Neuropeptide S inhibits gastrointestinal motility and increases mucosal permeability through nitric oxide

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Abstract

Neuropeptide S (NPS) receptor (NPSR1) polymorphisms are associated with enteral dysmotility and inflammatory bowel disease (IBD). This study investigated the role of NPS in conjunction with nitrergic mechanisms in the regulation of intestinal motility and mucosal permeability. In rats, small intestinal myoelectric activity and luminal pressure changes in small intestine and colon, along with duodenal permeability were studied. In human intestine, NPS and NPSR1 were localized by immunostaining. Pre- and postprandial plasma NPS was measured by ELISA in healthy and active IBD humans. Effects and mechanisms of NPS were studied in human intestinal muscle strips.

In rats, NPS 100-4000 pmol/kg·min had effects on the small intestine and colon. Low doses of NPS increased myoelectric spiking \( (p<0.05) \). Higher doses reduced spiking and prolonged the cycle length of the migrating myoelectric complex, reduced intraluminal pressures \( (p<0.05-0.01) \) and increased permeability \( (p<0.01) \) through NO-dependent mechanisms. In human intestine, NPS localized at myenteric nerve cell bodies and fibers. NPSR1 was confined to nerve cell bodies. Circulating NPS in humans was tenfold below the ~0.3 nmol/l dissociation constant \( (K_d) \) of NPSR1, with no difference between healthy and IBD subjects. In human intestinal muscle strips pre-contracted by bethanechol, NPS 1-1000 nmol/l induced NO-dependent muscle relaxation \( (p<0.05) \) that was sensitive also to tetrodotoxin \( (p<0.01) \).

In conclusion, NPS inhibits motility and increases permeability in neurocrine fashion acting through NO in the myenteric plexus in rats and humans. Aberrant signaling and up-regulation of NPSR1 could potentially exacerbate dysmotility and hyperpermeability by local mechanisms in gastrointestinal functional and inflammatory reactions.

KEYWORDS

Inflammation, inflammatory bowel disease, migrating motor complex, NO, peristalsis.
INTRODUCTION

Neuropeptide S (NPS) is localized to the brainstem and gastrointestinal tract. NPS activates the G protein-coupled receptor NPSR1 (also named GPR154) and activates adenylate cyclase to increase cAMP (16). NPS is regarded as an excitatory neurotransmitter and is involved in stress responses with regulation of arousal, wakefulness and anxiety in mammals (1, 13). The presence of an Asn-Gly (NG) sequence is a defining feature of a phylogenetically ancient family of neuropeptides called “NG peptides” (4). Rodent and human NPS possesses this NG sequence (17).

NPS and NPSR1 exist in gastrointestinal mucosal epithelial cells (3, 9, 20). An NPSR1 polymorphism was reported along with higher mucosal epithelial immunoreactivity of NPSR1 in inflammatory bowel disease (IBD) with expression in leukocytes (3). Multiple NPSR1 polymorphisms have since been associated with IBD (1). These publications have driven efforts focused on NPSR1 signaling in the context of inflammation. However, NPS also increases mRNA expression of gastrointestinal peptides that act on motility (e.g., CCK, VIP, PYY and somatostatin) (2), suggesting that in addition to a role in inflammation, NPSR1 signaling can influence gastrointestinal motor and sensory disturbances such as hastening of colonic transit, pain, gas and urgency sensations (2).

There is a deficit in current literature regarding NPS action in gastrointestinal motility or mucosal permeability, both of which are relevant to IBD and functional gastrointestinal disorders. The aim of this study was to study the localization of NPS and its receptor, NPSR1, in the gut as well as its effects on gastrointestinal motility and permeability. Therefore, we examined NPS effects on the gastrointestinal tract using the rat as an in vivo model of small intestinal and colonic motility and mucosal permeability. Conscious rats were used to study NPS effects on the myoelectric activity of the small intestine utilizing the recurring migrating myoelectric complex (MMC) to unveil motility effects through changes of the characteristic
phase III (activity front) of the MMC. Anesthetized rats were then used to study NPS effects on intestinal luminal pressure changes and mucosal permeability by employing Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) to rule out involvement of nitric oxide (NO) in the response to NPS. To this end, human gastrointestinal muscle strips were examined for immunostaining of NPS, NPSR1 and inducible nitric oxide synthase (iNOS), and for use in organ baths as an *in vitro* motility model that excludes contribution from brain-vagus nerve axis and other extraintestinal factors.

