Central apelin mediates stress-induced gastrointestinal motor dysfunction in rats

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Running Head: Central apelin and gastrointestinal motor functions

Abbreviations; Functional gastrointestinal disorders (FGID), gastric emptying (GE), colon transit (CT), acute stress (AS), chronic homotypic stress (CHS), chronic heterotypic stress (CHeS) corticotropin releasing factor (CRF), supraoptic nucleus (SON), paraventricular nucleus (PVN), hypothalamus-pituitary-adrenal (HPA) axis.

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ABSTRACT

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 non-stressed (NS) conditions in conscious rats. Prior to solid gastric emptying and colon transit measurements, APJ receptor antagonist F13A was centrally (icv) administered under NS conditions and following AS (acute stress), CHS (chronic homotypic stress) and CHeS (chronic heterotypic stress). 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 CRF9-41 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 seem like partly CRF-dependent. Apelin-13 inhibits colon motor functions through a CRF-independent pathway.

KEYWORDS: apelin, stress, corticotropin releasing factor, gastrointestinal motility, microdialysis
INTRODUCTION

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 (13, 40, 41, 54). It is well known that stressful and traumatic life events trigger exacerbations of symptoms in FGID patients (13, 62). 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, 25, 50, 51). Interestingly, gastrointestinal (GI) motor disorders may develop in some individuals, however others are able to adapt to a stressful conditions without developing GI symptoms.

Corticotrophin-releasing factor (CRF) is known to act in the brain to influence the GI motor functions via autonomic neural pathways (6, 30, 50, 51). It has been shown that CRF is up-regulated upon stress in hypothalamic paraventricular nucleus (PVN), which in turn accelerates colonic motility; whereas, inhibits gastric emptying via CRF receptor type-1 (CRF1R) and type-2 (CRF2R), respectively (35, 39). In rodents, increased hypothalamic CRF mRNA expression induced by an acute stressor was found to be down-regulated following chronic homotypic stress (CHS) for five consecutive days; whereas, the increased CRF mRNA expression did not significantly alter upon chronic heterotypic stress (CHeS) loading (4, 10, 67). Likewise, impaired gastric emptying and accelerated colonic transit was completely restored following chronic homotypic stress for five consecutive days, but not upon 7-day chronic complicated stress loading (1-5, 9, 66, 68). 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 (56). Apelin gene encodes the pre-proapelin molecule which contains 77 amino acids with a signal peptide in the N-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 (24, 56). 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 (12, 33). Apelin and APJ receptor m-RNA
expression has been shown in a variety of tissues including the heart, lung, alimentary tissues, placenta, ovary, adipose, thyroid gland, mammary gland and costal cartilage (18, 20, 60, 61).

Besides the peripheral tissues, apelinergic system is also widely expressed in brain suggesting that apelin may be involved in regulation of certain CNS functions (24, 44, 45, 48). 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 (38). Apelin has been shown to stimulate the release of hypothalamic AVP and CRF in in-vivo and in-vitro studies (37, 52). The Increase in plasma corticosterone following central apelin administration is partly blocked by central pre-treatment of CRF antagonist α-helical CRF9-41, suggesting that CRF-producing neurons are involved in apelin-induced stimulation of HPA axis (22, 37). In recent years, there has been a growing body of evidence regarding the involvement of central apelinergic system in GI motor functions. Centrally administered apelin-13 has been shown to inhibit gastric emptying and gastrointestinal transit, while accelerating colonic motility in mice (28, 65). 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 non-stressed (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 current study clearly demonstrates the alterations in hypothalamic CRF and apelin under various stress models.

