Intranasal Administration of Neuropeptide Y Significantly Antagonized Chronic Stress Responses via Central Gabaa Receptors in Male Rats

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Research Article

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Abstract

Stress is widely believed to play a major role in the pathogenesis of many diseases. Central neuropeptide Y (NPY) counteracts the biological actions of corticotropin-releasing factor (CRF), and in turn attenuates stress responses. Administration (intracerebroventricular, ICV) of NPY, significantly antagonized the inhibitory effects of chronic complicated stress (CCS) on gastrointestinal (GI) dysmotility in rats. However, ICV administration is an invasive technique. The effect of intranasal administration of NPY on the hypothalamus-pituitary-adrenal (HPA) axis and GI motility in CCS conditions have not been studied, and the inhibitory mechanism of NPY on CRF through the gamma-aminobutyric acid $(GABA)_A$ receptor needs to be further investigated. A CCS rat model was set up, NPY was intranasal administered every day prior to the stress loading. Further, a $GABA_A$ receptor antagonist was ICV injected daily. Central CRF and NPY expression were evaluated, serum corticosterone and NPY levels were analyzed, and colonic motor functions was assessed. CCS rats showed significantly increased CRF expression and corticosterone levels, which resulted in enhanced colonic motor functions. Intranasal NPY significantly increased central *NPY* mRNA expression and reduced central *CRF* mRNA expression as well as the plasma corticosterone level, helping to restore colonic motor functions. However, ICV administration of the $GABA_A$ receptor antagonist significantly abolished these effects. Intranasal administration of NPY upregulates the hypothalamic NPY system. NPY may, through the $GABA_A$ receptor, significantly antagonize the overexpressed central CRF and attenuate the HPA axis activities in CCS conditions, exerting influences and helping to restore colonic motor function.

Introduction

Functional gastrointestinal disorders (FGIDs), such as irritable bowel syndrome (IBS), are one of the common pathologies of the gut. The pathogeneses of IBS are highly associated with stress in humans [1, 2]. Gastrointestinal (GI) dysmotility might develop as a result of the accumulation of repeated stress in some individuals. Corticotropin releasing factor (CRF) in the central nervous system plays a significant role in mediation of stress-induced GI dysmotility [3, 4]. Accelerated colonic transit induced by Acute restraint stress (ARS) is mediated via central CRF type 1 (CRF_1) receptors and parasympathetic pathways in rats [5]. Accelerated colonic transit was also detected in the rat model of chronic complicated stress (CCS), when rats received 7 days different types of stressors, with raised *CRF* mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus, resulting in triggering of the hypothalamus-pituitary-adrenal (HPA) axis [6, 7].

Although peripheral CRF receptor antagonists have been developed, the efficiency of the antagonists to treat for stress-induced GI dysmotility remains to be investigated [8, 9]. Neuropeptide Y (NPY), a 36 amino acids peptide, is highly expressed in the mammalian nervous system [10, 11]. NPY is also synthesized and released from peripheral sympathetic nerve system [12, 13]. NPY is involved in modulating stress related disorders, such as anxiety, depression, and post-traumatic stress disorder (PTSD) [14, 15], and may even the epilepsy [16]. NPY is involved in the termination of the stress and anxiety response by

inhibiting the biological actions of CRF [17]. Endogenous NPY is potently anxiolytic, acting as a buffer that promotes behavioral adaptation to cope with stress [18, 19]. The actions of NPY are mediated through at least five G protein coupled receptors [20]. The anxiolytic effect of NPY is mediated primarily through the Y1 receptor [17, 19]. Our previous study also showed central NPY via the Y1 receptor plays an important role in mediating the adaptation mechanism against repeated restraint stress in rats [21]. Furthermore, in the study of the inhibition mechanism of NPY on CRF, it was found that central NPY could regulate the excitability of central *nucleus* of amygdala (CeA) through gamma-aminobutyric acid $(GABA)_A$ receptor and improve the adaptability of organism to stress response [22]. In addition, NPY and GABA were co-expressed in the arcuate nucleus of the hypothalamus (ARC) and projected to PVN [23]. The regulation of NPY on feeding function is also mediated by $GABA_A$ receptor [24]. Therefore, it is necessary to further explore the regulatory and inhibitory mechanism of NPY on CRF through $GABA_A$ receptor under chronic stress.

ICV administration is an invasive technique, which will produce a stress response, and may affect the results. Intranasal infusion represents a non-invasive approach for the rapid delivery of NPY to the brain that can avoid the stress response. Recently, NPY application via the intranasal administration route has been shown to exert a reduction of anxiety and depression-like behavior in a rat model of PTSD [25, 26]. The intranasal administration method allows peptides to rapidly penetrate into the brain, bypassing the blood brain barrier to effectively interact with their receptors in multiple brain regions. Furthermore, in therapeutic study design, intranasal NPY reduced the perceived severity of stress and prevented stress-induced dysregulation of the HPA axis and noradrenergic activity [27, 28].

However, the effect of intranasal administration of NPY on HPA axis and GI motility has not been investigated. Therefore, the present study sought to evaluate in chronic complicated stress rat model, whether intranasal administration of NPY attenuated the HPA axis activity in response to stress and restored stress-induced GI dysmotility. Further, it is necessary to explore the inhibitory mechanism of NPY on CRF through $GABA_A$ receptor under chronic stress. Our work will contribute to the further studies on stress-induced abnormal GI motility and will help to seek a new approach (intranasal administration of NPY) for treatment of FGIDs.

Material And Methods

Animals

Adult male Sprague-Dawley rats (laboratory animal center of China Medical University, Shenyang, China), weighing 260 - 300g, were housed in individual cages under conditions of controlled temperature (22 - 24°C) and illumination (12-hour:12-hour light-dark cycle starting at 6 ante meridiem [AM]) for at least 7 days before the experiment. Rats were fed with commercial pelleted feed from Xietong Organism Institute (Nanjing, China) and water ad libitum. All experiments were started at 9 AM each day. Animal procedures were reviewed and approved by the Animal Care and Use Committee of China Medical University and conducted according to the guidelines of the laboratory animal ethical standards of China Medical

University, and conform to NIH guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Chemicals

NPY was from NeoBioSci (Cambridge, MA, USA). Bicuculline methiodide (BMI) and Evans blue was from Sigma Aldrich (MO, USA). Drugs were prepared as stock solutions in the appropriate solvent (water and saline).

Chronic complicated stress loading

For CCS loading, the rats (n = 6-8) received different types of stressors for 7 consecutive days, as previously reported [29]. The stress paradigms used were fasten restraint stress (FRS), force swimming stress (FSS), cold restraint stress (CRS), and water avoidance stress (WAS). The specific conditions for each type of stress are as follows:

(1) FRS: rats were placed on a wooden plate with their trunks wrapped in a confining harness for 90 minutes. For the control group (n = 6-8), the rats were housed in original individual cages for 90 minutes, but limited access to food and water.

(2) FSS: rats were placed individually in a plastic tank (52 × 37 × 20 cm) filled with room temperature (RT) water to the depth of 15 cm for 20 minutes. The depth of the water forced the animal to swim or float without hindlimbs touching the bottom of the tank. Control rats were placed individually in a waterless container tank for 20 minutes.

(3) CRS: rats were kept restrained at 4°C for 45 minutes. Control rats were kept at RT for 45 minutes.

(4) WAS: rats were placed on a platform (6 × 8 cm) in the middle of a plastic container (50 × 30 × 20 cm) filled with RT water to 1 cm below the height of the platform for 60 minutes. Control rats were placed on the same platform in a waterless container for 60 minutes.

Rats were exposed to different stressors each day for 7 days:

(1) Day 1: FRS (90 minutes, AM), FSS (20 minutes, post meridiem [PM]); Day 2: CRS (45 minutes, AM); Day 3: FRS (90 minutes, AM), WAS (60 minutes, PM); Day 4: CRS (45 minutes, AM); Day 5: FRS (90 minutes, AM), FSS (20 minutes, PM); Day 6: CRS (45 minutes, AM); Day 7: FRS (90 minutes, AM).