**METHODS**

**Animal studies**

For studies of small intestinal myoelectric activity in conscious animals, 42 male Sprague-Dawley rats (300-350 g) were purchased from Scanbur AB (Sollentuna, Sweden). For studies of small and large intestinal motility and mucosal paracellular permeability in depth under anesthesia, 54 male Sprague-Dawley rats (300-350 g) were obtained from Taconic (Ejby, Denmark). Animals were maintained under standardized temperature and light-dark cycle (21-22 °C, 12:12 h). They were acclimatized for at least a week before experiments and caged in groups of two or more with *ad libitum* water and chow (R36, Lantmännen, Kimstad, Sweden). The rats were fasted overnight for 16 h prior to experiments or surgery. Ethical approvals were obtained from Uppsala Ethics Committee for Experiments with Animals (C309/10 and C147/13) and Northern Stockholm Animal Ethics Committee (N348/09 and 353/09).

**Surgical procedures**

For studies on small intestinal myoelectric activity, surgery was performed under anesthesia with a mixture of midazolam (5 mg/ml; Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanylcitrate, 0.315 mg/kg plus fluanisone 10 mg/kg; Janssen-Cilag, Oxford, USA) injected
subcutaneously (SC) in a volume of 1.5-2.0 ml/kg body weight. Buprenorphine (Temgesic® 0.05 mg/kg; Schering-Plough, Stockholm, Sweden) was given SC after surgery to alleviate post-operative pain.

The abdomen was opened by midline incision. Animals were then supplied with three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instr., Reading, UK) implanted into the muscular wall of the small intestine, 5 (D), 10 (J1) and 15 (J2) cm distal to the pylorus. All animals were supplied with an indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) inserted into the external jugular vein for administration of NPS. The electrodes were pierced through the abdominal muscle wall and together with the vein catheter tunneled SC to exit at the back of the neck. After implantation, the animals were housed individually and given at least 7 days recovery before experiments were undertaken.

For motility and duodenal mucosal permeability studies in anesthetized rats, surgical and experimental procedures are described elsewhere (12, 15, 18). The venous port was used for drug and NPS dosing, as well as for infusion of saline and $^{51}$Cr-EDTA (1.0 ml/h). For motility studies on the duodenum and colon, the proximal tubing was connected to a peristaltic pump (Gilson Minipuls 3, Villiers, Le Bel, France). The isolated intestinal segment was continuously perfused with saline (154 mM NaCl; 300 mOsm/kg) at a rate of ~0.4 ml/min. After surgery, ~60 min was allowed for cardiovascular, respiratory and intestinal functions to stabilize before experiments were undertaken. For colonic motility studies, contractile activity was stimulated with bethanechol (bolus dose 2.5 mmol/kg followed by 2.5 mmol/kg·h as continuous IV infusion).

**Experimental procedures**

**Migrating myoelectric complex**

Experiments were performed in animals equipped with implanted electrodes after overnight
fasting. The rats were placed in Bollman cages and the electrodes connected to
electroencephalography preamplifiers (7P5B) operating a Grass Polygraph 7 B (Grass Instr.,
Quincy, MA, USA). The key feature of the myoelectric activity of the small intestine in the
fasted state, phase III of the MMC, was identified by a period of clearly distinguishable
intense spiking activity with an amplitude at least twice that of the preceding baseline and a
frequency of at least 40 spikes/min, propagating aborally through the whole recording
segment and followed by a period of quiescence, phase I of MMC. This period was followed
by gradually increasing sporadic spiking activity, phase II of MMC, until phase III recurred.
The MMC cycle length, reflecting the interval between propagated phase IIIs of MMC, and
the duration of phase III were calculated at the J2 recording site. Propagation velocity was
calculated between the J1 and J2 recording sites. The MMC cycle length, the duration and
propagation velocity of phase III of the MMC were calculated as mean of the study period.
All experiments started with a control recording of basal myoelectric activity over a period of
~60 min with four regular MMCs propagating over all three recording sites, during which a
continuous IV infusion of saline solution (NaCl 154 mM; 300 mOsm/kg H2O, Fresenius Kabi,
Halden, Norway) was given using a microinjection pump (CMA 100; Carnegie Medicine,
Stockholm, Sweden). As the fifth activity front had vanished at the first electrode site, an IV
infusion of NPS (NeoMPS) at 100, 300, 1000, 2000 or 4000 (each dose n = 8) pmol/kg·min
was started through the microinjection pump and continued for 60 min, after which the
experiment continued until the basal MMC pattern returned (within a total experiment time of
6 h). Two animals were discarded due to broken electrode connection.

