Neuropeptide S inhibits stress-stimulated faecal output in the rat

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ABSTRACT

Neuropeptide S (NPS) is a recently identified bioactive peptide that activates an orphan G-protein coupled receptor, called the NPS receptor (NPSR). In rats, NPS and NPSR constitute a novel neuropeptide system expressed both in the central nervous system and in peripheral tissues, controlling visceromotor, neuroendocrine, nociceptive and behavioural responses. To improve the knowledge of the role of the NPS–NPSR system in the gastrointestinal (GI) tract, we investigated: 1- the supraspinal effect of NPS on motor functions of the upper (gastric emptying and gastrointestinal transit) and lower (distal colonic transit and faecal output) GI tract under basal conditions, 2- during pathological states (restraint stress and corticotropin releasing factor (CRF)-induced defecation) in the rat, and 3- the receptor type involved in treatment with NPS using NPS, tachykinin NK_3 and opioid receptor antagonists (([D-Cys(tBu)^5]NPS), SR142801 and naloxone, respectively).

Intracerebroventricular injection of NPS failed to modify basal gastric emptying, gastrointestinal transit and distal colon propulsion, but significantly and dose-dependently reduced faecal pellet excretion and weight stimulated by restraint stress and CRF. The inhibitory effect of NPS on stress-induced defecation was unmodified by pre-treatment with either the tachykinin or opioid receptor antagonists, but was counteracted by a NPSR antagonist.

The present study demonstrates, for the first time, that the supraspinal NPS system, which does not participate in the physiological control of GI motility, plays an inhibitory role on defecation stimulated by restraint stress and CRF. The combination of the ability of NPS to inhibit faecal output together with its known anxiolytic effect may be promising, especially in pathological conditions such as irritable bowel syndrome, where stress and the hyperactivity of the CRF system contribute to the co-morbidity of anxiety with colonic motor symptoms such as diarrhoea.

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1. Introduction

Neuropeptide S (NPS) is a bioactive 20 amino acid peptide recently identified via the reverse pharmacology technique [1]. Across all species thus far examined, its primary sequence is highly conserved and its N-terminal residue is serine (S), hence the peptide is named neuropeptide S [2–4]. NPS selectively binds and activates an orphan G-protein coupled receptor, called the NPS receptor (NPSR), and induces an elevation of intracellular Ca^{2+} and cAMP, thereby acting as an excitatory neurotransmitter [4,5].

In rats, NPS and NPSR mRNA are expressed both in the central nervous system and in the peripheral tissues. The NPS precursor mRNA in the brain displays a very limited distribution with the exception of high expression in a few discrete brain areas such as a group of neurons located between the locus coeruleus and Barrington's nucleus and the lateral parabrachial nucleus of the brainstem. The highest NPSR mRNA levels are found in the cortex, thalamus, hypothalamus, amygdala, periaqueductal gray matter, and it is found in low levels in the brainstem, such as the ventral tegmental area and the substantia nigra [4,6–9]. In peripheral tissues, NPSR mRNA is expressed in gastrointestinal (GI) enteroendocrine cells and the enteric nervous system [4], suggesting a modulatory role of this system in GI motor and sensory functions. A functional polymorphism in the human NPSR gene is known and is associated with asthma [10,11], inflammatory bowel disease [12], panic disorder

[13], celiac and allergic diseases [14]. In addition, NPS and NPSR expression is regulated by treatment with caffeine [15] and nicotine [16].

NPS and NPSR constitute a novel neuropeptide system that has been shown to play a role in controlling visceromotor, neuroendocrine, nociceptive and behavioural responses essential for survival, especially arousal, anxiety, learning and memory. In fact, NPS, administered centrally, increases locomotor activity [4,6], produces anxiolytic-like effects [17–19], potently promotes wakefulness and prevents oxidative stress damage [20,21]. NPS and NPSR are also involved in drug abuse [22,23], stress, control of fear expression [24,25] and the macrophage immune response [26]. Very recently, NPS has been reported to produce antinociception in mice through the activation of supraspinal NPSR [27] but not opioid receptors, suggesting that a central NPS–NPSR system could be a potential target for developing new analgesic drugs.