Intranasal administration of NPY

Intranasal administration was performed once a day, 30 min prior to the stress loading (AM), rats (n = 6 - 8) were infused with 150 μg NPY (NeoBioSci, Cambridge, MA) freshly dissolved in 20 ul saline, and 10 ul NPY was infused into each nare with pipetteman and disposable plastic tip under light isoflurane anesthesia (2%, RWD life science, Shenzhen, China), as previously described [27, 28]. Care was taken to avoid contact with the intranasal mucosa. Following intranasal administration, the head of the animal

was held in a tilted back position for approximately 15s to prevent loss of solution from the nares. For the control groups, 10 µl saline was infused into each nare. Intranasal administration of NPY (150 µg) has been shown to reduce depressive-like behavior, and attenuate HPA axis activity in rats stress model, as reported recently [25, 30].

Blood collection and hormone assays

The experimental rats were euthanized immediately by pentobarbital sodium (200 mg/kg intraperitoneal injection; Sigma-Aldrich, St. Louis, MO, USA) after fasten restraint stress, the 7th day of CCS loading.

At the time of rats' death, blood was collected immediately via a cardiac puncture, and then the samples were allowed to clot in tubes and centrifuged at 4°C for 10 min at 3000 rpm to separate out the serum. The serum fraction was stored at −80°C for further analysis. Corticosterone concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a corticosterone ELISA kit (Cat# ADI-900-097, Enzo Life Sciences, Plumoth meeting, PA, USA, detection level 32-20,000 pg/ml, sensitivity 27.0 pg/ml), as previously reported [31]. NPY concentrations were measured by NPY EIA kit (Cat**#** EK-049-03, Phoenix Pharmaceuticals, Belmont, CA, USA, detection level 0.09-1.43 ng/ml, sensitivity 0.09 ng/ml), as previously reported [21, 27]. All procedures were carried out according to the manufacturer's instructions. *The* experiments *wererunintriplicate* and *the* results represent their average values.

Quantitative real-time polymerase chain reaction

The rat brain tissue micropunching technique was applied for acquiring hypothalamus tissue samples from specific regions with micro-punchers. Briefly, after fasten restraint stress, the 7th day of CCS loading, the experimental rats were euthanized and the brains were removed immediately and cut into 450 µm coronal sections. Punches were collected from the left and right PVN (1.8 mm caudal to bregma; 0.4 mm lateral to midline; 7.6 mm ventral to the brain surface), as previously reported [7, 21]. All coordinates were based on the rat brain atlas and hypothalamic images of previously reported [32]. Samples were stored at −80 ◦C until use. Total RNA was extracted from the brain tissues using Trizol (Invitrogen, Carlsbad, CA, USA), and trace DNA contamination was removed by DNase digestion (Promega, Madison, WI, USA). Complementary DNA was synthesized from 3 µg total RNA by use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

The following primers were designed to amplify rat CRF: sense primer 5'-CCAGGGCAGAGCAGTTAGCT-3', antisense primer 5'-CAAGCGCAACATTTCATTTCC-3'. And the following were designed to amplify rat NPY: sense primer 5'- CAGAGGCGCCCAGAGCAG -3', antisense primer 5'- CAGCCCCATTCGTTTGTTACC -3'. For an internal control, the following were designed to amplify rat ß −actin: sense primer 5'-TGGCACCACACCTTCTACAATGAG-3', antisense primer 5'-GGGTCATCTTTTCACGGTTGG- 3', as previously reported (Yang et al. 2018; 2019). Quantitative polymerase chain reaction (PCR) was performed by using SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China). Amplification reactions were performed using ABI 7500 Real-time PCR instrument (Applied Biosystems, San Mateo, CA, USA). Initial template denaturation was performed for 30 seconds at 95°C. The cycle profiles were programmed as follows: 5 seconds at

95°C (denaturation), 20 seconds at 60°C (annealing), and 15 seconds at 72°C (extension). Forty-five cycles of the profile were run, and the final cooling step was continued for 30 seconds at 40°C. Quantitative measurement of each messenger RNA (mRNA) sample was achieved by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. Amplicon size and specificity were confirmed by melting curve analysis. The relative amount of each mRNA was normalized by the amount of ß-actin mRNA, as our previously reported [21, 33].

ICV cannulation and administration of $GABA_A$ receptor antagonist

The rats were anesthetized with isoflurane (2%, RWD life science, Shenzhen, China) and placed in a stereotaxic apparatus (RWD life science, Shenzhen, China). After the skin and muscles of the head were dissected, a 24-gage plastic sterile cannula was implanted into the right lateral ventricle (1.5 mm caudal, 2 mm lateral from the bregma; 6 mm ventral from the skull surface), as previously reported [21, 29]. The cannula was fixed with cement (Kyowa, Tokyo, Japan) and acrylic resin (Shofu, San Marcos, CA, USA). After cannulation, the rats were allowed to recover for 1 week.

To investigate whether $GABA_A$ receptor is involved in NPY mediated restoration following CCS, bicuculline methiodide (BMI, Sigma Aldrich, MO, USA) (100 ng/5 µl, ICV) was injected daily, 15 minutes prior to the stress loading. Saline (5 µl, ICV) was daily injected as controls. It has been shown that ICV administration of BMI (100 ng) was effective in antagonization of $GABA_A$ receptor subtypes in rats, as reported before [34, 35]. At the end of the experiment, the implantation site of ICV-cannula was confirmed by the presence of Evans blue (Sigma-Aldrich, St. Louis, MO, USA, 5%; 1 µl) after injection via the cannula, as previously reported [21, 33].

Monitoring of colonic motility

Rats were anesthetized with isoflurane (2%). Strain gauge transducers were implanted on the distal colon for colonic contractions. All wires were tunneled subcutaneously to exit at the back of the rat's neck and protected by a protective jacket (Star Medical, Tokyo, Japan). The abdominal wall was closed, and rats were allowed to recover for 7 days, as previously reported [7, 21]. The wires from the transducers were connected to a recording system (Power Laboratory 8SP; AD Instruments, Colorado Springs, CO, USA). Colonic circular smooth muscle contractions were monitored before, during, and after fasten restraint stress in the 7th days of CCS loading as mentioned above. For the non-stressed (NS) groups, the rats were housed in original individual cages for 90 min, but limited access to food and water. Quantification of colonic motility was studied by calculating motility index (MI). The MI was defined as MI log e (sum of amplitudes × total number of contraction waves + 1) that is equivalent with the area under the contractility recording curve and the baseline, for the 90-minute duration of colonic contractions in non-stressed rats as control (MI as 100%, saline 10 µl intranasal administered). MI was calculated using a computer-assisted system (Power Laboratory; AD Instruments), as previously reported [21, 29].

Measurement of fecal pellet output (FPO)

Rats were exposed to restraint stress treatment for 90 min as mentioned above, and the FPO numbers were counted after stress loading as measure of colonic transit function. For control group, the rats after finishing the feeding were back to their original cages for 90 min, and the FPO were also counted, as previously reported [21, 33].

Experimental design

Experiment 1

To study the effects of intranasal administration of NPY under CCS condition, in non-stressed (NS) rats and CCS rats model, NPY were intranasal administered (daily, 30 min before stress loading). For the control groups, the rats were saline intranasal administered. Central *CRF* and *NPY* mRNA expression (in PVN) were analyzed, serum corticosterone and NPY concentrations were measured, and colonic motor function was evaluated by motility recording system.

Experiment 2

To study whether the inhibitory mechanism of NPY on CRF via $GABA_A$ receptor under CCS condition. In ICV cannulated NS rats and CCS rats model, with or without intranasal administration of NPY, $GABA_A$ receptor antagonist bicuculline methiodide (BMI, Sigma Aldrich, MO, USA) was ICV injected daily, 15 min prior to the stress loading. Saline (5 μl, ICV) was daily injected as controls. The central CRF expression and the serum corticosterone concentrations were measured, and colonic motor function was evaluated by motility recording system.

