56, 57, 61). The robust expression of APJ receptor in the medial parvocellular and magnocellular regions of PVN suggest that apelin may be involved in stress response. Increased APJ mRNA expression in PVN in response to acute and chronic stress has been shown in rats (49). Apelin has been shown to stimulate the release of hypothalamic vasopressin and CRF in vivo and in vitro studies (48, 67). The increase in plasma corticosterone following central apelin administration is partly blocked by central pretreatment of CRF antagonist α-helical CRF9-41, suggesting that CRF-producing neurons are involved in apelin-induced stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (25, 48). In recent years, there has been a growing body of evidence regarding the involvement of the central apelinergic system in GI motor functions. Centrally...
administered apelin-13 has been shown to inhibit GE and GI transit and to accelerate CT in mice (34, 82). Taken together, these results suggest that central apelin may regulate stress-induced changes in GI motor functions and play a role in maladaptation paradigm.

The aim of the present study is to investigate the role of central apelin in impaired GI motor function under nonstressed (NS), AS, CHS, and CHeS conditions. Many animal studies have been done to investigate the effects of an acute and chronic exposure of a stressor on GI motility. However, relatively few studies have been done on chronic complicated stress in rats. Using a microdialysis method, particularly for neuropeptides, the present study clearly demonstrates the alterations in hypothalamic CRF and apelin under various stress models.

MATERIALS AND METHODS

Animals. Wistar rats weighing 280–300 g were housed under conditions of controlled temperature (22–24°C) and illumination (12-h light cycle starting at 6:30 AM). Rats were allowed ad libitum access to food and water. This study was approved by the Animal Ethical Committee of Akdeniz University and performed with standard guidelines for care and use of laboratory animals. All efforts were made to mitigate animal suffering and to reduce the number of animals in experiments. To minimize the stress, animals were acclimatized to handling for 7 days prior to the surgical or experimental procedures. All animals including the NS (control) rats were housed singly to protect the implanted experimental stuff (probes, cannulas, tubing, etc.) from their cagemates.

Stress protocols. For AS, rats were loaded with restraint stress for 90 min. Briefly, rats were placed on a wooden plate with their trunks wrapped in a confining harness, as previously reported (10, 84, 85). Rats were able to move their limbs and head but not their trunks. This restraint stress model has been used as a physical and psychogenic stress model in rodents (30, 79). For CHS, rats were exposed the restraint stress for 5 consecutive days. The CHeS protocol was comprised of restraint stress, water avoidance stress (WAS), force swimming stress (FSS), and cold restraint stress (CRS). The rats were exposed to two different stressors on the 1st, 3rd, and 5th days, whereas a single stressor was applied on 2nd, 4th, 6th, and 7th days of the CHeS protocol. Morning stressors were applied between 9:00 AM and afternoon stressors were applied at time 2:00 PM (Table 1). For CRS loading, rats were restrained and kept at 4°C for 45 min. The WAS procedure involves placing the animals on a cylindrical platform (10 cm × 20 cm) in the middle of a plastic container (50 cm × 30 cm × 30 cm) filled with water at room temperature to 1 cm below the height of the platform for 60 min. For FSS loading, plastic tanks (50 cm × 30 cm × 30 cm) were filled with water at room temperature to the depth of 25 cm. The depth of the water required the animal to swim or float without the hind limbs touching the bottom of the tank. Rats were placed individually in a tank for 20 min.

Table 1. The 7-day CHeS protocol

<table>
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<tr>
<th>Day</th>
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<tr>
<td>1</td>
<td>RS (90 min)</td>
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<tr>
<td>2</td>
<td>RS (90 min)</td>
<td>CR5 (45 min)</td>
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<tr>
<td>3</td>
<td>RS (90 min)</td>
<td>WAS (60 min)</td>
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CHeS, chronic heterotypic stress; RS, restraint stress; CRS, cold restraint stress; FSS, forced swimming stress; WAS, water avoidance stress.

Neuropeptide microdialysis. Rats were anesthetized by xylazine (10 mg/kg ip) and ketamine (90 mg/kg ip). A stainless steel guide cannula (0.70 mm OD/0.62 mm ID) was stereotaxically implanted into hypothalamic PVN according to the coordinates (2.1 mm caudal, 0.3 mm lateral from the Bregma; 7 mm ventral from the skull surface) calculated by using the rat brain atlas of Paxinos and Watson (54). The guide cannula was fixed to the skull by use of two anchor screws and dental cement. Each cannula was fitted with a 33-G dummy cannula that extended 0.5 mm beyond the tip to prevent blockage of the guide cannula. The rats were allowed to recover for 5 days. The dummy cannula was placed inside the guide throughout the recovery period. To obtain CRF and apelin from hypothalamic PVN, we utilized a novel in vivo brain microdialysis method that allows recovery of large molecules from interstitial regions in conscious and freely moving rats, as described elsewhere (68).