**Intestinal motility, mucosal permeability and inflammatory response**

Intraluminal pressure changes were recorded to quantify intestinal wall contractions. The inlet
perfusion tubing was connected via a T-tube to a pressure transducer. The outlet tubing was
positioned at the same level as the inlet tubing. An upward deflection of at least 2 mm Hg above baseline was defined as a motor response. Changes in luminal pressure were recorded via a digitizer on a computer using PowerLab® and the software Labchart7® (ADInstruments Ltd. Hastings, East Sussex, UK). Intestinal motility was assessed as the pressure-activity curve over baseline (AUC) in 10-min intervals. This "motility index" was expressed as the mean AUC/10 min for any specified period.

To study mucosal permeability $^{51}$Cr-EDTA was simultaneously administered IV as a bolus of ~75 µCi followed by continuous infusion at a rate of ~50 µCi/h for 30-60 min to permit equilibration in the tissue. Two blood samples (~0.3 ml each) were drawn during the experiment. The first was collected 10 min before starting, the second after ending the experiment. The blood volume loss was compensated for by injection of a 0.3 ml 7% BSA solution. After centrifugation of samples, 50 µl plasma was removed for measurements of $^{51}$Cr-EDTA. The duodenal segment was perfused with saline at a rate of ~0.4 ml/min and the perfusate collected in 10-min samples. The luminal perfusate and plasma were then analyzed for $^{51}$Cr-EDTA. Quantification was done using a gamma counter (Compugamma 1282 CS, Pharmacia, Uppsala, Sweden). A linear regression analysis of the plasma samples was made to obtain a corresponding plasma value for each perfusate sample. The clearance of $^{51}$Cr-EDTA from blood-to-lumen was calculated as described previously (5, 18) and expressed as ml/min·100 g at 10-min intervals.

Control experiments were performed by measuring basal duodenal motility and mucosal permeability simultaneously for 150 min with saline perfusion of the duodenal segment (n = 9). In a parallel group animals were challenged with NPS. The experimental protocol was the same as under control conditions with addition of NPS administered IV after a 30-min baseline period either as bolus injections at 30 min with 0.5 nmol/kg and at 70 min with 5.0 nmol/kg (n =10) after onset of experiment, or as NPS infusions IV at doses 8, 83 and
833 (n = 10) pmol/kg·min.

In studies of colonic motility, baseline studies during 30 min were carried out with an infusion of bethanechol 2.5 mmol/kg·min to induce motility for a total period of 60 min (n = 5). In a parallel group a continuous infusion of NPS at 833 pmol/kg·min IV was added at 30 min and motility examined for another 30 min (n = 5).

In a separate set of experiments, NO dependency of the NPS effects on duodenal motility and permeability was examined. Similar to control experiments with saline, L-NAME was added as a 3 mg/kg IV bolus dose followed by continuous infusion 4 µg/h for a baseline period of 30 min and throughout the experiments. One group was pre-treated with L-NAME alone (n = 6) and a parallel group with added challenge of NPS (n = 9) at doses of 8, 83 and 833 pmol/kg·min IV for a total period of 120 min.