The role of the NPSR–NPS system in the GI tract is still poorly understood. Central injection of NPS was reported to regulate food intake in rats [28–33]. Recent studies have indicated that central NPS inhibits colonic motor functions in mice, while the peripheral administration of the peptide did not influence these activities [34]. NPS has also been associated with susceptibility to inflammatory bowel disease [12]. In addition, in vitro models have provided indirect evidence that signalling through NPSR can induce an increase in peptides and hormones also involved in the control of physiological motor and sensory functions in the GI tract [3,14]. Therefore, it is plausible that NPS–NPSR signalling may play a role in the complex gut–brain interactions modulating inflammatory responses, anxiety, nociception and gut functions [35].

To further clarify the poorly understood physio-pathological GI profile of NPS in the rat, in the present study, we sought to first investigate the effects of NPS at the supraspinal level on some motor functions of the upper (gastric emptying and GI transit) and lower (distal colonic transit and faecal output) GI tract under basal conditions, and on faecal pellet output during pathological states (restraint stress and CRF-induced defecation), and, second, to define the receptor type involved by using treatment with NPS [20], tachykinin NK_3 and opioid receptor antagonists [36–39].

2. Materials and methods

2.1. Animals

Adult male Wistar rats (180–200 g), were housed individually in plastic boxes under standard controlled environmental conditions with 12 h light/dark cycles and food and water ad libitum. For intracerebroventricular (i.c.v.) injections, rats were anaesthetised with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg), and implanted with a permanent Akulon cannula (Linca, Tel Aviv, Israel) stereotaxically inserted through a skull hole drilled over the left lateral ventricle (AP = −0.5 mm and L = ±1.8 mm relative to the bregma; V = −1.0 mm relative to the skull surface, calculated from the rat brain atlas of Paxinos and Watson [40]) and secured to the bone with dental cement. Accurate placement of the guide cannula was verified, at the end of the experimental period, by injecting the rats with 5 μl of methyl ethylene blue dye solution and checking post mortem for ventricular system perfusion. Data from injection sites not within the ventricle were discarded. Rats were euthanised by 70% CO_2 and each study was conducted according to the guidelines of the Italian Ministry of University and Research (D.L.116, 27/01/92) and the European Communities Council Directive (86/609/EEC). The experimental protocol was authorised by the Italian Ministry of Health. All possible efforts were made to minimise the number of animals used (about six to eight for each experimental group) and their discomfort.

2.2. Drugs and administration

Neuropeptide S (NPS) kindly made available by the Chemical Laboratories at the Department of Pharmaceutical Sciences and Biotechnology Centre, University of Ferrara, Italy, and corticotrophin-releasing factor (CRF) purchased from Sigma Chemicals (St. Louis, MO), were dissolved in distilled water. The agonists were i.c.v. injected at various doses and times as indicated in each assay, whereas the NPSR ([D-Cys(tBu)5]NPS, 60 nmol/rat) and tachykinin NK_3 antagonist (SR142801, 16 nmol/rat) were centrally administered at the same time of NPS and the opioid antagonist (naloxone, 1 mg/kg i.p.) was peripherally injected 15 min before NPS.

2.3. Gastric emptying assay

In the gastric emptying assay [41], a test meal (1.5 ml per rat) consisting of a 50 mg phenol red solution in 100 ml aqueous methylcellulose (1.5%) was administered by gavage through an oro-gastric polyethylene catheter to 24 h fasted rats, but with free access to water. Rats were administered either saline (control) or test compounds in a constant volume (5 μl per rat) by the i.c.v. route immediately before the liquid and acaloric meal and euthanised 10 min after the test meal, as at this time point the maximal differences between the saline and peptide groups were observed [42–44]. The stomach was then exposed by laparotomy, quickly ligated at the pylorus and the cardia and then removed. The stomach and its contents were homogenised with 100 ml of NaOH 0.1 N. Phenol red was assayed according to a previously described procedure [42]. The suspension was allowed to settle for 1 h at room temperature and 5 ml of the supernatant were added to 0.5 ml of 20% trichloroacetic acid (w/v) and then centrifuged at 3000 rpm for 20 min. The supernatant was mixed with 4 ml of 0.5 N NaOH and the absorbance of the sample was read at a wavelength of 560 nm with a spectrophotometer (LKB-Biochrom). Phenol red recovered from animals euthanised immediately after the administration of the test meal was used as the standard (0% emptying). Percent gastric emptying in the 10 min period was calculated according to the following formula: gastric emptying (%) (GE%) = (1 − A560 sample/A560 reference) × 100, where the A560 sample was the absorption at 560 nm of the gastric content at 10 min and A560 reference was the absorption at 560 nm of the gastric content at zero emptying time. Each value is expressed as percent changes with respect to percentage of gastric emptying in saline treated rats (68.6 ± 2.3 = 100%).