Statistical analysis

Analysis was performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). Results were shown as mean ± standard error (SEM). Statistical analyses were performed using a two-way classification ANOVA with Tukey's post hoc tests to determine the significant interaction between different stress groups and drug treatment. Differences with $P < 0.05$ were considered statistically significant.

Results

Experiment 1

Effects of intranasal administration of NPY on central CRF and NPY mRNA expression in response to CCS.

In the NS groups, *CRF* mRNA expression in the PVN showed very low level, intranasal administration of NPY (150 μg, daily, before stress loading) did not change the *CRF* mRNA expression significantly, compared to that of saline intranasal administered NS rats (saline 10 μl as a control, n = 6). In the CCS groups, *CRF* mRNA expression increased significantly compared to that of saline intranasal administered

NS rats (n = 6, F (1, 20) = 26.663, p<0.001). Intranasal administration of NPY significantly decreased the *CRF* mRNA expression compared to that of saline intranasal administered CCS rats (n = 6, P = 0.001, saline 10 µl intranasal administered as a control; Figure. 1A).

In the NS groups, *NPY* mRNA expression in the PVN showed low level, intranasal administration of NPY (daily, before stress loading) did not change the *NPY* mRNA expression significantly, compared to that of saline IN administered NS rats (saline 10 µl as a control, n = 6). In the CCS groups, *NPY* mRNA expression increased significantly compared to that of saline intranasal administered NS rats (n = 6, F (1, 20) = 17.486, p=0.027). Intranasal administration of NPY further significantly increased the *NPY* mRNA expression compared to that of saline intranasal administered CCS rats (n = 6, p=0.011, saline10 µl intranasal administered as a control; Figure. 1B).

Effects of intranasal administration of NPY on serum corticosterone and NPY levels in response to CCS.

In the NS groups, serum corticosterone concentration showed low level. Intranasal administration of NPY (daily, before stress loading) did not change the serum corticosterone levels significantly (64.6 ± 6.3 ng/ml) compared to that of saline intranasal administered rats (67.2 ± 6.4 ng/ml, saline 10 µl intranasal administered as a control, n = 6). In the CCS groups, serum corticosterone concentration significantly increased to 146.3 ± 11.2 ng/ml compared to that of saline intranasal administered NS rats (n = 6, F (1, 20) = 18.215, p<0.001). Intranasal administration of NPY significantly decreased the corticosterone level to 93.8 ± 10.4 ng/ml, compared to that of saline intranasal administered CCS rats (n = 6, p=0.002, saline 10 µl intranasal administered as a control; Figure. 2A).

In the NS groups, serum NPY concentration showed very low level. Intranasal administration of NPY (daily, before stress loading) did not change the serum NPY levels significantly (1.83 ± 0.15 ng /ml), compared to that of saline intranasal administered rats (1.75 ± 0.13 ng/ml, saline 10 µl intranasal administered as a control, n = 6). In the CCS groups, serum NPY level decreased to 1.47 ± 0.17 ng /ml, but did not show significant change (n = 6, F (1, 20) = 1.666, p=0.206). However intranasal administration of NPY increased the NPY level to 1.92 ± 0.25 ng/ml, but also did not show significant change (n = 6, p=0.185, saline 10 µl intranasal administered as a control; Figure. 2B).

Effects of intranasal administration of NPY on colonic motility recording, MI changes and FPO in response to CCS.

In the NS groups, intranasal administration of NPY (daily, before stress loading) had no effects on the amplitude of distal colonic contraction, saline 10 µl intranasal administered as a control. In the CCS groups, restraint stress enhanced the amplitude of distal colonic contraction in the saline group (saline 10ul intranasal administered). However, Intranasal administration of NPY helped to partially restore the amplitude of distal colonic contraction groups. (n = 6, saline 10ul intranasal administered as a control; Figure. 3A).

In the NS groups, intranasal administration of NPY (daily, before stress loading) did not significantly alter the colonic MI change (95 ± 9%, n=6) compared to that of saline intranasal administered rats (100 ± 10%, saline 10 µl intranasal administered as a control, n = 6; F (1, 20) = 16.856, p<0.001, Figure. 3B). In the CCS groups, the enhanced colonic MI change in the saline group, was significantly attenuated by intranasal administration of NPY (from 196 ± 14% to 131 ± 11%, p=0.003, n=6, saline 10ul intranasal administered as a control; Figure. 3B).

In the NS groups, intranasal administration of NPY (daily, before stress loading) did not change the FPO significantly 3.7 ± 0.8 (number/90 min, n = 6) compared to that of saline intranasal administered rats (3.5 ± 0.7, saline 10 µl intranasal administered as a control, n = 6). In the CCS groups, FPO was significantly increased to 9.7 ± 0.9 (number/90 min, n = 6) compared to that of saline intranasal administered NS rats (n = 6, F (1, 20) = 13.842, p<0.001). Intranasal administration of NPY significantly decreased the FPO to 5.3 ± 0.7 (number/90 min, n = 6), compared to that of saline intranasal administered CCS rats (n = 6, p=0.004, saline 10 µl intranasal administered as a control; Figure. 3C).

Experiment 2

Effects of intranasal administration of NPY and $GABA_A$ receptor antagonist BMI on central CRF mRNA expression and NPY mRNA expression in response to CCS.

In the non-stressed (NS) groups, *CRF* mRNA expression showed very low level (saline 10 µl intranasal administered, and saline 5 µl ICV injected as a control), both intranasal administration of NPY (150 µg, daily) and ICV administered $GABA_A$ receptor antagonist BMI (100 ng/5 µl, for 7 consecutive days) did not change the *CRF* mRNA expression significantly (n = 6). In the CCS conditions, the *CRF* mRNA expression in the nasal saline groups was highly elevated (n = 6, F (1, 40) = 34.970, p<0.001). ICV administered BMI (100 ng/5 µl, 15 min prior to the stress loading for 7 consecutive days) did not change the *CRF* mRNA expression significantly (n = 6, saline 5 µl ICV injected as a control). However, in the nasal NPY groups, the decreased *CRF* mRNA expression was significantly increased by ICV administered BMI (n = 6, p=0.001, nasal saline and ICV saline as a control; Figure. 4A).

In the NS groups, *NPY* mRNA expression showed low level (saline 10 µl intranasal administered, and saline 5 µl ICV injected as a control), both intranasal administration of NPY (150 µg, daily) and ICV administered $GABA_A$ receptor antagonist BMI (100 ng/5 µl, for 7 consecutive days) did not change the *NPY* mRNA expression significantly (n = 6).

In the CCS conditions, the *NPY* mRNA expression increased significantly in the nasal saline groups (n = 6, F (1, 40) = 9.569, p=0.030). ICV administered of BMI did not change the *NPY* mRNA expression significantly (n = 6, p=1.000). However, in the nasal NPY groups, the further elevated *NPY* mRNA expression was significantly inhibited by ICV administered BMI (n = 6, p=0.021, nasal saline and ICV saline as a control; Figure. 4B).

Effects of intranasal administration of NPY and $GABA_A$ receptor antagonist BMI on serum corticosterone and NPY levels in response to CCS.

In the NS groups, the serum corticosterone concentration showed low level (67.4 ± 6.8 ng/ml, saline 10 μl intranasal and saline 5 μl ICV as a control), ICV administered BMI (100 ng/5 μl) increased the corticosterone level to 74.2 ± 6.4 ng/ml, but did not show significant change (n = 6). In the nasal NPY groups, the serum corticosterone level also did not show significant change by ICV administered saline or BMI (66.9 ± 6.6 ng/ml and 69.6 ±5.7 ng/ml, respectively, n = 6). In the CCS conditions, the serum corticosterone concentration in the nasal saline groups was significantly elevated (142.4 ± 9.5 ng/ml, saline 5 μl ICV injected, n = 6, $F(1, 40) = 20.716$, $p<0.001$, compared with NS groups). ICV administered BMI did not change the corticosterone level significantly (147.8 ± 10.4 ng/ml, n = 6, p=1.000). However, in the nasal NPY groups, the decreased serum corticosterone level was significantly increased by ICV administered BMI (from 97.1 ± 7.8 ng/ml to 140.3 ± 9.9 ng/ml, n = 6, p=0.011, nasal saline and ICV saline as a control; Figure. 5A).