**MATERIALS & METHODS**

*Animals*

Wistar rats weighing 280-300 g were housed under conditions of controlled temperature (22-24°C) and illumination (12-hr 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 minimize animal suffering and to reduce the number of animal in experiments.
**Stress Protocols**

For acute restraint stress (ARS) loading, rats were placed on a wooden plate with their trunks wrapped in a confining harness for 90 min, as previously reported (10, 67, 68). 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 (25, 63). For CHS, rats were exposed the restraint stress for 5 consecutive days. CHS protocol was comprised of restraint stress (RS), water avoidance stress (WAS), force swimming stress (FSS) and cold restraint stress (CRS). The rats were exposed to two different stressors on the 1\(^{st}\), 3\(^{rd}\) and 5\(^{th}\) days; whereas, a single stressor was applied on 2\(^{nd}\), 4\(^{th}\), 6\(^{th}\) and 7\(^{th}\) days of the CHS protocol. Morning stressors were applied between at 09:00 AM, while afternoon stressors were applied at time 02:00 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 x 20 cm) in the middle of a plastic container (50 cm x 30 cm x 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 x 30 cm x 30 cm) were filled with water at room temperature to the depth of 25 cm. The depth of the water made 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.

**Neuropeptide Microdialysis**

Rats were anesthetized by xylazine (10 mg/kg, ip) and ketamine (90 mg/kg, ip). A permanent stainless steel guide cannula (0.70/0.62 mm OD/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 using rat brain atlas of Paxinos and Watson (43). The guide cannula was fixed to the skull with a pair of anchor screw and dental cement. Each cannula was fitted with a 33G dummy cannula that extended 0.5 mm beyond the tip in order 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. In order 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 (53).

On the experiment day, a microdialysis probe with 8 mm shaft and 2 mm, 1000 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 which serves to equalize and stabilize pressure with outside atmospheric pressure. On the experiment day, rats were placed in plexiglass microdialysis cages 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, USA). 0.15% bovine serum albumin (BSA) was added to aCSF in order 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, 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⁻¹ prior to the baseline sample collection. After insertion of the probe, samples were collected in polypropylene tubes at 0.5 µl min⁻¹ 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 (1.5g kg⁻¹) and then perfused intracardially with isotonic saline and 10% formalin. For histological verification of the guide cannula and probe were performed as reported elsewhere (26). Briefly, the brains were were then 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 (icv) cannulation

Rats were placed in a stereotaxic apparatus under ketamine/xylazine (90 mg kg⁻¹/10 mg kg⁻¹) anesthesia. A 26G 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 using atlas of Paxinos and Watson(43). The cannula was fixed with a pair of anchor screw and dental cement onto skull surface. After the surgery, rats were housed individually and allowed to recover for 5 days.
In order to verify the cannula placement in the lateral ventricle, rats were intracerebroventricularly injected with 100 ng human angiotensin II. Immediately after the 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 sec were excluded from experiments, as previously reported (48). 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 hrs. Pre-weighed pellets (1.6 g) were given, as previously reported (10, 67, 68). Immediately after completion of feeding, rats were subjected to ARS for 90 min. The rats that did not consume 1.6 g of food within 10 min were excluded from the study. After ARS loading, rats were sacrificed by exsanguination under isoflurane anesthesia. The stomach was surgically removed and gastric content was recovered, dried, and weighed. The control rats that did not undergo restraint stress were sacrificed 90 min after completion of feeding, while in AS group, restraint stress loading started immediately after completion. In 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 (90mg/kg, ip), a silicon catheter with 2.3 mm outer diameter (Cole-Parmer, Vernon Hills, IL, USA) 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, i.m.) and housed individually. Three days after surgery, the rats underwent stress protocols. On experiment day, 1.5 ml of saline containing 0.75 mg non-absorbable 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 sacrificed 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 a 560 nm using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The non-stressed control rats that did not undergo restraint stress were sacrificed 90 min after injection of phenol red solution, while in 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. Colonic transit 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 120Ω 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 towards 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 hr 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, USA). Antral and distal colonic contractions were measured in conscious, freely moving rats. Pre-prandial 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 (AUC) was calculated and expressed as a motility index. The motility index was evaluated before and after central apelin-13 administrations.