2.4. Upper gastrointestinal transit assay

Upper gastrointestinal transit was measured with the charcoal meal test [45]. Briefly, rats received 1 ml of a 20% (w/v) charcoal suspension in a 5% (w/v) gum Arabic solution via a stomach tube. Immediately afterwards, the rats were injected i.c.v. with NPS at doses of 1 and 4 nmol/rat and were killed (70% CO_2) 10 min [42] after receiving the charcoal meal; the small intestine was removed en bloc. Small bowel propulsion was determined by calculating the ratio between the distance travelled by the charcoal meal and the total length of the small bowel for each rat. The data are presented as the percent gastrointestinal transit measured as a quotient of the propulsion value in drug-treated rats and that in saline-treated rats (42.5 ± 3.2 = 100%).

2.5. Distal colonic propulsion assay

Distal colonic propulsion was measured according to the method of Raffa et al. [46]. In brief, immediately after rats were injected i.c.v. with NPS or saline, a single 5 mm diameter glass bead was inserted into the distal colon to a distance of about 2 cm from

Table 1
Effect of i.c.v. injected NPS (4 nmol/rat) on gastric emptying, gastrointestinal transit, colonic propulsion and faecal output in the normal rat.

Treatment	Saline	NPS (4 nmol/rat, i.c.v.)
Gastric emptying (%)	100 ± 10.13	96.8 ± 2.95
Gastrointestinal transit (%)	100 ± 8.22	99.4 ± 2.03
Colonic propulsion (MET)	1.41 ± 0.23	2.31 ± 1.08
Faecal output (no./h)	0.54 ± 0.05	0.6 ± 0.03

The data are expressed as % gastric emptying and gastrointestinal transit, as the mean glass bead expulsion time (MET) and number of faecal pellets $\pm$ S.E.M. for at least six rats.

the anus of each rat. After bead insertion, the rats were replaced individually without food or water in their home cages and thereafter showed normal behaviour. The time required for expulsion of the glass bead, mean expulsion time (MET), was determined (to the nearest 0.1 min) for each rat and the inhibition of colonic propulsion was measured as the increase in MET vs. saline-treated rats. The higher the MET value, the stronger the inhibition of colonic propulsion.

2.6. CRF-stimulated faecal pellet output

Rats were accustomed to single housing and handled for 2 days before the experiment. CRF at dose of 210 pmol/rat i.c.v., known to stimulate faecal pellet output [47], was injected 5 min after i.c.v. injected saline or NPS, and the total number of pellets excreted was measured at 60 min after treatment. Each value is expressed as the cumulative faecal pellet output recorded at 60 min after treatment.

2.7. Restraint stress (RS) stimulated faecal pellet output

RS was induced by maintaining rats for 60 min in a plastic tube (6 cm in diameter and 20 cm in length) with perforated holes for adequate ventilation. The narrow tube effectively restrained the rats, preventing them from turning around and moving forwards or backwards. The rats were placed in the plastic tube immediately after NPS or saline were injected i.c.v., and the number of faecal pellets excreted was measured at 60 min after treatment.

2.8. Statistics

Unless otherwise specified, all values are expressed as mean $\pm$ SEM. Data were analysed with the Statistic Software Package using one-way analysis of variance (ANOVA) and post hoc Student's t-test or Fisher's exact test. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of i.c.v. NPS on gastric emptying, gastrointestinal transit, colonic propulsion and faecal pellet output under basal conditions

NPS, i.c.v. injected at doses of 0.5 and 1 nmol (data not shown) and 4 nmol/rat (Table 1) did not significantly affect the gastric emptying of a phenol red meal, the gastrointestinal transit of a charcoal meal, the distal colon propulsion of a glass bead or faecal output, compared with i.c.v. saline group.