On the experiment day, a microdialysis probe with 8-mm shaft and 2-mm, 1,000-kDa molecular weight cutoff polyethylene membrane was connected to a push-pull perfusion system. On the outlet side of the probe, a ventilation hole with 0.7-mm diameter served to equalize and stabilize pressure with outside atmospheric pressure. On the experiment day, rats were placed in a Plexiglas microdialysis cage and a probe (0.44 mm OD) was inserted through the guide cannula. Before use, the probe was conditioned by briefly dipping it in ethanol and then washed with artificial cerebrospinal fluid (aCSF) (148 mM NaCl, 4 mM KCl, 1.2 mM CaCl2, 0.85 mM MgCl2) that was filtered through a 0.22-μm-pore-size membrane (Corning Life Sciences, Union City, CA). We added 0.15% BSA to aCSF to prevent fluid loss into tissue and to improve total volume recovery through membranes with a higher molecular weight cutoff. The preconditioned probe’s outlet and inlet were connected to a peristaltic pump (ERP-10, Eicom, Kyoto, Japan) and microsyringe pump (ESP-32, Eicom, Kyoto, Japan), respectively, by using fluorinated ethylene propylene (FEP) tubing with 0.25-mm inner diameter. Before insertion, the probe and connecting tubes were perfused with 0.15% BSA-aCSF for 60 min at a flow rate of 2 μl/min prior to the baseline sample collection. After insertion of the probe, samples were collected in polypropylene tubes at 0.5 μl/min flow rate for 120 min.

Histological verification of microdialysis cannula. At the end of the microdialysis sampling, 1 μl of 2% methylene blue solution was injected through a special microinjection cannula made of fused silica (Eicom, Kyoto, Japan). Rats were given an overdose of urethane by intraperitoneal injection (1.5 g/kg) and then perfused intracardially with artificial cerebrospinal fluid (aCSF) and then perfused intracardially with isotonic saline and 10% formalin. Histological verification of the guide cannula and probe were performed as reported elsewhere (31). Briefly, the brains were removed and fixed in 10% formalin, and 50 μm coronal sections were stained with neutral red. Rats showing injection sites within a distance larger than 500 μm from the PVN were excluded from the study.

Intracerebroventricular cannulation. Rats were placed in a stereotaxic apparatus under ketamine/xylazine (90 mg kg−1/10 mg kg−1) anesthesia. A 26-G guide cannula made of FEP polymer was implanted into the right lateral ventricle according to coordinates (0.8 mm caudal, 1.4 mm lateral from the Bregma; 4 mm ventral from the skull surface) calculated by using the atlas of Paxinos and Watson (54). The cannula was fixed with a pair of anchor screws and dental cement onto skull surface. After the surgery, rats were housed individually and allowed to recover for 5 days.

To verify the cannula placement in the lateral ventricle, rats were centrally injected with 100 ng human angiotensin II (ANG-II). Immediately after the intracerebroventricular cannulation (icv) injection, rats were returned to their home cage with access to a water bottle. The latency to drink was recorded; rats that failed to drink within 120 s were excluded from experiments, as previously reported (61). At the end of the experimental protocol, proper cannula placement was also verified by injecting of methylene blue (10 μl icv) through each icv cannula. Brains were removed and cut sagittally, and spread of the dye in ventricles was macroscopically examined.
Measurement of solid GE. For the evaluation of GE, rats were fasted for 24 h. Preweighed pellets (1.6 g) were given, as previously reported (10, 84, 85). In GE measurements, drugs were administered 20 min before giving the pellets and rats were allowed to finish eating within 10 min. The rats that did not consume 1.6 g of food within 10 min were excluded from the study in order not to exceed 30 min postinjection period. Immediately after completion of feeding, rats were subjected to AS for 90 min. At the end of AS loading, rats were euthanized by exsanguination under isoflurane anesthesia. The stomach and distal colon were exposed and two 120-mm outer diameter (Cole-Parmer, Vernon Hills, IL) cannulas were inserted via the cecum (1 cm proximal to the cecocolic junction) into the proximal colon (1 cm distal to the cecocolic junction) and fixed with sutures. The catheter was tunneled through the anterior abdominal wall subcutaneously and placed outside the neck skin of rats. Postoperatively, rats were treated with tramadol hydrochloride (40 mg/kg im) and ketamine (90 mg/kg ip), a silicon catheter with 2.3-mm outer diameter (Kyowa Electronic Instruments, Tokyo, Japan) was inserted via the cecum (1 cm proximal to the cecocolic junction) into the proximal colon (1 cm distal to the cecocolic junction) and fixed with sutures. The catheter was tunneled through the anterior abdominal wall subcutaneously and placed outside the neck skin of rats. Postoperatively, rats were treated with tramadol hydrochloride (40 mg/kg im) and housed individually. Three days after surgery, the rats underwent stress protocols. On experiment day, 1.5 ml of saline containing 0.75 mg nonabsorbable phenol red was injected via the colonic catheter, and the catheter was flushed with 0.5 ml of saline. Ninety minutes later, the rats were euthanized under isoflurane anesthesia and the entire colon was immediately removed and divided into six segments of equal length. The contents released from the anus were collected and referred to as segment 7 for the measurement of possible phenol red. The content of each segment was placed in 100 ml of 0.1 N NaOH and homogenized. The homogenate was allowed to keep for 1 h at room temperature. Five milliliters of the supernatant was added to 0.5 ml of 20% trichloroacetic acid solution to precipitate the protein. After centrifugation at 10,000 g for 30 min, 4 ml of 0.5 N NaOH was added to the supernatant. Phenol red was determined by measuring the absorption at 560 nm by use of a spectrophotometer (Shimadzu, Kyoto, Japan). The NS control rats that did not undergo restraint stress were euthanized 90 min after completion of feeding. In the AS group, restraint stress loading started immediately after completion. In the CHS and CHeS groups, GE measurement was performed following restraint stress loading on the 5th and 7th days of CHS and CHeS, respectively. The solid GE was calculated as follows:

\[
% \text{GE} = 1 - \left( \frac{\text{weight of dried content}}{\text{weight of pellet}} \right) \times 100
\]