**Chemicals and icv administration**
Human angiotensin II (Sigma Chemical, St Louis, MO, USA), [Pyr^1]Apelin-13 Santa Cruz (Biotechnology, Dallas, TX, USA), F13A (i.e. [Ala^13]Apelin-13) (Phoenix Pharmaceuticals, Burlingame, CA, USA) and alpha-helical CRF_{9-41} (Sigma-Aldrich, St Louis, MN, USA) were dissolved in sterile artificial cerebrospinal fluid (aCSF) on experimental days. F13A was administered 30 min prior to restraint stress loading, while α-helical CRF_{9-41} 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 33G infusion needle linked to a polyethylene tube was introduced through the guide cannula. An infusion volume of 5 μl was delivered manually using a 10 μl Hamilton syringe over the course of 1 min. The injection needle protrudes 0.5 mm beyond the guide cannula and remained in place for at least 30 sec immediately after injection in order 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 for overnight, rinse with phosphate-buffered saline (PBS) and embedded. Serial sections were collected on SuperFrost Plus slides (Novoglas, Berne, Switzerland). After re-hydrating, 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% (v/v) H_2O_2 prepared in methanol for 20 min and subsequently rinsed with PBS. Non-specific binding was decreased by normal multi-species serum Ultra V Block (Thermo TA-125-UB) at room temperature for 7 min. Anti-goat apelin (SantaCruz; sc-33469) diluted in dilution buffer (1/200) were applied for overnight at 4^0C in a humidified chamber. For negative controls the primary antibodies were replaced by normal goat IgG serum (Vector Lab. Burlingame, CA, USA) at the same concentration. After several washes in PBS, sections were incubated with biotinylated anti-goat IgG secondary antibody (1/400 dilution, BA-9500; Vector Lab. Burlingame, CA, USA) for 1h followed by LSAB streptavidin-peroxidase complex (Sensitek, SHP-125) incubation for 40 min and were rinsed with PBS. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) 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-cfos (1/300 dilution, Abcam; ab7963) antibody for 2h at room
temperature in a humidified chamber. Following the washing steps in PBS, sections were incubated with polyvalent anti-rabbit secondary antibody (Thermo Scientific; TS-125-BN) for 30 min at room temperature. Labelling was visualized 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; 11 496 549 001) for 10–15 min as a chromogene. All sections were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany) and mounted with Kaiser’s glycerol gelatin. Pictures were taken with a Zeiss-Axioplan (Zeiss, Oberkochen, Germany) microscope.

Semi-quantitative analysis of staining intensities

The intensities for apelin, c-Fos and apelin/c-Fos double staining immunoreactivity were scored by a blind count of immunoreactive cells. A semi-quantitative evaluation was performed by using the following intensity categories: weak but detectable staining (+), moderate or distinct staining (++), strong (+++), very strong or intense staining.

Quantification of Apelin and CRF in microdialysates

All microdialysis samples were kept at -80°C prior to the analysis. Apelin and CRF assays were performed with commercially available EIA kits (Phoenix Pharmaceuticals, CA, USA). Microdialysis samples were extracted using Sep-Pak C-18 (Phoenix Pharmaceuticals, CA, USA) 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 ventral tail artery 30 min before and after the central apelin-13 administration (100 μg, icv). Whole blood was centrifuged at 10,000g and plasma samples were aliquoted and frozen at -80°C. In order to measure corticosterone, a commercially available EIA kit was used (Enzo Life Sciences, Plymouth Meeting, PA, USA). The assay procedure was carried out according to the protocol supplied by the manufacturer.
Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA or Kruskal Wallis test followed by Student’s t-test or Mann Whitney-U test were used to determine the significance among groups, as appropriate. The plasma corticosterone results following apelin-13 administration were compared with the baseline results by using 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 (46, 55), following AS increased number of neurons expressing c-Fos immunoreactivity in their nuclei was observed in hypothalamic PVN (Fig. 1). Increased apelin and c-fos coexpression were detected in a group of PVN neurons in ARS-loaded rats. The stress-induced effects on apelin and c-Fos expressions were observed more robust in CHeS rats compared to the CHS-loaded rats. The intensity for apelin/c-Fos double staining was more pronounced in CHeS group (Table. 2).