3.2. Effect of i.c.v. NPS on restraint stress (RS) stimulated faecal pellet output and weight

Saline i.c.v. injected control rats defecated only occasionally during the 60 min observation period (the number and weight of
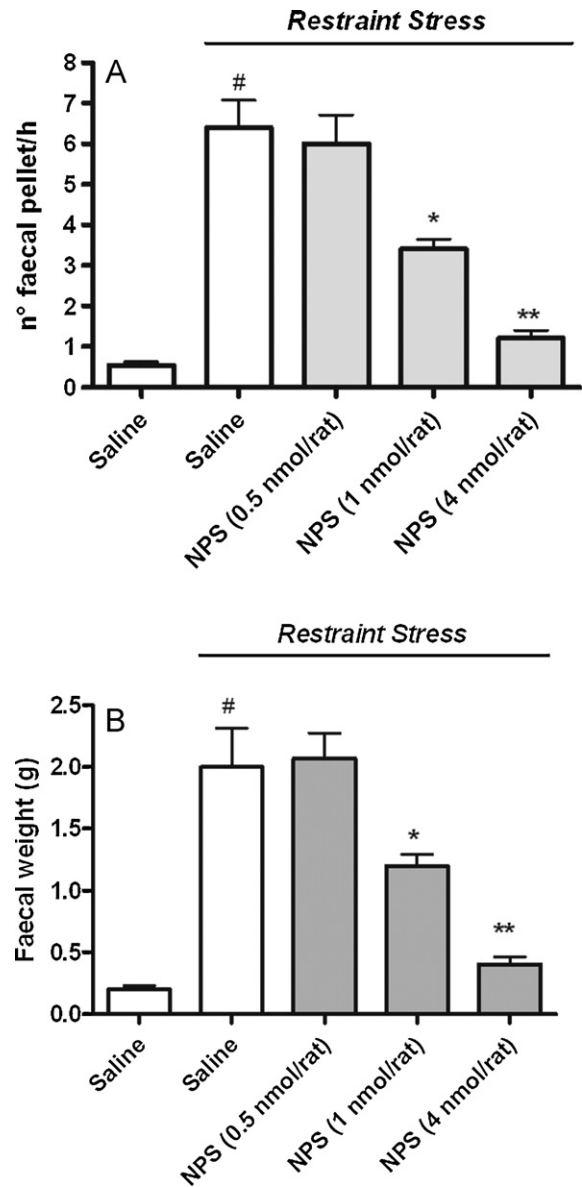
Fig. 1. Effect of i.c.v.-injected NPS (0.5–1–4 nmol/rat) on number (A) and weight (B) of faecal pellets in 1 h-restrained stressed rats. Each column represents the mean $\pm$ SEM for at least eight rats. #$P < 0.001$ vs. rats injected with saline without stress *$P < 0.01$ and **$P < 0.001$ vs. the control saline-injected rats with stress.

faecal pellets per hour were 0.54 ± 0.28 and 0.2 ± 0.08 g, respectively). When i.c.v. saline-injected control rats were exposed to RS for 60 min, the number and weight of faecal pellets per hour increased significantly compared with the values from unstressed control rats (the number and weight of faecal pellets per hour were 6.0 ± 1.12 and 2.2 ± 0.1 g, respectively) (Fig. 1A and B). The i.c.v. injection of NPS (0.5, 1 and 4 nmol/rat), significantly inhibited the RS-induced increase in the number and weight of faecal pellets in a dose-dependent manner (Fig. 1A and B). The highest dose of peptide yielded the maximal inhibition (about 80%).

Central pre-treatment with a NPS receptor antagonist, [D-Cys(tBu)^5]NPS, at a dose of 60 nmol/rat, that alone had no effect on the RS-induced increase of faecal pellet output and weight vs. saline-treated rats, attenuated the inhibitory effect of centrally injected NPS at a dose of 4 nmol/rat (molar ratio antagonist/agonist 15/1) (Table 2).

3.3. Effect of pre-treatment with tachykinin NK₃ and opioid receptor antagonist on NPS-induced inhibition of RS-stimulated faecal pellet output and weight

Pre-treatment with both SR142801, a selective tachykinin NK₃ antagonist (at an i.c.v. dose of 16 nmol/rat, known to prevent many effects due to the activation of NK₃ receptors), and naloxone, the non-selective opioid antagonist (at an i.p. dose of 1 mg/kg), did not modify the inhibitory effect of i.c.v. injected NPS (4 nmol/rat) on RS-stimulated faecal pellet output and weight (Table 2).