Measurement of CT. Under general anesthesia with xylazine (10 mg/kg ip) and ketamine (90 mg/kg ip), a silicone catheter with 2.3-mm outer diameter (Cole-Parmer, Vernon Hills, IL) was inserted via the cecum (1 cm proximal to the cecocolic junction) into the proximal colon (1 cm distal to the cecocolic junction) and fixed with sutures. The catheter was tunneled through the anterior abdominal wall subcutaneously and placed outside the neck skin of rats. Postoperatively, rats were treated with tramadol hydrochloride (40 mg/kg im) and housed individually. Three days after surgery, the rats underwent stress protocols. On experiment day, 1.5 ml of saline containing 0.75 mg nonabsorbable phenol red was injected via the colonic catheter, and the catheter was flushed with 0.5 ml of saline. Ninety minutes later, the rats were euthanized under isoflurane anesthesia and the entire colon was immediately removed and divided into six segments of equal length. The contents released from the anus were collected and referred to as segment 7 for the measurement of possible phenol red. The content of each segment was placed in 100 ml of 0.1 N NaOH and homogenized. The homogenate was allowed to keep for 1 h at room temperature. Five milliliters of the supernatant was added to 0.5 ml of 20% trichloroacetic acid solution to precipitate the protein. After centrifugation at 10,000 g for 30 min, 4 ml of 0.5 N NaOH was added to the supernatant. Phenol red was determined by measuring the absorption at 560 nm by use of a spectrophotometer (Shimadzu, Kyoto, Japan). The NS control rats that did not undergo restraint stress were euthanized 90 min after injection of phenol red solution. In the AS group, restraint stress loading started immediately after phenol red injection. In CHS and CHeS groups, CT measurement was performed following the last restraint stress loading on the 5th or 7th days of CHS and CHeS, respectively. CT was calculated as the geometric center of distribution of phenol red described as follows:

\[
\text{Geometric center} = \sum \text{(optic density of phenol red per segment} \times \text{segment number)}
\]

Recording of GI motility. Through a midline laparotomy, the stomach and distal colon were exposed and two 120-μm strain gage transducers (Kyowa Electronic Instruments, Tokyo, Japan) were implanted on the serosal surface of the gastric antrum and distal colon. The wires from transducers were exteriorized through abdominal wall and ran under skin toward the back and protected by a special jacket. Rats were housed individually with access to a standard diet and tap water, and they were allowed to recover for 7 days prior to the experiments. On the experiment day, following 18–24 h of fasting with free access to water, the wires from the transducer were connected to a Wheatstone bridge amplifier and data acquisition system (MP100, Biopac Systems, Santa Barbara, CA). Antral and distal colonic contractions were measured in conscious, freely moving rats. Preprandial GI contractions were monitored for 1–2 h and then rats were given 1.6 g of pellet. After the completion of feeding, postprandial contractions were recorded. The area under the curve was calculated and expressed as a motility index (MI). The changes in MI were evaluated before and after central apelin-13 administrations and expressed as percentage of aCSF-induced changes.

Chemicals and icv administration. Human ANG-II (Sigma Chemical, St. Louis, MO), [Pyr5]apelin-13 (Santa Cruz Biotechnology, Dallas, TX), F13A (i.e., [Ala13]apelin-13) (Phoenix Pharmaceuticals, Burlingame, CA), and α-helical CRF9–41 (Sigma-Aldrich, St. Louis, MO) were dissolved in sterile aCSF on experimental days. F13A was administered 30 min prior to restraint stress loading and α-helical CRF9–41 pretreatment was performed 30 min prior to apelin-13 administration. All icv injections were performed by hand to the lightly restrained rats gently wrapped in a soft cloth. A 33-G infusion needle linked to a polyethylene tube was introduced through the guide cannula. An infusion volume of 5 μl was delivered manually via a 10-μl Hamilton syringe over the course of 1 min. The injection needle protruded 0.5 mm beyond the guide cannula and remained in place for at least 30 s immediately after injection to prevent backflow.