Verification of the cannula placements

The data of 5 rats used for microdialysis were excluded from the study due to 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. 2A. Verification of the icv cannula was performed by ANG-II drinking behavior test and macroscopic observation of dye in ventricles (Fig. 2B and 2C). Among the icv-cannulated animals, 2 rats were excluded from the experiments due to latency of drinking behavior over 120 sec following central ANG-II injection and absence of methylene blue within 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 (1060.44±85.04 ng/ml, n=8, \( p<0.001 \)). The AS-induced increase remained unchanged (981.27±57.14 ng/ml, n=8, \( p<0.001 \))
vs NS) in CHeS-loaded rats, while in CHS-load rats, plasma corticosterone levels were found significantly lower (666.94±51.03 ng/ml, n=8, p<0.01) compared to the CHeS rats (Fig. 3).

**Stress exposure increases hypothalamic release of apelin and CRF**

Compared to the NS rats (0.83±0.43 ng/ml, n=6), exposure to AS for 90 min significantly increased the apelin levels (8.15±2.45 ng/ml, n=6, p<0.05) in hypothalamic microdialysates. CHS loading significantly (p<0.05, n=6) reduced the AS-induced apelin concentrations to 2.35±1.03 ng/ml. In CHeS-loaded rats, apelin concentrations remained significantly (p<0.05) higher (9.56±2.76 ng/ml, n=6, p<0.05) compared to those in NS and CHS groups (Fig. 4A).

Under NS conditions, CRF concentration was found 0.43±0.28 ng/ml (n=6). AS significantly increased CRF release (8.59±3.99 ng/ml, n=6, p<0.05). CHS loading restored the CRF concentrations (0.66±0.27 ng/ml, n=6) to the NS levels; whereas, in CHeS group, CRF concentrations were found significantly higher (10.22±3.29 ng/ml, n=6, p<0.05) compared to the rats in NS and CHS groups (Fig. 4B).

**Central F13A abolishes stress-induced changes 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), while 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 to the NS rats.

Central F13A administration (10 µg, icv) did not affect the solid GE (54.51%±1.9, n=7) under NS conditions, while 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. 5).

**Central F13A abolishes stress-induced changes 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 to (4.4±0.2, n=9), while it was 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. 6).

**Correlations between apelin/GE and apelin/CT under chronic stress**

Following chronic stress protocols (CHS and CHeS), there was a significant positive correlation (R²= 0.75, n=6, p<0.01) was found between apelin level in microdialysates and geometric center of CT (Fig. 7A). Also, a significant negative correlation (R²= 0.82, n=6, p<0.01) was observed between apelin level and GE (Fig. 7B).

**Central apelin-13 inhibits gastric and colonic motor functions**

In order to test whether apelin-13 alters solid GE under NS rats, different doses of apelin-13 were centrally (icv) administered 30 min prior to GE measurements. 10 µg apelin-13 administration (57.9±2.9%, n=7) did not change GE (61.9±4.3%, n=7), compared to vehicle-injected rats. 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 to those in vehicle group (Fig. 8A).

To test the effects of central apelin-13 treatment on CT, same doses of apelin-13 in GE measurements were administered 30 min prior to CT measurements. In line with GE measurements, centrally injected 100 µg dose of apelin-13 significantly inhibited CT (2.7±0.1%, n=7, p<0.05), while 10 µg (3.9±0.1, n=7) and 50 µg (3.8± 0.4, n=7) doses did not alter CT compared to vehicle-injected rats (4.0±0.2, n=7) (Fig. 8B).