In the NS groups, the serum NPY concentration showed very low level (1.76 ± 0.13 ng/ml, saline 10 μl intranasal and saline 5 μl ICV as a control), both intranasal administration of NPY (150 μg, daily) and ICV administered BMI (100 ng/5 μl) did not change the corticosterone level significantly (1.80 ± 0.16 ng/ml, 1.81 ± 0.14 ng/ml and 1.85 ± 0.15 ng/ml, respectively, n = 6). In the CCS conditions, the serum NPY level in the nasal saline groups decreased to 1.62 ± 0.14 ng /ml, but did not show significant change (n = 6, $F(1, 40) = 0.670$, p=0.996). ICV administered BMI also did not change the serum NPY level significantly (1.65 ± 0.12 ng/ml, n = 6). In the nasal NPY groups, the serum NPY level also did not show significant change by ICV administered saline or BMI (1.91 ± 0.11 ng/ml and 1.95 ± 0.15 ng/ml, respectively, n = 6, p=1.000, nasal saline and ICV saline as a control; Figure. 5B).

Effects of the $GABA_A$ receptor antagonist on colonic motility recording, motility index (MI) changes and FPO in response to CCS.

In the non-stressed (NS) groups, both intranasal administration of NPY (150 μg, daily) and ICV administered $GABA_A$ receptor antagonist BMI (100 ng/5 μl) had no effects on the distal colonic contractions (data not shown). In the CCS condition, restraint stress enhanced the amplitude of distal colonic contraction in the nasal saline group. ICV administered $GABA_A$ receptor antagonist BMI (100 ng/5 μl, 15 min prior to the stress loading for 7 consecutive days) did not change the distal colonic contraction obviously. However, in the nasal NPY groups, the attenuated distal colonic contraction was enhanced by ICV administered BMI.

(n = 6, saline 5 μl ICV injected as a control; Figure. 6A).

In the NS groups, the colonic MI change is 100 ± 10% (nasal saline and saline 5 μl ICV administered as a control, n=6). The combination of nasal saline or nasal NPY with ICV administered saline or BMI (100 ng/5 μl) did not alter the colonic MI change significantly (102± 10%, 94 ± 9%, and 97 ± 11%, respectively, n = 6). In the CCS condition, the colonic MI change was highly elevated (198 ± 12%, n=6, $F(1, 40) =$

17.760, p<0.001). ICV administered $GABA_A$ receptor antagonist BMI did not change colonic MI change significantly (195 ± 14%, n=6, p=1.000). However, in the nasal NPY groups, the decreased colonic MI change was significantly increased by ICV administered BMI (from 130 ± 11% to 201 ± 15%, nasal saline and ICV saline as a control; n = 6, p=0.003, Figure. 6B).

In the NS groups, the FPO is 3.5± 0.4 (number/90 min, nasal saline and saline 5 μl ICV administered as a control, n=6). The combination of nasal saline or nasal NPY with ICV administered saline or BMI (100 ng/5 μl) did not alter the FPO significantly (3.7 ± 0.6, 3.5 ± 0.6, and 3.8 ± 0.6, number/90 min, respectively, n = 6). In the CCS condition, the FPO was highly elevated 9.7 ± 0.7 (number/90 min, n = 6, F (1, 40) = 24.525, p<0.001). ICV administered $GABA_A$ receptor antagonist BMI did not change the FPO significantly 9.6 ± 0.6 (number/90 min, n = 6, p=1.000). However, in the nasal NPY groups, the FPO was significantly increased by ICV administered BMI (from 5.8 ± 0.6 to 9.5 ± 0.6 number/90 min, nasal saline and ICV saline as a control; n = 6, p=0.002, Figure. 6C).

Discussion

In the present study, we found that accelerated colonic motor function were also observed when rats received different types of stressors for 7 days, and hypothalamic *CRF* mRNA expression and serum corticosterone level was also highly elevated. These results are consistent with previous studies [6, 7]. In stress conditions, CRF accelerates colon transit via central CRF 1 receptors and parasympathetic nervous system in rats [5]. Although peripheral CRF receptor antagonists have been developed, as the possible candidates for the treatment of anxiety and depression like behavior, but the effects are still controversial [8, 9].

Our present study, in CCS rat model, central *NPY* mRNA expression increased after received different types of stressors, supported the results that NPY may usually react to acute stress that of more than moderate degree or chronic stress as mentioned above [19]. In the peripheral nervous system, NPY is found in sympathetic nerves, the platelets, and adrenal medulla. The circulating plasma NPY levels are in the low range under resting conditions; however, in many stress conditions, the release of NPY is dependent on the intensity and duration of stress, as well the pattern of sympathetic nerve activation [36]. NPY as a co-transmitter with norepinephrine (NE) for the peripheral sympathetic system, is also released during stress, but the proportions of these two transmitters vary depending on the type of stress. NPY requires a more prolonged and/or intense stimulation, while the release of NE is the mildest acute stress condition [37, 38]. Our previous study also found that the serum NPY levels did not increase significantly in response to acute fasten restraint stress (moderate stress stimulus), but were significantly increased at repeated restraint stress [21]. However, our present study, in CCS condition, serum NPY level decreased lower to 1.47 ± 0.17 ng /ml, but did not show significant change. This result supported the theory that in higher intensity chronic stress condition, NPY system will be exhaust gradually, and central NPY failed to play an important role to produce an adaptation [36, 37]. Thus, external administration of NPY were expected to terminate the stress responses in CCS condition.

Previous studies have shown that ICV-injection of NPY significantly reduces anxiety levels and stress response induced in rats [39, 40]. However, ICV administration is an invasive technique, which will produce stress response, and may affect the results. Intranasal infusion represents a non-invasive approach for the rapid delivery of peptides to the brain that can avoid side effects elicited by peripheral administration. This method allows peptides to enter the central nervous system rapidly and directly via intracellular neuronal olfactory and extracellular trigeminal-associated pathways bypassing the blood–brain barrier to affect multiple sites within the brain [26]. Recently, NPY application via the intranasal administration route has been shown to exert reduction of anxiety and depression-like behavior, in a rat model of PTSD [25, 26]. Furthermore, intranasal NPY reduced the perceived severity of stress and prevented stress-induced dysregulation of the HPA axis and noradrenergic activity [27, 28]. Intranasal administration of NPY was found to elevate NPY in the CSF to a similar degree as ICV NPY administration that was found effective to reduce anxiety-like behavior [27]. Further, delivery of NPY to specific brain areas was shown after intranasal administration of fluorescent-labeled NPY (FAM-NPY). FAM-NPY was found in many brain regions, including olfactory bulbs, hypothalamus, hippocampus and amygdala 30 min later [15].

Our present study also found that in the CCS conditions, the elevated central *CRF* mRNA expression and serum corticosterone level was significantly attenuated by intranasal administration of NPY (150 μg) and helped restore the distal colonic dysmotility was that induced by CCS. Intranasal administration of NPY also significantly increased the *NPY* mRNA expression and serum NPY level, the effects may due to accumulation of anxiolytic effect by daily administration of NPY, NPY might interact with the HPA axis, thereby counteracting the biological actions of CRF, and involved in the termination of the stress response.