Histology. Immunohistochemistry for c-Fos and apelin was performed on paraffin-embedded sections of the brain tissues. The samples were fixed in 4% formaldehyde overnight, rinsed with PBS, and embedded. Serial sections were collected on SuperFrost Plus slides (Novoglas, Bern, Switzerland). After rehydration, samples were transferred to 0.01 M citrate buffer (pH 6) and subsequently heated in a microwave oven for 2.5 min at 750 W for retrieving antigen. After cooling for 20 min at room temperature, the sections were washed with PBS. To remove endogenous peroxidase activity, sections were kept in 3% (vol/vol) H2O2; prepared in methanol for 5 min and subsequently rinsed with PBS. Non-specific binding was decreased by normal mulsisspecies serum Ultra V Block (Thermo TA-125-UB) at room temperature for 7 min. Control antibodies (Santa Cruz; sc-33469) diluted in dilution buffer (1/200) were applied for overnight at 4°C in a humidified chamber. For negative controls the primary antibodies were replaced by normal goat IgG serum (Vector Laboratories, Burlingame, CA) at the same concentration. After several washes in PBS, sections were incubated with biotinylated anti-goat IgG secondary antibody (1/400, Vector Laboratories) for 1 h followed by labeled streptavidin-biotin complex (Sensitex, SHP-125) incubation for 40 min and then rinsed with PBS. Antibody complexes were visualized by incubation with diaminobenzidine chromogen. Until the primary antibody step, the same protocol was applied to the slides for double staining as described above. Following the blocking of non-specific binding, the sections were incubated with rabbit polyclonal anti-c-Fos antibody (1/3000 dilution, Abcam; ab7963) for 2 h at room temperature in a humidified chamber. Following the washing steps in PBS, sections were incubated with polyclonal anti-rabbit secondary antibody (Thermo Scientific; TS-125-BN) for 30 min at room temperature. Labeling was visualized by using the alkaline phosphatase kit (ThermoScientific; TS-125-AP) according to the instructions of the manufacturer. After rinsing in PBS, alkaline phosphatase was developed with Fast Red (Roche; 11496 549 001) for 10–15 min as a chromogene. All microdialysis samples were kept at −80°C prior to the analysis. Apelin and CRF assays were performed with commercially available enzyme immunoassay (EIA) kits (Phoenix Pharmaceuticals). Microdialysis
samples were extracted by using Sep-Pak C-18 columns prior to the EIA assay.

Measurement of plasma corticosterone. Blood samples were collected from abdominal aorta at the end of the stress protocols. To assess the effect of central apelin-13 administration on plasma corticosterone, blood samples were withdrawn through a 24-G catheter from the ventral tail artery. Blood samples were withdrawn before and 90 min after the central apelin-13 administration (100 μg icv). Whole blood was centrifuged at 10,000 g and plasma samples were aliquoted and frozen at −80°C. To measure corticosterone, a commercially available EIA kit was used (Enzo Life Sciences, Plymouth Meeting, PA). The assay procedure was carried out according to the protocol supplied by the manufacturer.

Statistical analysis. Data were expressed as means ± SE. One-way ANOVA or Kruskal-Wallis test followed by Student’s t-test or Mann-Whitney U-test was used to determine the significance among groups, as appropriate. The plasma corticosterone results following apelin-13 administration were compared with the baseline results by paired t-test. Pearson’s correlation was used to assess a correlation between GE, CT, and apelin levels in microdialysates. P < 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS v13.0 software.

RESULTS

Stress exposure enhances hypothalamic coexpression of apelin and c-Fos. As previously reported (59, 70), following AS, increased number of neurons expressing c-Fos immunoreactivity was observed in hypothalamic PVN. Apelin immunostaining was detected in cytoplasm of some neurons in PVN, and c-Fos protein expression was observed as nuclear immunoreactivity in a group of PVN neurons. Increased apelin and c-Fos coexpression were detected in a group of PVN neurons in AS-loaded rats. The stress-induced effects on apelin and c-Fos expressions were observed more robust in CHes rats compared with the CHS-loaded rats (Fig. 1).
Compared with NS rats, AS significantly increased number of cells showing apelin (P < 0.01), c-Fos (P < 0.01), and apelin+c-Fos (P < 0.05) immunoreactivity. AS-induced increase in apelin immunoreactivity was significantly (P < 0.05) attenuated following CHS for 5 days. The strongest immunoreactivities for apelin, c-Fos, and apelin+c-Fos were found in CHeS group. Compared with the CHS, the intensity of staining was observed significantly (P < 0.01 apelin and c-Fos; P < 0.05 apelin+c-Fos) higher following CHeS (Fig. 2).

**Verification of the cannula placements.** The data of five rats used for microdialysis were excluded from the study because of improper placement of the lesions induced by probe insertion. A proper placement of guide cannula and microdialysis probe in hypothalamic PVN is represented in Fig. 3A. Verification of the icv cannula was performed by ANG-II drinking behavior test and macroscopic observation of dye in ventricles (Fig. 3, B and C). Among the icv-cannulated animals, two rats were excluded from the experiments because of latency of drinking behavior over 120 s following central ANG-II injection or absence of methylene blue within the cerebral ventricular system.

Stress exposure increases plasma corticosterone levels. Plasma corticosterone level was 468.59 ± 46.83 ng/ml (n = 8) in NS rats. AS significantly increased the plasma corticosterone concentration (1,060.44 ± 85.04 ng/ml, n = 8, P < 0.01). The AS-induced increase remained unchanged (981.27 ± 57.14 ng/ml, n = 8, P < 0.01 vs. NS) in CHeS-loaded rats. Compared with the CHeS rats, plasma corticosterone levels were found significantly lower (666.94 ± 51.03 ng/ml, n = 8, P < 0.01) in CHS-load rats.

A single dose of apelin antagonist F13A (10 μg icv) was administered 30 min prior to the stress loading (on 5th day of CHS and 7th day of CHeS). F13A administration did not have any effect on plasma corticosterone in NS and CHS-loaded rats. However, AS-induced increase in plasma corticosterone was completely abolished (668.1 ± 99.3 ng/ml, n = 8, P < 0.05) by a single pretreatment of F13A. In contrast, F13A failed to alter the increased corticosterone in CHeS group (Fig. 4).
Stress exposure increases hypothalamic release of apelin and CRF. Under NS conditions, CRF concentration was found 19.73 ± 7.26 pg/ml (n = 6). AS significantly increased CRF release (219.32 ± 32.51 pg/ml, n = 6, P < 0.05). CHS loading restored the CRF concentrations (40.15 ± 15.74 pg/ml, n = 6) to the NS levels, whereas in CHeS group, CRF concentrations were found significantly higher (363.97 ± 61.53 pg/ml, n = 6, P < 0.05) compared with the rats in NS and CHS groups (Fig. 5A).