### 3.4. Effect of i.c.v. injection of NPS on CRF-stimulated faecal pellet output and weight

When CRF was i.c.v. injected at 210 pmol/rat, the number and weight of faecal pellets per hour significantly increased in comparison with the values from saline-injected control rats (6.0 ± 0.3 and 2.0 ± 0.2 g faecal pellets output and weight per hour, respectively). NPS (4 nmol/rat) i.c.v. pre-treatment significantly reduced the CRF-induced increase in faecal pellet output and weight (Fig. 2A and B).

4. Discussion

Collectively, this study suggests that although the central NPS–NPSR system plays no apparent role in normal GI motility, a role for this system becomes apparent when the gut is subjected to unusual or stressful conditions in rats. Our results show that i.c.v. injection of NPS up to the dose of 4 nmol/rat had no effect on basal gastric emptying, GI transit, distal colon propulsion or faecal output, but significantly reduced the stress-stimulated defecation in rats. These findings taking together suggest that when a stimulus is more intense than that required to evoke normal defecation, NPS influences faecal output by modulating neuronal pathways controlling this function.

The fact that NPS failed to modify GI activities under basal conditions contradicts recent studies showing that, in mice, central NPS inhibits distal colonic transit and spontaneous defecation through the activation of central NPSR [34]. Although an explanation for these different findings is not clear, it is not the first time that opposing results have been found between rat and mice, found probably related to pharmacokinetic and/or pharmacodynamic differences (NPSR localisation or density) in the brains of rats and mice.

The lack of a role of this peptidergic system in the control of physiological GI activities does not exclude its involvement under pathological conditions, where disturbed colonic motor function is the main consistent pattern. An experimental model to study these gastrointestinal dysfunctions as an increase in colonic transit and
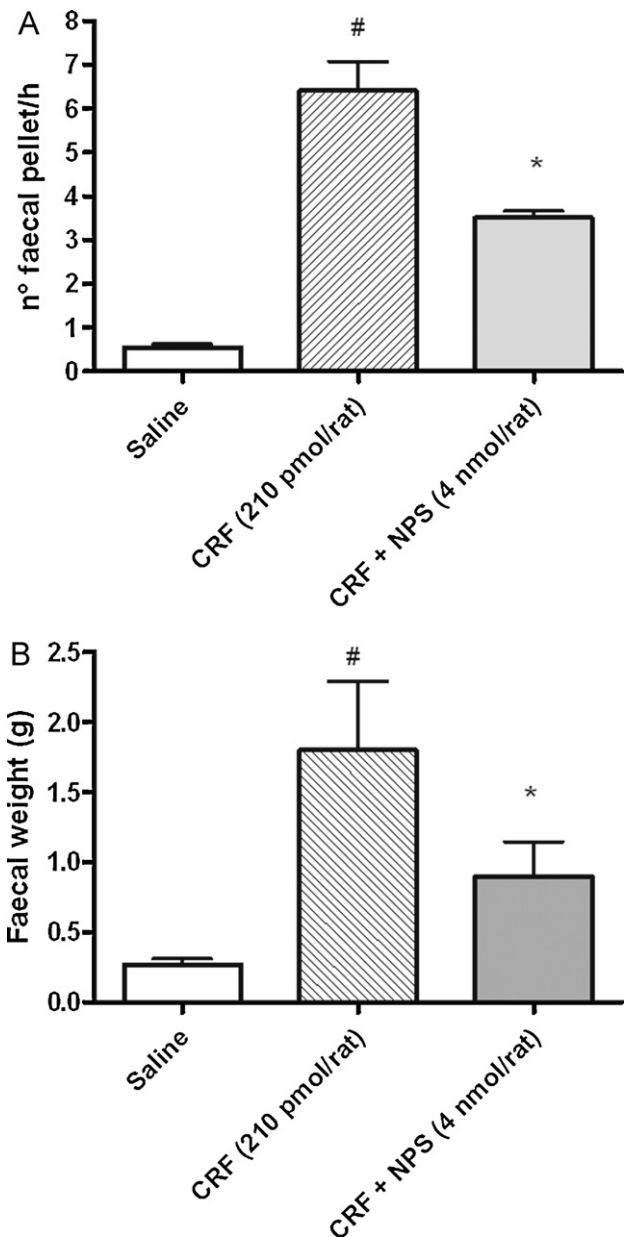
Fig. 2. Effect of i.c.v.-injected NPS (4 nmol/rat) on number (A) and weight (B) of faecal pellet stimulate by i.c.v.-injected CRF (210 pmol/rat). Each column represents the mean ± SEM for at least eight rats. #P < 0.001 vs. rats injected saline; *P < 0.01 vs. rats injected with CRF alone.