In contrast to daily intranasal administration of NPY in CCS conditions, single intranasal NPY infusion failed to elevate the plasma NPY level in PTSD rat model, but can protect from over-activation of both central noradrenergic and HPA systems [27]. And further investigate is needed on this issue. However, intranasal administration of NPY, which was ineffective on central *CRF* mRNA expression and serum corticosterone level, also on the colonic motility in normal conditions, suggested that central NPY may play a predominant role in regulating stress-induced GI dysmotility, but not in non-stressed conditions.

The anxiolytic behavioral effect of NPY is mediated primarily through post-synaptic Y1 receptors have been well documented [13, 41]. Our previous study also found that central NPY via the Y1 receptor plays an important role in mediating the adaptation mechanism against chronic stress [21]. However, NPY as one of the most potent orexigenic peptides found in the brain, feeding is depending critically on the function of NPY system, and the orexigenic effect is primarily mediated by Y1 receptors, as previous reported [42, 19]. Thus, in the current study, we did not study the effect of intranasal administration of NPY on the gastric motor function, but the colonic function, and further investigate is needed to clarify this issue.

Further, we evaluated the distal colonic motility by a recording system, and we found that in the non-stressed groups, intranasal administration of NPY had no effects on the colonic contraction. However, in the CCS groups, intranasal administration of NPY significantly attenuated the enhanced colonic motor function induced by restraint stress.

In experiment 2 of our current study, a further study based on the results of experiment 1 was performed, to show whether the inhibitory mechanism of NPY on CRF *via* the $GABA_A$ receptor under CCS condition, in the ICV cannulated CCS rat model, $GABA_A$ receptor antagonist BMI was ICV injected. In the nasal NPY groups, the attenuated distal colonic contraction was enhanced by ICV administered BMI. Also, the decreased *CRF* mRNA expression and serum corticosterone level was significantly increased by ICV administered BMI. In the trace the timing of dosing in addition to the stress period, as well the *NPY* mRNA expression and plasma concentrations should be measured in the further investigation.

However, in the study of the inhibition mechanism of NPY on CRF, it was found that central NPY could regulate the excitability of CeA through $GABA_A$ receptor and improve the adaptability of organism to stress response [22]. In addition, NPY and GABA were co-expressed in ARC neurons of hypothalamus and projected to PVN [23]. The regulation of NPY on feeding function is also mediated by $GABA_A$ receptor [24]. GABA is the major inhibitory amino acid transmitter of the mammalian central nervous system. GABA exerts its effects through $GABA_A$ and $GABA_B$ receptors. GABA-projecting neurons into the PVN are known to inhibit CRF expression via $GABA_A$ receptors [35]. Released corticosterone in response to acute stress inhibits CRF release via a feedback mechanism, which is mediated via $GABA_A$ receptors in the PVN [43]. Our present study found that ICV administered $GABA_A$ receptor antagonist BMI (100 ng, 15 min prior to the stress loading for 7 consecutive days) did not change the *CRF* mRNA expression and the serum corticosterone level significantly, as well as the distal colonic motility in CCS condition. However, in the nasal NPY groups, the decreased *CRF* mRNA expression and the serum corticosterone level were significantly antagonized by ICV administered BMI, while the attenuated distal colonic contraction was enhanced by BMI. The results may support the theory that in higher intensity chronic stress condition, NPY system was exhausted, and central NPY failed to via GABAergic system play an important role to produce anxiolytic effects, the only external administration of NPY were expected in the termination of the stress response of CCS condition.

Previous studies have shown that central administration of BMI (50, 100, and 200 pmol) produced significant, dose-dependent effects on the sympathetic nerve activity in rats [34], and previous study also found that ICV administration of BMI 100 ng (200 pmol) was effective in antagonization of $GABA_A$ receptor subtypes in rats [35]. In the current study, we also found that ICV administration of BMI 100 ng significantly abolished the effects that induced by intranasal administration of NPY, suggesting that the GABAergic system is also involved in the inhibitory mechanism of intranasal administration of NPY on *CRF* mRNA expression, in chronic stress conditions.

In conclusion, intranasal administration of NPY significantly antagonized the overexpressed central CRF and colonic dysmotility in response to CCS, NPY is only effective under the stressful conditions. GABAergic system is also involved in the inhibitory mechanism. Our study may contribute to a better understanding of the mechanism and the treatment strategies in GI dysmotility of stress in daily life. Intranasal administration of NPY may be a new approach for treatment of stress-induced GI motility disorders.

Declarations

Author contribution

YY, HY and BS performed the experiment; RB, WS and XZ were involved in the study supervision and critical revision of the manuscript, JZ designed the experiment, analyzed the data and wrote the paper.

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Data Availability

The dataset generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no conflicts of interest exist.

Ethics Approval

All research procedures were carried out in accordance with the guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892-2018 and approved by the Animal Care and Use Committee of China Medical University.

Consent to Participate None.

Consent for Publication None.

Code Availability None.