Compared with the NS rats (185.06 ± 44.79 pg/ml, n = 6), exposure to AS for 90 min significantly increased the apelin levels (1,435.83 ± 190.68 pg/ml, n = 6, P < 0.05) in hypothalamic microdialysates. CHS loading significantly (P < 0.05, n = 6) reduced the AS-induced apelin concentrations to 237.75 ± 61.35 pg/ml. In CHeS-loaded rats, apelin concentrations remained significantly (P < 0.05) higher (1,633.78 ± 208.72 pg/ml, n = 6, P < 0.05) compared with those in NS and CHS groups (Fig. 5B).

Central F13A abolishes stress-induced alterations in GE. In NS rats, solid GE was found 60.3 ± 4.2% (n = 9). AS significantly delayed GE (31.9 ± 2.9%, n = 9, P < 0.01), whereas AS-induced delayed GE was completely restored (55.0 ± 4.8%, n = 9) following CHS. In contrast, in CHeS-loaded rats, GE was still delayed (31.6 ± 2.9%, n = 9, P < 0.01) compared with the NS rats.

Central F13A administration (10 µg icv) did not affect the solid GE (54.51% ± 1.9, n = 7) under NS conditions, whereas F13A significantly restored delayed GE in AS-loaded (50.5 ± 5.7%, n = 9, P < 0.05) and CHeS-loaded (49.5 ± 4.2%, n = 9, P < 0.05) rats. However, central F13A administration did not alter GE (54.8 ± 4.8%, n = 9) in CHS-loaded rats (Fig. 6).

Central F13A abolishes stress-induced alterations in CT. Under NS conditions, CT was found 4.1 ± 0.1 (n = 9). AS loading for 90 min significantly accelerated CT (6.1 ± 0.1, n = 9, P < 0.01). Following CHS, the accelerated CT was completely restored (4.4 ± 0.2, n = 9), whereas it remained significantly accelerated (6.16 ± 0.2, n = 9, P < 0.01) in CHeS-loaded rats.

In NS rats, central F13A treatment did not have any effect on CT (3.8 ± 0.1, n = 9). However, single administration of central F13A (10 µg icv) significantly restored the accelerated CT in AS (4.2 ± 0.3, n = 9, P < 0.05) and CHeS (4.3 ± 0.2, n = 9, P < 0.05) groups. In contrast, F13A did not change the CT (4.4 ± 0.2, n = 9) in CHS-loaded rats (Fig. 7).

Correlations between apelin/GE and apelin/CT under chronic stress. Following chronic stress protocols (CHS and CHeS), there was a significant positive correlation (R² = 0.20, n = 6) between apelin level and GE (Fig. 8A). Also, a significant negative correlation (R² = 0.64, n = 6, P < 0.05) was observed between apelin level and GE (Fig. 8B).

Central apelin-13 inhibits gastric and colonic motor functions. To test whether apelin-13 alters solid GE under NS rats, different doses of apelin-13 were centrally (icv) administered 30 min prior to the GE measurements. Compared with vehicle-injected rats, 10 µg apelin-13 administration (57.9 ± 2.9%, n = 7) did not change GE (61.9 ± 4.3%, n = 7), and a 50 µg dose of apelin-13 (48.9 ± 2.2%, n = 7) caused a slight but not statistically significant decline in GE. However, 100 µg apelin-13 administration significantly inhibited GE (42.2 ±
2.4%, n = 7, P < 0.05), compared with those in the vehicle group (Fig. 9A).

To test the effects of central apelin-13 treatment on CT, the same doses of apelin-13 in GE measurements were administered 30 min prior to the CT measurements. In line with GE measurements, a centrally injected 100 µg dose of apelin-13 significantly inhibited CT (2.7 ± 0.1%, n = 7, P < 0.05). However, 10-µg (3.9 ± 0.1, n = 7) and 50-µg (3.8 ± 0.4, n = 7) doses did not alter CT compared with vehicle-injected rats (4.0 ± 0.2, n = 7) (Fig. 9B).

Central apelin-13 administration increases plasma corticosterone. Compared with basal (preinjection) level (391.84 ± 16.35 ng/ml), the plasma corticosterone concentration was measured significantly (747.03 ± 42.12 ng/ml, P < 0.01, n = 9) higher 90 min after the single icv administration of 100 µg apelin-13 (Fig. 10).

Central apelin-13 administration inhibits gastric and colonic postprandial motility. Antral and distal colonic postprandial contractions were inhibited by icv administration of 100 µg apelin-13 (Fig. 11A).
The inhibitory effect of apelin-13 was observed immediately after administration and persisted 60 min (Fig. 11). Pretreatment of aCSF (5 μl icv) did not affect the apelin-induced effects both on antral and colonic postprandial contractions, whereas the inhibitory effect of apelin-13 on antral contractions was completely abolished by CRF9–41 (10 μg icv). In contrast, contractions in distal colon were slightly diminished but not significantly inhibited by pretreatment of CRF9–41 (Fig. 12). MI changes were evaluated before and after central apelin-13 administrations and expressed as percentage of aCSF-induced changes. Pretreatment of CRF9–41 significantly (n = 6, P < 0.05) attenuated the inhibitory effect of apelin-13 on MI in antrum, but not in distal colon (Fig. 13).