Table 2
Effect of pre-treatment with NPS ([D-Cys(tBu)⁵]NPS) antagonist and opiod (naloxone)- and tachykinin NK₃ (SR142801)-receptor antagonist on the inhibition of faecal pellet number and weight induced by NPS in stressed rats.

Treatment	Stress-induced faecal output			
	Faecal pellet (no./h)		Faecal weight (g)	
	+ Saline	+ NPS (4 nmol/rat, i.c.v.)	+ Saline	+ NPS (4 nmol/rat, i.c.v.)
Saline	6.0 ± 1.1	1.2 ± 0.5#	2.2 ± 0.1	0.4 ± 0.1#
[D-Cys(tBu)⁵]NPS(60 nmol/rat, i.c.v.)	6.0 ± 0.3	4.0 ± 0.6*	2.2 ± 0.3	1.8 ± 0.3*
SR142801 (16 nmol/rat, i.c.v.)	6.9 ± 0.5	0.7 ± 0.3#	2.6 ± 0.6	0.6 ± 0.2#
Naloxone(1 mg/kg, i.p)	6.2 ± 0.3	0.9 ± 0.6#	2.1 ± 0.2	0.7 ± 0.5#

The data are expressed as the number and the weight of faecal pellets ± S.E.M for at least six rats.

# P < 0.01 in comparison with the corresponding group injected with saline alone.

* P < 0.05 vs. saline + NPS-treated rats.

faecal excretion is the acute restraint stress model. Restraint stress appears to change the central nervous system output signal to the colon and to alter the colonic smooth muscle response in manner that facilitates colonic evacuation. The results reported here, showing that i.c.v. administered NPS inhibits faecal excretion stimulated by restraint stress in rats, indicate that a NPSR system in the brain is an important mediator of the stress-induced colonic response. Moreover, there is evidence that an increased emotional response is often associated with disturbances in colonic responses [48,49]. Psychological stress is widely believed to play a major role in irritable bowel syndrome (IBS), in which the colonic motor response to stress is exaggerated [50,51]. A recent study demonstrated that NPSR mRNA is expressed in various stress-related brain regions [9], and that NPS produces anxiolytic-like effects in animals exposed to different stressful paradigms, confirming the relationship between NPS and stress [4,5].

To characterise the site of the *in vivo* central action of NPS on pathological distal colonic function, we evaluated its effect in the presence of the NPSR antagonist, [D-Cys(tBu)^5]NPS, whose antagonistic activity has been demonstrated in a wide variety of animal tests [1,20].

Data reporting that [D-Cys(tBu)^5]NPS, when centrally injected alone, had no effect on colonic function, suggest no endogenous role of the NPSR system in the control of this function, evaluated under the same experimental conditions. When injected before the agonist, [D-Cys(tBu)^5]NPS partially but significantly reduced the efficacy of NPS in stress-stimulated defecation, providing evidence that the action of this peptide is solely due to selective NPSR activation.

In terms of the possible underlying mechanisms through which i.c.v. NPS reduces stress-stimulated defecation, it is well known that a number of neuropeptides, which play an inhibitory role in the control of colonic motility, might participate in the NPS-induced delay in faecal output during restraint stress. The central opioid receptor system modulates colonic motility [52] and is involved in stress responses [37–39]. Therefore, we investigated whether pre-treatment of rats with naloxone, a peripherally administered opioid receptor antagonist that generally crosses the blood-brain barrier and may therefore modify central actions, would change the inhibitory action of NPS. Because it failed to do so, we concluded that NPS-induced anti-defecation is not mediated by central NPS-opioid receptor system interactions. Our previous paper reports that the central tachykinin NK$_3$ receptor system also imposes inhibitory control on colonic propulsion [36]. Accordingly, neurokinin B, the tachykinin NK$_3$ receptors and the mRNA for both are expressed in key brain areas of the stress circuitry [53]. Pre-treatment of rats with SR142801, a centrally administered tachykinin NK$_3$ receptor antagonist, did not change the NPS inhibitory effect on stress-stimulated faecal pellet output, thereby suggesting that the central tachykinin NK$_3$ receptor system is not involved in the NPS-induced colonic stress response.