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Figures

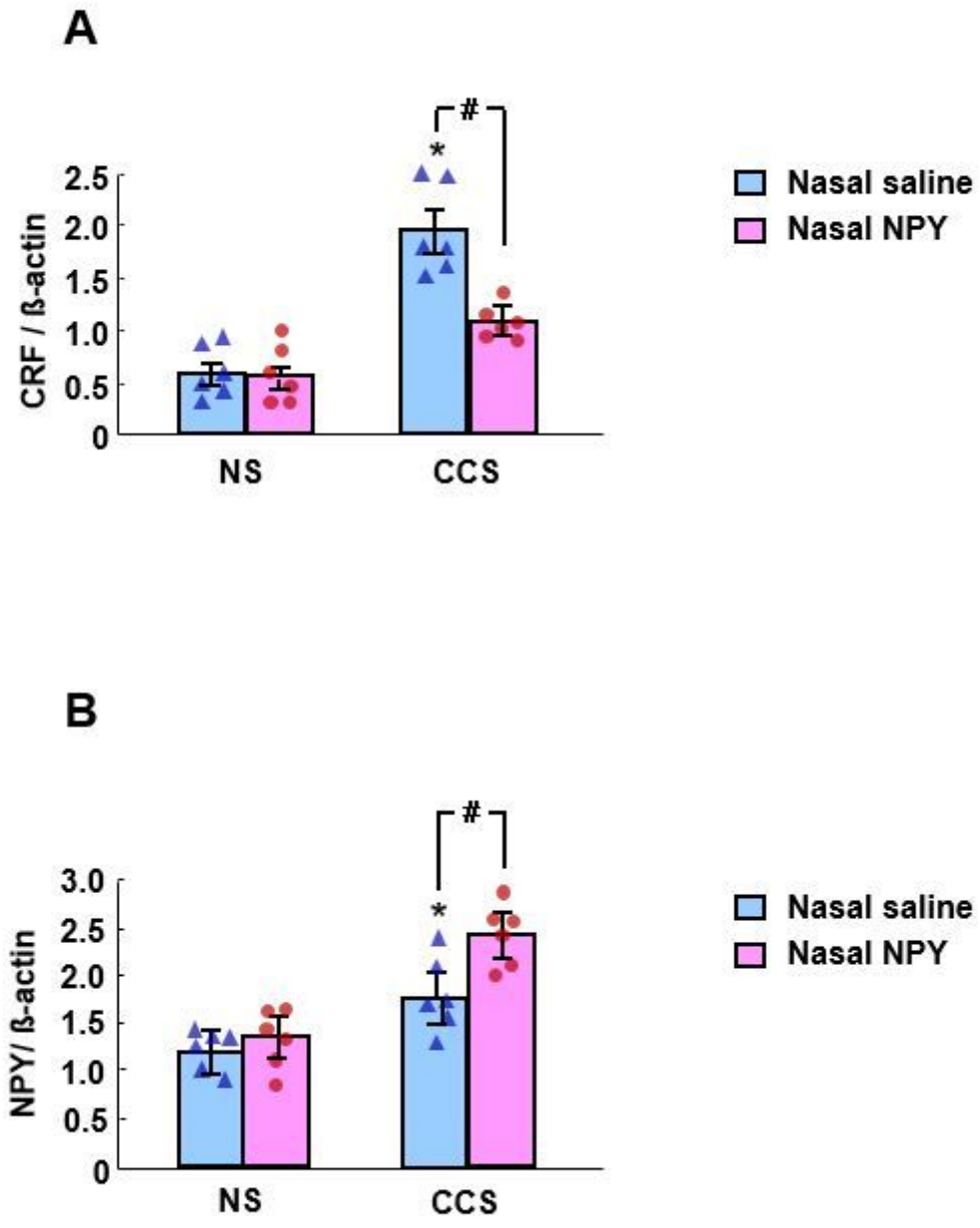

Figure 1

Effects of intranasal administration of NPY on central CRF (A) and NPY (B) mRNA expression in response to CCS. A. In the non-stressed (NS) groups, nasal NPY (150 µg, daily, before stress loading), had no effect on the CRF mRNA expression. In the CCS groups, CRF mRNA expression increased significantly (nasal saline), however, nasal administration of NPY significantly decreased the CRF mRNA expression. B. In the NS groups, nasal NPY had no effect on the NPY mRNA expression. In the CCS groups, NPY mRNA expression significantly increased (nasal saline). Nasal administration of NPY further significantly increased the NPY mRNA expression. The mRNA expression was standardized with the ratio of internal

control of β-actin. (n =6, *P <0.05 compared with NS nasal saline group, #P < 0.05 compared with CCS nasal saline group)

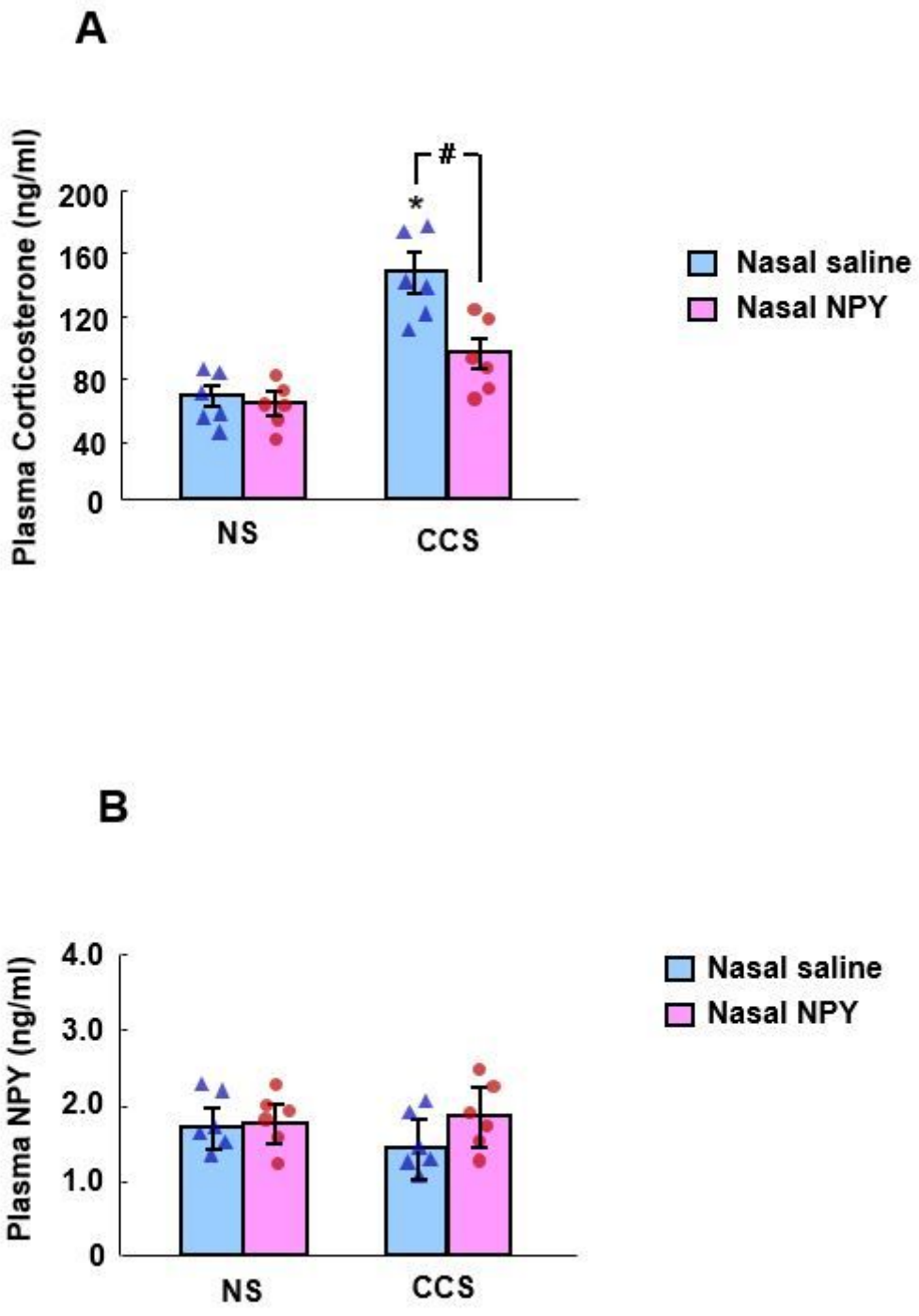

Figure 2

Effects of intranasal administration of NPY on serum corticosterone (A) and NPY (B) levels in response to CCS. A. In the non-stressed (NS) groups, nasal NPY (150 μg, daily, before stress loading), had no effect on the serum corticosterone level. In the CCS groups, corticosterone level increased significantly (nasal saline), however, intranasal administration of NPY significantly decreased the serum corticosterone level.

B. In the NS groups, nasal NPY had no effect on the NPY level. In the CCS groups, NPY level decreased, but not shown significantly changed (nasal saline). Nasal administration of NPY also did not change the NPY level significantly. (n =6, *P <0.05 compared with NS nasal saline, #P < 0.05 compared with CCS nasal saline)