**DISCUSSION**

Accumulating evidences have raised the consideration that apelin plays a regulatory role in stress response. Following a stress exposure, CRF is secreted from the hypothalamus, which in turn activates the HPA axis, resulting in the secretion of corticosterone from the adrenal cortex (19, 21, 62). Besides neuroendocrine functions, CRF also acts in the brain to influence the GI motor functions via autonomic neural pathways (6, 36, 63, 64). Similarly, central CRF administration delays GE and stimulates CT in rodents (37, 46, 60). Many of the central effects of apelin are attributed to its expression in hypothalamic PVN, the neuronal circuits that are key structures in the regulation of endocrine and autonomic responses for the maintenance of homeostasis (47). Recent findings brought the possibility that central apelin may mediate stress-induced changes in GI motor functions besides its role in stress-related neuroendocrine functions. In rodents, central exogenous apelin treatment has been shown to increase c-Fos expression in hypothalamic PVN (26) and plasma ACTH and corticosterone levels in plasma (25, 67). In the present study, we have shown the increased apelin and c-Fos coexpression detected in hypothalamic PVN neurons. In the ChES group, coexpression of apelin and c-Fos was found more pronounced compared with other rats.

In rodents, the brain microdialysis technique has an advantage over other sampling techniques in that it is possible to continuously sample the dynamic time profile changes of a specific molecule in a target tissue over an extended period of time without the collection of whole tissue samples. On the other hand, each animal can serve as its own intrinsic control that eliminates the variability (73). Direct measurement of neuropeptides in the hypothalamus is essential for neuroendocrine studies. However, small quantities of peptides released at their neuroterminals and relatively large molecular sizes make these measurements difficult (17). In the present study, we have utilized a large-molecule microdialysis system that allows recovery of brain neuropeptides following stress protocols. Specifically, our study revealed that, in response to stress, the expression pattern of apelin accompanied CRF. The 5-day CHS protocol involves a predictable and repetitive stress exposure that leads to habituation of stress response. However, the 7-day ChES protocol was loaded to create an unpredictable and nonhabituating stress protocol that yields maladaptation. In parallel with recent reports (1, 4, 10, 85), our data demonstrated that AS remarkably increased the CRF in microdialysates, compared with NS rats. Following CHS, increased CRF and apelin were completely recovered to the basal levels. The increased concentrations of CRF and apelin were still observed in ChES group rats. In agreement with CRF, the upregulated

![Graph](https://example.com/graph.png)

*Fig. 10. Alteration in plasma corticosterone concentration 90 min after central administration of apelin-13 (100 μg icv). Data are means ± SE (n = 9). **P < 0.01 vs. preinjection.***

![Graph](https://example.com/graph2.png)

*Fig. 11. Effect of central administration of apelin-13 (100 μg icv) on antral (A) and distal colonic (B) postprandial contractions under nonstressed conditions. Apelin caused a remarkable inhibition in antral and distal colonic postprandial contractions.*
apelin expression induced by AS was completely attenuated to the basal levels following CHS. However, increased hypothalamic apelin remained unchanged following CHeS.

HPA function is regulated by negative feedback, whereby circulating glucocorticoids inhibit their own release by acting at pituitary and hypothalamic levels via the corticosteroid receptors. The neuroendocrine response to many stressors is reduced after repeated or chronic exposure (18, 39). The HPA axis exhibits marked adaptation of responsiveness following exposure to a single stressor, which in turn can lead both to facilitation of the subsequent response and habituation after following exposures. Nevertheless, the habituation phenomenon is considered stressor specific, such that subsequent exposure to a heterotypic stressor restores or potentiates the release of glucocorticoids (12, 15, 18, 39). In the present study, the plasma corticosterone levels were found significantly higher in AS and CHeS-loaded rats compared with the rats under NS and CHS conditions. It was not surprising that CRF exhibited the same pattern in microdialysates, since we have previously reported that acute stressor-induced increased CRF expression is attenuated back to the basal levels following chronic exposure to the same stressor for 5 consecutive days (10, 84). The AS-induced increase in plasma corticosterone was abolished by central apelin antagonist F13A administration, whereas CHeS-induced alterations were not altered. These results suggest that a single F13A injection may not be adequate to antagonize the sustained activation of CRF neurons induced by 7-day CHeS. Therefore, daily injection of apelin antagonist during chronic nonadaptive stress models may help to elucidate the influence of apelinergic neurons on HPA axis at hypothalamic level.

Apelin is known to stimulate ACTH secretion indirectly by increasing CRF release at the hypothalamic level or directly at the anterior pituitary level in autocrine and paracrine fashions. The presence of apelin-immunoreactive fibers and APJ receptor in parvocellular neurons of PVN suggests that apelin may modulate ACTH release via an indirect action on the hypothalamus. Additionally, APJ receptor mRNA was shown in pituitary corticotroph cells, indicating a local interaction between apelin and ACTH (56). In rodents, centrally administered apelin was shown to induce the hypothalamic-pituitary-adrenal axis neuroendocrine function and increase the release of ACTH and corticosterone release (25, 47, 48, 67). Newson and colleagues (48) showed a twofold increase in plasma corticosterone by administering less than half of our dose (1 mg/kg), in mice. In parallel to the latter findings, we found a quite similar pattern with twofold increase in plasma corticosterone in response to 100 μg of central apelin-13, which could be expected more pronounced. Besides the species difference, a plausible explanation for these results is that Newson and colleagues obtained the plasma samples 30 min after the injection. However, our animals were subjected to 90 min of postinjection period, which is the time interval used in GE and CT measurements.