Colonic functions are directly mediated by the enteric nervous system which, in turn, is regulated by specific brain nuclei. The most important is Barrington's nucleus which projects to the lumbosacral preganglionic neurons that provide direct parasympathetic innervation of the visceral pelvis and impacts on distal colon function by relaying pelvic visceral information to the forebrain [54]. Interestingly, NPS is expressed in a previously undefined cluster of cells located between the locus coeruleus and Barrington's nucleus [9,8]. Moreover, with respect to their response to colon distention and their immunoreactivity, Barrington's nucleus neurons are endowed with CRF, the stress-related neuropeptide [55]. Out of 30 labelled neurons in the central part of Barrington's nucleus, 53% were activated by colon distension and 63% of these were immunoreactive for CRF. CRF/CRF1 signalling pathways in these nuclei may physiologically regulate the behavioural and autonomic responses to stress that influence colonic function as part of the brain–gut axis [56]. These pathways may play a role in diarrhoea-predominant IBS patients with psychic comorbidities such anxiety and depression [57]. Based on these findings, we supposed that inhibition of stress-stimulated faecal pellet output by NPS could be, at least to some extent, due to a NPS interaction with the CRF system. In a group of rats treated with central NPS prior to i.c.v. CRF administration, which increased faecal pellet output, we observed that the peptide significantly reduced CRF-induced faecal output. The data suggest that a CRF-sensitive circuit might be the target of NPS inhibitory control. However, NPS, which completely inhibited stress-induced faecal pellet output, exerts a significant but partial decrease in CRF-stimulated faecal excretion. The differential efficacy of NPS in reducing defecation during stress and CRF-induced stimulation may be due to the pharmacological doses of CRF administered to stimulate colonic motility, which were probably higher than endogenous CRF levels released during stress, which could be more easily surmountable. However, we cannot exclude that NPS neurons may also interact with other distinct peptidergic systems (e.g. N/OFQ, Oxytocin, Orexin) known as modulators of stress or of response to stress [39,58,59]. The NPS inhibitory effect on CRF pathways (interactions between NPS and CRF) evidenced here could appear to be in disagreement with previous studies showing that i.c.v. NPS stimulates the HPA axis through the release of CRF [28–30]. However, the stimulation of colonic transit and defecation by restraint or in response to centrally injected CRF is not modified after hypophysectomy or adrenalectomy [50,60], indicating that the CRF-induced stimulation of colonic motor activity occurs independently from the activation of the HPA axis in rats and is neurally mediated through the activation of parasympathetic cholinergic pathways. Transneuronal labelling showed that the locus coeruleus/sublocus coeruleus/Barrington complex sends direct projections to the intermediolateral column at the SI segment of the rat sacral spinal cord which provides parasympathetic innervation of the colon [61]. Based on these findings, central CRF may activate sacral parasympathetic centres through the pontine nuclei upon exposure to acute stressors, and central NPS may inhibit the activation of such neuronal pathways from the brain to the gut.

In conclusion, the present study demonstrates, first, that NPS, known to promote anxiolytic and arousal effects at the same dose range has no effects on motility of the entire gastrointestinal tract under physiological conditions. These data therefore suggest that the NPSR system may represent an interesting target for the development of new treatments for anxiety without adverse gastrointestinal effects. Second, the activation of central NPS receptors induces a marked inhibition of defecation only when this function is stimulated by restraint stress and CRF. Thus, the combination of the ability of NPS to inhibit stress-stimulated faecal output together with its anxiolytic effect is particularly promising, especially for diarrhoea predominant IBS patients, where the hyperactivity of the CRF system, presumably through CRF$_1$-dependent pathways, contributes to the co-morbidity of anxiety with colonic motor symptoms.

Acknowledgement

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