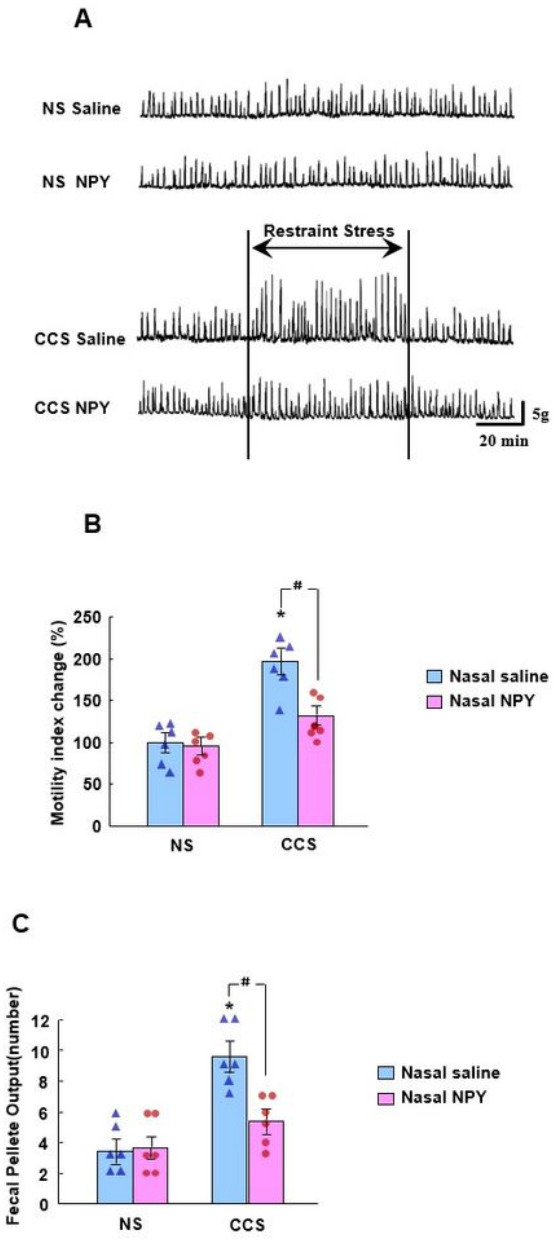

Figure 3

Effects of intranasal administration of NPY on distal colonic motility (A), MI changes (B) and FPO (C) in response to CCS. A. The distal colonic contractions in the non-stressed groups (NS Saline). Nasal NPY (150 μg, daily, before stress loading) had no effect on the distal colonic contractions (NS NPY). CCS strongly enhanced the amplitude of distal colonic contractions in the nasal saline group (CCS Saline). Intranasal administration of NPY attenuated the distal colonic contractions. B. In the NS group, nasal NPY did not significantly alter the colonic MI change. In the CCS groups, colonic MI change was significantly increased (Nasal saline). Intranasal administration of NPY significantly decreased the MI change. C. In the NS groups, nasal NPY did not significantly change the FPO. In the CCS groups, the FPO was significantly increased. Intranasal administration of NPY significantly decreased the FPO. (n =6, *P <0.05 compared with NS nasal saline, #P < 0.05 compared with CCS nasal saline)

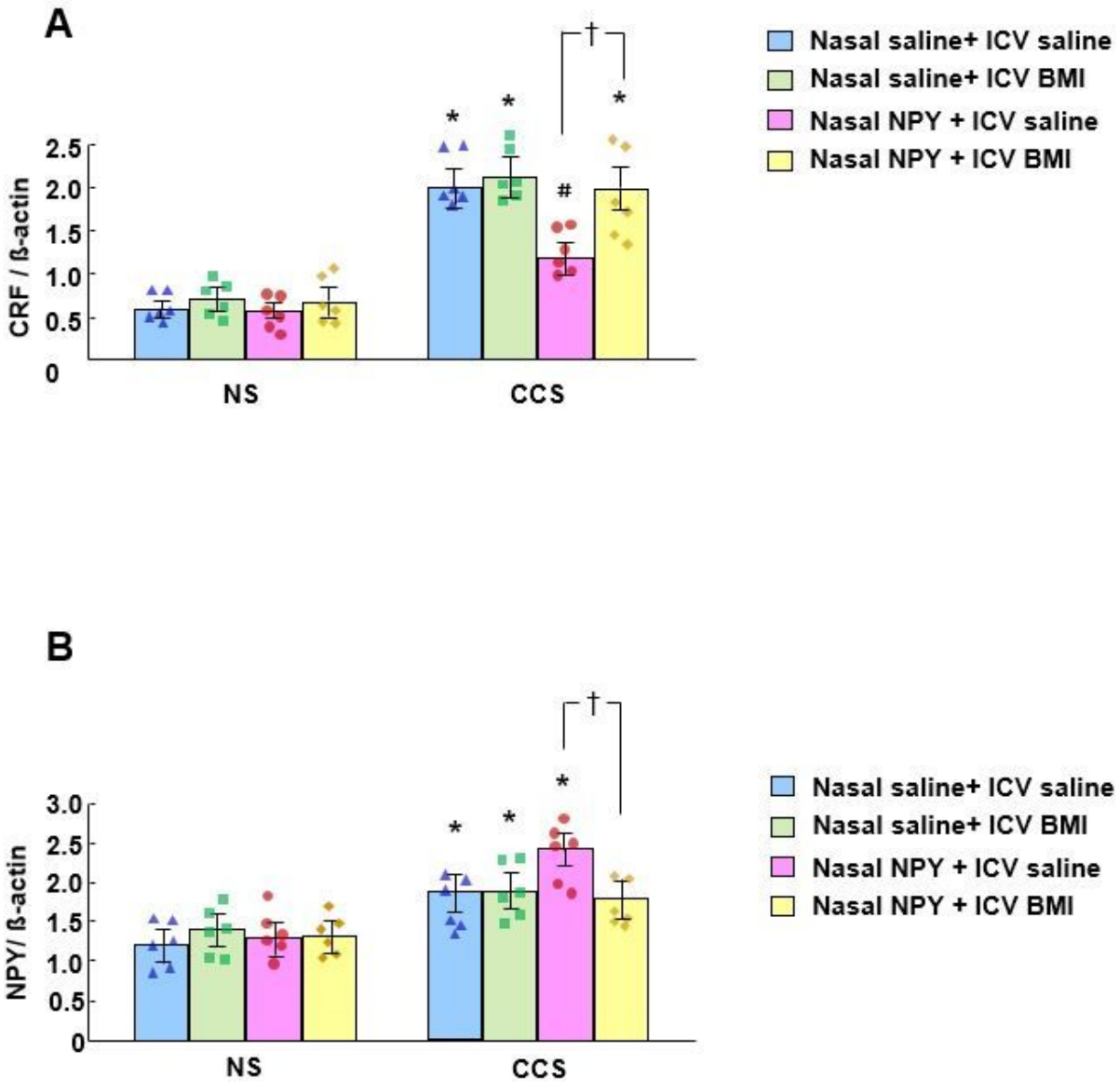

Figure 4

Effects of ICV injection of an GABAA receptor antagonist on central CRF mRNA expression (A) and NPY mRNA expression (B) in response to CCS. A. In the NS groups, CRF mRNA expression showed low level, intranasal administration of saline (with ICV administered saline or BMI) did not change the CRF mRNA expression significantly. However, intranasal administration of NPY (with ICV administered saline or BMI) also did not change the CRF mRNA expression significantly. In the CCS conditions, CCS highly elevated the CRF mRNA expression in the sham group (nasal saline and ICV administered saline). In the nasal saline group, ICV administered BMI did not change the CRF mRNA expression significantly. However, in the nasal NPY groups, the reduced CRF mRNA expression was significantly increased by ICV administered BMI. B. In the NS groups, NPY mRNA expression showed low level, intranasal administration of saline (with ICV administered saline or BMI) did not change the NPY mRNA expression significantly. However, intranasal administration of NPY (with ICV administered saline or BMI) also did not change the NPY mRNA expression significantly. In the CCS conditions, CCS significantly elevated the NPY mRNA expression in the sham group (nasal saline and ICV administered saline). In the nasal saline group, ICV administered BMI did not change the NPY mRNA expression significantly. However, in the nasal NPY groups, the further elevated NPY mRNA expression was significantly decreased by ICV administered BMI. (n =6, *P <0.05 compared with NS nasal saline+ ICV saline group, †P < 0.05 compared with CCS nasal NPY+ICV saline group)