Additional experiments were carried out to study inflammatory properties of NPS. Conscious rats were challenged with NPS 4 nmol/kg·min IV (n = 6) or saline solution (n = 6) during 60 min. Animals were then euthanized with pentobarbital (Apoteksbolaget, Solna, Sweden). Tissue specimens from stomach, duodenum, jejunum and colon were immediately frozen at -80° C for protein analysis. The protein analysis was done by homogenizing the tissue on ice in lysis buffer (200 mM NaCl, 5 mM EDTA, 100 mM Tris (pH 7.4), 10% glycerol, and SigmaFAST protease inhibitor (Sigma, St Louis, MI) using a 1 ml glass Dounce tissue grinder (Wheaton, Millville, NJ) with 30 strokes for each of the loose and tight pestles. Then, homogenates were incubated 30 min on ice, centrifuged at 10 000 g for 10 min at 4° C to pellet remaining debris. The protein concentration was measured in the supernatants using Bradford reagent (Bio-Rad, Hercules, CA, USA). A total amount of 50 µg of each sample was assayed for the content of seven inflammatory cytokines and chemokines (interferon-γ, interleukin (IL)-1β, IL-4, IL-5, IL-13, chemokine (C-X-C motif) ligand 1 (CXCL1), and tumor necrosis factor (TNF)-α) on a multispot array using an electrochemiluminescence
sandwich immunoassay (MesoScale Diagnostics, Gaithersburg, MA). Data are presented as fold changes in arbitrary units relative to the mean of control.

Human studies

Smooth muscle specimens were obtained from the middle portion of the greater curvature of the gastric corpus of normal human stomach (n = 10), from the jejunum 70 cm distal to the pylorus (n = 15) in conjunction with gastric bypass surgery, and from the free resection margin in the jejunum 30 cm orally of the ileo-cecal valve (n = 24) and midportion of the transverse colon within 40 cm distal of the ileo-cecal valve (n = 24) from patients undergoing elective surgery for non-obstructive colorectal cancer. The experiments were approved by the Regional Ethics Committee at Uppsala University (2010/157 and 2010/184). Informed consent was obtained from all study subjects.

Immunostaining for NPS, NPSR1 and nNOS

Paraffin embedded sections of normal human gastric corpus, jejunum, ileum and colon (each n = 3) were immunostained by HRP-DAB (mouse primary Ab’s) or AP (alkaline phosphatase)-Fast Red (rabbit primary Ab’s). Primary Ab’s were: mouse monoclonal clone 7C5 against NPSR1 (GPRA-A, cat# 501-100, C-terminal selective, antigen: CREQRSQDSRMTFRERTER from accession number Q6W5P4-1, the canonical isoform 1 sequence, 1:1000) from Icosagen (Tartu, Estonia) rabbit polyclonal against NPS from Abcam® (1:1000, cat# ab92424, Cambridge, UK) and rabbit polyclonal against neuronal NOS (nNOS) from Santa Cruz Biotechnology Inc (1:400, NOS1, cat# sc-648, Dallas, TX, USA). Neuron-specific staining with this nNOS primary Ab was confirmed using rabbit monoclonal primary Ab against neuron-specific enolase from Cell Signaling Technology® (Beverly, MA, USA) (1:1000, clone D20H2, cat# 8171). Double staining was done using HRP-DAB and AP-Fast
Red simultaneously on same sections.

Plasma concentrations of NPS

Adult subjects were fasted at least 6 h. In healthy volunteers, plasma was prepared from blood collected immediately before and 30, 60 and 180 min after a mixed meal (n = 5). This was compared to plasma from a biobank of active IBD cases (n = 14) of which lipopolysaccharide and C-reactive protein were elevated relative to healthy controls. Active IBD was defined as partial Mayo score $\geq 6$ for ulcerative colitis or Harvey-Bradshaw index $\geq 6$ for Crohn’s disease. SigmaFAST protease inhibitor was added to blood samples to mitigate degradation of NPS. A commercial ELISA kit was used to assay NPS (cat# EZHFGF21-19K Millipore, Billerica, MA, USA) according to product insert. The detection limit was 18 pM. Intra-assay coefficient of variation was 3.9%. Spike recovery experiments did not reveal any marked degradation.