Following the 5-day CHS protocol, we have found that both the AS-induced delayed GE and the accelerated CT were completely recovered, whereas the 7-day CHeS-loaded rats...
failed to adapt. The correlation analysis was performed between central apelin, CT, and GE in chronically stressed rats. There was a significant ($P < 0.001, n = 6$) positive correlation between hypothalamic apelin synthesis and CT. In addition, a significant ($P < 0.01$) negative correlation was found between apelin and GE in CHS and ChEs rats. To treat stress-induced GI dysmotility, we administered APJ receptor antagonist F13A (10 µg icv). The delayed GE and the increased geometric center observed in AS and CHS groups were completely abolished by central administration of F13A, indicating the involvement of central apelin in stress-induced GI motor dysfunction via APJ receptor. Interestingly, central F13A did not alter GE and CT in NS rats. These results indicate that endogenous central apelin mediates stress-induced changes in GI motility, although it does not have a regulatory function on GI motor activity under normal conditions. It is likely that increased hypothalamic apelin elicited by stress mediates the stress-induced alterations in GI motor functions in addition to or in cooperation with CRF in hypothalamic stress circuitry.

The data of recent reports indicate that upregulation of hypothalamic oxytocin plays a pivotal role in mediating the adaptation of GI motor functions to chronic stress exposure. In rats, recovery of accelerated CT following CHS was reversed by central administration of oxytocin antagonist and the accelerated CT elicited by CHS was attenuated by central oxytocin treatment (83). It was previously demonstrated that compared with the day 1, oxytocin release from PVN was significantly increased on the day 3 and the day 5 of CHS in rats (2). On the other hand, following CHS the accelerated CT was attenuated in WT mice, but not in oxytocin-knockout mice. It was also found that upregulated CRF mRNA expression in hypothalamic supraoptic nucleus (SON) was much greater in oxytocin-knockout mice (4). Furthermore, electrophysiological recordings from hypothalamic SON neurons demonstrated that apelin-13 administration increased the firing rates of vasopressinergic neurons but had no effect on the firing rate of oxytocin neurons. The direct excitatory effect of apelin-13 on vasopressinergic cell activity was also supported by in vitro studies showing depolarization of membrane potential and increase in action potential firing (72). On the other hand, in situ hybridization and immunohistochemistry studies demonstrated the presence of apelin receptor mRNA in hypothalamic oxytocin neurons and colocalization of apelin with oxytocin in hypothalamic oxytocin-positive neurons. Apelin was also shown to inhibit these neurons in an autocrine and paracrine manner (7).

Besides CNS, apelin and APJ receptor mRNA were shown in enteric tissues, including GI smooth muscle, enteroneurocrine cells, and enteric neurons (40, 55, 74, 75). However, there is still no direct evidence showing that peripheral apelin alters GI motility acting through enteric APJ receptors. In mice, central injection of apelin-13 was shown to decrease gastric emptying, whereas it was not affected by intraperitoneal administration (34). Furthermore, Yang and colleagues (82) tested the effects of apelin on distal colonic contractions using the longitudinal muscle from the mouse distal colon and demonstrated that apelin-13 did not alter the distal colonic contractions even when applied at high concentrations ($10^{-8}$ - $10^{-6}$ M). On the other hand, increased gastric mucosal apelin upon acute water-immersion stress has been recently shown in rats (24). Recently, we have detected increased apelinergic immunoreactivity in antral and duodenal myenteric plexus neurons following 90-min restraint stress in rats (unpublished observations). Hence we cannot exclude the possibility that apelin mediates stress-induced alterations in GI motor functions through peripheral pathways in addition to central autonomic pathways.

CRF and its the closely related family of neuropeptides urocortins are known as ancient paracrine-signaling peptides secreted in both the central and peripheral neural circuits (28). Endogenous CRF ligands are expressed in gastrointestinal GI tissues pointing to local expression of CRF receptors (11, 32, 45). Recent studies indicate that peripheral CRF-related mechanisms also contribute to stress-induced changes in gut motility and intestinal mucosal function. It was shown that peripheral administration of CRF or urocortin inhibits gastric emptying and promotes colonic motility via CRF2 and CRF1, respectively (28, 38, 65). In addition, intraperitoneal CRF administration was shown to increase c-Fos expression in colonic myenteric neurons in rats (41). On the other hand, peripheral administration of CRF antagonist α-helical CRF₉₋₄₁ (50 µg iv) was reported to prevent the stress-induced increase in large intestinal transit and the associated increase in fecal excretion in rats (78). Likewise, Tache and Perdue (66) reported that peripherally given CRF receptor antagonists prevent acute restraint and WAS-induced delayed gastric emptying, stimulation of colonic motor function, and mucosal permeability. These results indicate that activation of peripheral CRF and CRF receptors is involved in stress-related alterations of gut physiology. Besides CNS, there is no evidence indicating that apelin stimulates CRF-producing neurons in enteric nervous system. Although not addressed in this study, the involvement of stress-induced peripheral apelin and enteric CRFergic system has yet to be determined.