**Central apelin-13 administration increases plasma corticosterone**

Compared to pre-injection levels (391.84±16.35 ng/ml, n=9), single administration of central apelin-13 (100 µg, icv) significantly increased the plasma corticosterone (747.03±42.12 ng/ml, n=9, p<0.01) under NS conditions (Fig. 9).

**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. The inhibitory effect of apelin-13 was observed immediately after administration and persisted approximately 60 min (Fig. 10). Pretreatment of aCSF (5 µl, icv) did not affect the apelin-induced effects both on antral and colonic
postprandial contractions, while the inhibitory effect of apelin-13 on antral contractions was completely abolished by CRF$_{9-41}$ (10 µg, icv) (Fig. 11). In contrast, contractions in distal colon were slightly diminished but not completely inhibited by pretreatment of CRF$_{9-41}$. MI was calculated before and after apelin-13 injections. Icv administration of aCSF did not alter the apelin-induced changes both in antral and colonic MI. However, pretreatment of CRF$_{9-41}$ significantly (n=6, p<0.05) attenuated the inhibitory effect of apelin-13 on MI in antrum, but not in distal colon (Fig. 12).

DISCUSSION

Accumulating evidences have raised the consideration that apelin play a regulatory role in stress response. Following a stress exposure, CRF is secreted from the hypothalamus and which in turn activates the HPA axis resulting in the secretion of corticosterone from the adrenal cortex (17, 19, 49). CRF also acts in the brain to influence the GI motor functions via autonomic neural pathways (6, 30, 50, 51). Similarly, central CRF administration delays GE and stimulates colon transit in rodents (31, 35, 47). 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 (36). 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 (23) and plasma ACTH and corticosterone levels in plasma (22, 52). In the present study, we have shown the increased apelin and c-fos coexpression detected in hypothalamic PVN neurons. In CHeS group, coexpression of apelin and c-Fos was found more pronounced compared to 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 (58). 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 (15). 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 non-habituating stress protocol that yields maladaptation. In parallel with recent reports (1, 4, 10, 68), our data demonstrated that AS remarkably increased the CRF in microdialysates, compared to NS rats. Following CHS, increased CRF and apelin were completely recovered to the basal levels, while increased concentrations of CRF and apelin were still observed in CHeS group rats. In agreement with CRF, the upregulated apelin expression induced by acute stress was completely attenuated to the basal levels following CHS. However, increased hypothalamic apelin remained unchanged following CHeS.

In the present study, the plasma corticosterone levels were also found significantly higher in AS and CHeS-loaded rats compared to the rats under NS and CHS conditions. HPA function is regulated by negative feedback, whereby circulating glucocorticoids inhibit their own release by actions at corticosteroid receptors in the brain. The neuroendocrine response to many stressors is reduced after repeated or chronic exposure (16, 32). 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 (11, 14, 16, 32).

Following the 5-day CHS protocol, we have found that both AS-induced delayed GE and accelerated colon transit were completely recovered, while 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, while a significant (p<0.01) negative correlation was found between apelin and GE in CHS and CHeS rats. In order 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 CHeS groups, were completely abolished by central administration of F13A indicates 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, while 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 up-regulation 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, while the accelerated colonic transit elicited by CHSeS was attenuated by central oxytocin treatment (66). It was previously demonstrated that compared to 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 colonic transit 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 found much greater in oxytocin-knockout mice (4). Furthermore, electrophysiological recordings from hypothalamic SON neurons demonstrated that apelin-13 administration increased the firing rates of vasopressin cells but had no effect on the firing rate of oxytocin neurons. The direct excitatory effect of apelin-13 on vasopressin cell activity was also supported by in-vitro studies showing depolarization of membrane potential and increase in action potential firing (57). On the other hand, in situ hybridization and immunohistochemistry studies demonstrated the presence of apelin receptor mRNA in hypothalamic OXY neurons and co-localization 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, stress-induced upregulation of apelin has been in alimentary tract. In a recent report, increased gastric mucosal apelin upon acute water-immersion stress has been shown in Wistar rats (21). Furthermore, following 90-min restraint stress, we have detected increased apelinergic immunoreactivity in antral and duodenal myenteric plexus neurons in rats (unpublished observations). Nonetheless, we cannot exclude the possibility that apelin mediates stress-induced alterations in GI motor functions through peripheral pathways in addition to autonomic pathways. This possibility will be investigated in future studies by comparing intact and autonomic denervated animals under stressed conditions.