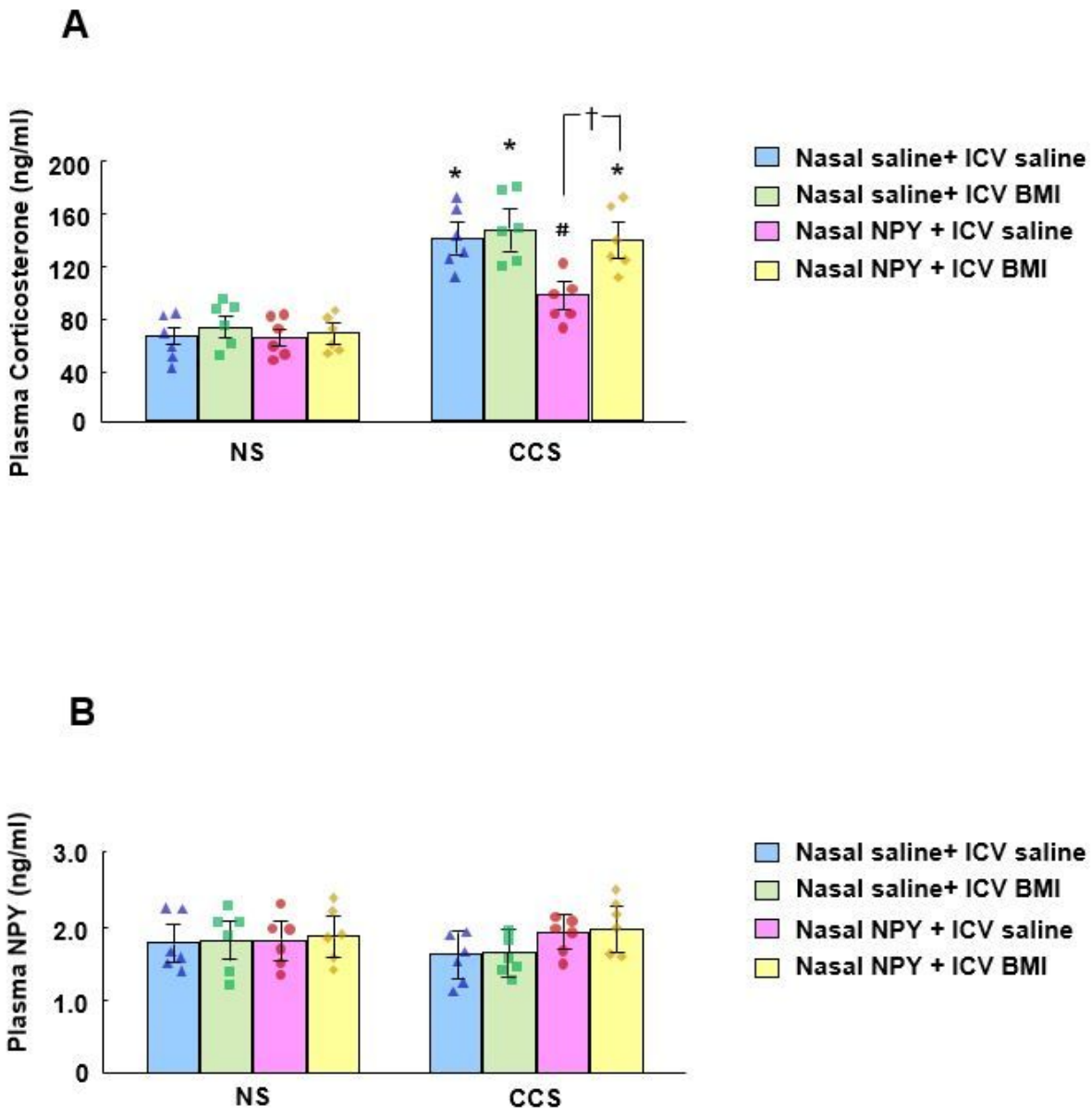

Figure 5

Effects of ICV injection of an GABAA receptor antagonist on serum corticosterone concentration (A) and NPY concentration (B) in response to CCS. A. In the NS groups, serum corticosterone concentration showed low level, intranasal administration of saline (with ICV administered saline or BMI) did not change the corticosterone level significantly. However, intranasal administration of NPY (with ICV administered saline or BMI) also did not change the corticosterone level significantly. In the CCS conditions, CCS highly elevated the corticosterone level in the sham group (nasal saline and ICV administered saline). In the nasal saline group, ICV administered BMI did not change the corticosterone level significantly. However, in the nasal NPY groups, the reduced corticosterone level was significantly increased by ICV administered BMI. B. In the NS groups, serum NPY concentration showed low level,

intranasal administration of saline (with ICV administered saline or BMI) did not change the NPY level significantly. However, intranasal administration of NPY (with ICV administered saline or BMI) also did not change the NPY level significantly. In the CCS conditions, CCS did not change the NPY level significantly in the sham group (nasal saline and ICV administered saline). In the nasal saline group, ICV administered BMI did not change the NPY level significantly. However, in the nasal NPY groups, the NPY level also did not change significantly (with ICV administered saline or BMI). (n =6, *P <0.05 compared with NS nasal saline+ ICV saline group, †P < 0.05 compared with CCS nasal NPY+ICV saline group)

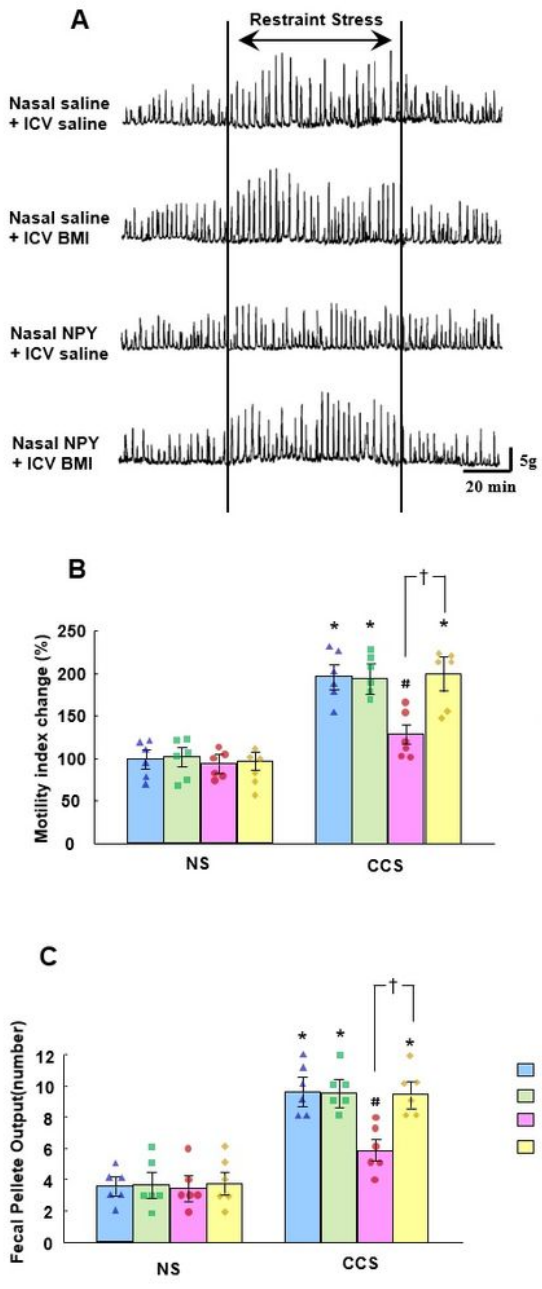

Figure 6

Effects of ICV injection of an GABAA receptor antagonist on colonic motility (A), colonic MI change (B) and FPO (C) in response to CCS. A. In the NS groups, both intranasal administration of NPY and ICV administered BMI had no effects on the distal colonic contractions (data not shown). In the CCS condition, CCS highly enhanced the amplitude of distal colonic contractions. In the nasal saline groups, ICV administered BMI had no effect on the colonic contractions. However, in the nasal NPY groups, the attenuated distal colonic contraction (ICV administered saline) was enhanced by ICV administered BMI. B. In the NS groups, the colonic MI change showed low value. Intranasal administration of saline (with ICV administered saline or BMI) had no effects on the colonic MI change. However, intranasal administration of NPY (with ICV administered saline or BMI) also had no impacts on the colonic MI change. In the CCS condition, CCS significantly increased the colonic MI change in the sham group (nasal saline and ICV administered saline), in the nasal saline group, ICV administered BMI did not change the colonic MI change significantly. However, in the nasal NPY groups, the decreased colonic MI change was significantly increased by ICV administered BMI. C. In the NS groups, the FPO showed low value. Intranasal administration of saline (with ICV administered saline or BMI) had no effects on the FPO. However, intranasal administration of NPY (with ICV administered saline or BMI) also had no impacts on the FPO. In the CCS condition, CCS significantly increased the FPO in the sham group (nasal saline and ICV administered saline), in the nasal saline group, ICV administered BMI did not change the FPO significantly. However, in the nasal NPY groups, the decreased FPO was significantly increased by ICV administered BMI. (n =6, *P <0.05 compared with NS nasal saline+ ICV saline group, †P < 0.05 compared with CCS nasal NPY+ICV saline group)