On the other side, peripherally administered exogenous apelin has been shown to stimulate cholecystokinin release in rats (16, 27, 76). CCK is well known for its inhibitory actions on gastrointestinal motor functions by diminishing central vagal outflow via activating CCK1 receptors on vagal afferents (22, 43, 44, 58, 81). Therefore, we speculate but cannot confirm that stress may increase the release of apelin from peripheral sources, so that CCK and CCK1 receptor may contribute to its inhibitory actions on GI motor functions. There is a need of additional studies to obtain enough evidence to support the enteric interaction between apelin and CCK under stressed conditions.

Recent reports have demonstrated that central apelin may influence GI motor functions in rodents. Central exogenous apelin has been shown to inhibit GE, GI transit, and CT in mice (34, 82). In our experiments, the rats were centrally given 10, 50, and 100 µg doses of apelin-13. Similar to the previous reports done in mice, our data demonstrate that centrally administered apelin-13 (100 µg icv) inhibited GE and CT by inhibiting the postprandial contractions in conscious rats under NS conditions. To corroborate our findings, we also performed motility recording experiments using strain gage transducers to monitor antral and colonic spontaneous contractions. In both antrum and distal colon, apelin-13 administration significantly inhibited the contractions and caused ~40% decline in MI.

To elucidate the mechanism of apelin-induced inhibition, rats were centrally pretreated with CRF antagonist α-helical CRF₉₋₄₁ prior to the apelin injection. We have found that the inhibitory effect of apelin-13 on antral postprandial contrac-
tions was completely abolished by α-helical CRF9–41, suggesting that apelin-induced inhibition of gastric motility is mediated through an CRF-dependent pathway. Surprisingly, after pretreatment of α-helical CRF9–41, apelin-13 no longer inhibited the spontaneous contractions in distal colon. The latter result implies that apelin-induced inhibition of GE, but not colon, is mediated through CRF-dependent pathway. We speculate but cannot confirm that apelin activates a distinct CRF-independent pathway, resulting in inhibition of colon motility. Another factor that may be relevant to the interpretation of our data relates to the type of CRF receptor. CRF in the brain is known to play a significant role in the CNS mediation of stress-induced inhibition of upper and lower GI motor functions through activation of distinct CRF receptors within the brain. The inhibition of GE by CRF may be mediated by interaction with the CRF2 receptor in rostral ventrolateral medulla, whereas CRF1 receptors in Barrington’s nucleus are involved in the colonic and anxiogenic responses to stress (42, 46, 63, 64). In the present study, we preferred administering a nonspecific antagonist α-helical CRF9–41 rather than specifically antagonizing by using NB127914 and astressin-2B, which are well-known antagonists for CRF2 and CRF1 receptors, respectively. Thus future experiments aimed at addressing the CRF receptor subtypes in brain stem may further elucidate the mechanism of apelin-elicited inhibition of upper and lower GI motor functions. Moreover, it has been suggested that the inhibitory effects of central apelin-13 were antagonized by central pretreatment of opioid receptor antagonist naloxone, indicating that central apelin inhibits GI motor functions through APJ and opioid receptor-mediated pathways (34, 82). The opioid peptides have a major physiological role in the control of gut motility and secretions (8, 35). In humans, opioid analgesics are the mainstay of therapy in patients with chronic cancer pain. Opioid bowel dysfunction is a common adverse effect that is commonly described as constipation (53). Especiably, the κ-opioid receptors are known to inhibit colonic contractions acting centrally (8). There is abundant evidence that indicates the involvement of apelin in central opioid signaling. Central administration of apelin-13 has been shown to potentiate the morphine-induced analgesia and this potentiated effect was reversed by opioid receptor antagonist naloxone (80). Moreover, it was demonstrated that APJ receptor forms a heterodimer with κ-opioid receptor and leads to increased PKC and decreased protein kinase-A activity (33). Although not addressed in this study, emerging evidence suggests that the CRF-independent inhibitory action of central apelin-13 on colonic motor functions is mediated by κ-opioid receptors. Taken together, our data for the first time have provided an evidence that apelin mediates stress-induced GI motor dysfunction in addition to or in cooperation with CRF in hypothalamic stress circuitry. Thus the upregulated CRF and apelin may act to influence each other in mediating the stress-induced GI motor dysfunctions in CHeS rats. It is also suggested that central apelin is only effective under stressed conditions. The alleviative effect of APJ receptor blocker on impaired GI motor functions under acute and maladaptive chronic stressed conditions, suggesting that central apelin may act as a regulator in hypothalamic stress circuitry and mediate the stress-induced effects on GI motor functions through brain-gut axis. In contrast, central exogenous apelin-13 was observed to inhibit colon motility. Therefore, another point that should be mentioned is that stress-induced central endogenous apelin and centrally administrated apelin in pharmacological doses seem to drive different pathways. Further studies are warranted to better clarify the neural pathways of exogenous apelin on upper and lower GI motility. The present study provides a consideration that apelin signaling and APJ receptor might be a novel pharmacological target for treatment of the FGIDs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.B. and V.I.-U. conception and design of research; M.B., O.S., I.B., and G.T. performed experiments; M.B. analyzed data; M.B. and V.I.-U. interpreted results of experiments; M.B. and O.S. prepared figures; M.B. drafted manuscript; M.B. edited and revised manuscript; M.B. approved final version of manuscript.

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