As previously reported, central apelin is known to stimulate the release of corticosterone from adrenal cortex by activating HPA axis (22, 37, 52, 59). Likewise, we have found that single central administration of apelin-13 significantly increased the plasma corticosterone under NS conditions. Recent reports have demonstrated that central apelin may
influence GI motor functions in rodents. Central exogenous apelin has been shown to inhibit gastric emptying, gastrointestinal transit and colon transit in mice (28, 65). 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 gastric emptying and colon transit by inhibiting the post-prandial contractions in conscious rats under NS conditions. To corroborate our findings, we also performed motility recording experiments using strain gage transducers in order to monitor antral and colonic spontaneous contractions. Both in antrum and distal colon, apelin-13 administration significantly inhibited the contractions and caused approximately 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 contractions was completely abolished by α-helical CRF9-41 suggesting that apelin-induced inhibition of gastric motility is mediated through 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 gastric emptying by CRF may be mediated by interaction with the CRF-2 receptors in rostral ventro-lateral medulla (RVLM); whereas, CRF-1 receptors in Barrington’s nucleus (BN) are involved in the colonic and anxiogenic responses to stress (34, 35, 50, 51).

In the present study, we preferred administering a non-specific antagonist α-helical CRF9-41 rather than specifically antagonizing by using NBI27914 and astressin-2B which are well-known antagonists for CRF2R and CRF1R, respectively. Thus, future experiments aimed at addressing the CRF receptor subtypes in brainstem 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 (28, 65). The opioid
peptides have a major physiological role in the control of gut motility and secretions (8, 29).

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 (42). Especially, the kappa opioid receptors are known to inhibit colonic contractions acting centrally (8). There is a bunch of 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 (64). Moreover, it was demonstrated that APJ receptor forms a heterodimer with kappa opioid receptor and leads to increased PKC and decreased protein kinase A activity (27). 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 kappa opioid receptors.

Taken together, our data for the first time provided a direct evidence that apelin mediates stress-induced GI motor dysfunction in addition to or in cooperation with CRF in hypothalamic stress circuitry. Thus, the up-regulated 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 mal-adaptive 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 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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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Representative figure of apelin/c-Fos double-staining of hypothalamic PVN neurons in NS, acute stress (AS), chronic homotypic stress (CHS) and chronic heterotypic stress (CHeS) groups. Apelin immunostaining was detected in cytoplasm of some neurons in PVN (red staining, arrow). The c-Fos protein expression was observed as nuclear immunoreactivity in a group of PVN neurons (brown staining, arrowhead). PVN neurons that exhibited both apelin and c-Fos expression by using double immunostainings are indicated by double arrowhead. Scale bar represents 50 μm.

**Fig. 2.** The histological section and macrophotographs showing the verification of cannulation for microdialysis (A) and icv injection. The icv cannulation site of right lateral ventricle and centrally injected methylene blue was observed within the cerebral ventricles in sagittal (B) and coronal (C) sections. GC: guide cannula, P: microdialysis probe, 3V: third ventricle.
Fig. 3. Plasma corticosterone concentrations following AS, CHS and CHeS. NS: non-stressed, AS: acute stress, CHS: chronic homotypic stress, CHeS: chronic heterotypic stress. Values are means ± SE; n=8 rats per group. ***p<0.001 vs NS; ##p<0.01 vs CHS.

Fig. 4. Apelin (A) and CRF (B) levels in microdialysates collected from hypothalamic PVN. NS: non-stressed, AS: acute stress, CHS: chronic homotypic stress, CHeS: chronic heterotypic stress. Values are means ± SE; n=6 rats per group. *p<0.05 vs NS; #p<0.05 vs CHS.

Fig. 5. Effect of AS, CHS, CHeS and central F13A administration on solid gastric emptying. NS: non-stressed, AS: acute stress, CHS: chronic homotypic stress, CHeS: chronic heterotypic stress. Values are means ± SE; n=9 rats per group. **p<0.01 vs NS; †p<0.05 vs AS; ‡p<0.05 vs CHeS.

Fig. 6. Effect of AS, CHS, CHeS and central F13A administration on colonic transit. NS: non-stressed, AS: acute stress, CHS: chronic homotypic stress, CHeS: chronic heterotypic stress. Values are means ± SE; n=9 rats per group. **p<0.01 vs NS; †p<0.05 vs ARS; ‡p<0.05 vs CHeS.

Fig. 7. Correlations between hypothalamic apelin/CT (A) and apelin/GE under CHS and CHeS conditions. CHS: chronic homotypic stress, CHeS: chronic heterotypic stress, GE: gastric emptying, CT: colon transit. n=6 rats per group.

Fig. 8. Effect of intracerebroventricular administration of apelin-13 on solid GE (a) and CT (b) under non-stressed condition. Values are means ± SE; n=9 rats per group. *p<0.05 vs vehicle.

Fig. 9. Plasma corticosterone concentrations following central administration of apelin-13 (100 µg, icv). Values are means ± SE; n=9 rats per group. **p<0.01 vs pre-injection.

Fig. 10. Effect of intracerebroventricular administration of apelin-13 on antral (a) and distal colonic (b) postprandial contractions under non-stressed conditions.
Fig. 11. Effect of intracerebroventricular pretreatment of α-helical CRF\textsubscript{9-41} on apelin-induced changes antral (a) and distal colonic (b) postprandial contractions under non-stressed conditions.

Fig. 12. Effect of intracerebroventricular apelin-13 and α-helical CRF\textsubscript{9-41} on antral (a) and distal colonic (b) motility index changes under non-stressed conditions. Values are means ± SE; n=6 rats per group. *p<0.05 vs aCSF+Apelin.

Table 1. Chronic heterotypic stress (CHeS) protocol. RS: restraint stress, CRS: cold restraint stress, FSS: forced swimming stress, WAS: water avoidance stress.

Table 2. Semi-quantitative evaluation of immunostaining intensities with apelin, c-Fos and apelin/c-Fos double staining. Staining intensity categories: (+) weak but detectable staining, (++) moderate or distinct staining, (++++) strong, (+++++) very strong or intense staining. NS: non-stressed, AS: acute stress, CHS: chronic homotypic stress, CHeS: chronic heterotypic stress.
Plasma Corticosterone (ng/mL)

Vehicle  F13A

NS  AS  CHS  CHeS

Plasma Corticosterone (ng/mL)
Gastric Emptying (%)

- **NS**
- **AS**
- **CHS**
- **CHeS**

F13A, 10 μg, icv
Geometric Center

F13A, 10 μg, icv

NS  AS  CHS  CHeS  NS  AS  CHS  CHeS
A

\[ R^2 = 0.6389 \]
\[ p < 0.05 \]

B

\[ R^2 = 0.6482 \]
\[ p < 0.05 \]
Motility Index Change (% of aCSF)

- **Apelin-13**
  - Antrum
  - Distal Colon

- **CRF$_{9-41}$+Apelin-13**
  - Antrum
  - Distal Colon

* Indicates a significant difference.
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