Organ bath motility studies

Excised tissue was placed in ice-cold Krebs solution (mM: 121.5 NaCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 4.7 KCl, 1.2 MgSO$_4$, 25 NaHCO$_3$, 5.6 D-glucose, equilibrated with 5% CO$_2$ and 95% O$_2$). The mucosa was removed. Strips were cut (2-3 mm wide, 12-14 mm long) along the circular axis, and mounted between two platinum ring electrodes in organ bath chambers (5 ml, Panlab, Barcelona, Spain) containing Krebs solution, continuously bubbled with 5% CO$_2$ and 95% O$_2$, maintained at 37 °C and pH 7.4. Tension was monitored using isometric force transducers (MLT0201, PanLab). Data acquisition was performed using Powerlab hardware and LabChart 7 software (ADInstruments, Oxford, UK). The tissues were equilibrated to a 2 g tension baseline for at least 60 min with the medium replaced every 15 min. After equilibration, the muscle strips were stimulated with bethanechol 10 µM (EC$_{50}$, see ref 8) for
8 min to establish a baseline. These contractions prior to experimental treatments were defined as 100% in normalized datasets. The effect of NPS 1-1000 nM (Bachem AG) was studied on smooth muscle strips pretreated with bethanechol (n = 6). To test for prejunctional effects, tissue contractions were evoked by electrical field stimulation (EFS) using biphasic square wave pulses (10 Hz, 50 V, 0.6 ms) with a Grass S88 stimulator (Grass Technologies, Astro-Med Inc., West Warwick, RI, USA) (n = 6). NPS was added to the muscle strip preparations about 30 seconds before EFS. The response to NPS was also tested in the presence of tetrodotoxin (TTX) 1 μM (n = 6), a voltage-dependent Na⁺-channel blocker, and L-NAME 1 μM, a non-selective NOS inhibitor (n = 6).

Chemicals and drugs

Sodium pentobarbital was obtained from Apoteksbolaget (Solna, Sweden). Parecoxib (Dynastat®, Pfizer, New York, NY, USA) was obtained through Apoteket AB (Uppsala, Sweden). ⁵¹Cr-labeled ethylenediaminetetraacetic acid (⁵¹Cr-EDTA) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Lidocaine (Xylocain® spray) was purchased from AstraZeneca AB (Södertälje, Sweden). NPS was purchased from Bachem AG (Bubendorf, Switzerland) and NeoMPS (Strasbourg, France). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Values are expressed as mean ± SEM. Student’s paired t-test was used when comparing the MMC cycle length, phase III duration and velocity. Duodenal and colonic motility, and mucosal permeability were assessed by repeat measures ANOVA followed by Tukey post-hoc test to assess differences within a group. The Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison’s test was applied to evaluate dose-response effects of NPS. The
Wilcoxon signed rank test was used to evaluate effects of NPS on the response to EFS as well as effects of TTX and L-NAME on the response to NPS. $P < 0.05$ was considered statistically significant.

RESULTS

During administration of NPS into conscious or anesthetized animals, no adverse events were observed regarding appearance, blood circulation, arterial blood pressure or breathing.

Migrating myoelectric complex

The MMC of small intestine in conscious rats was studied with IV infusions of NPS. NPS at low doses 100-1000 pmol/kg·min caused irregular spiking ($P < 0.05$), whereas higher doses exceeding 1000 pmol/kg·min dampened the myoelectric activity and prolonged the MMC cycle length and phase III duration in a dose-dependent manner ($P < 0.01$). Figure 1 is a representative trace showing the inhibitory effects of 2000 pmol/kg·min NPS. Effects of NPS on the characteristics of phase III of the MMC are summarized in Table 1.

Intestinal motility and mucosal permeability

Under baseline conditions, the duodenal motility index for the entire experiment was stable at $\approx 407 \pm 27$ AUC/10 min (Figures 2A and 2C) and mucosal paracellular permeability (blood-to-lumen clearance of $^{51}$Cr-EDTA) gradually decreased from start to end of experiments (Figure 2B and 2D, $P < 0.01$). Compared to control rats, injection of NPS 5 nmol/kg IV significantly reduced duodenal motility (Figure 2A, $P < 0.05$) and net paracellular permeability failed to decrease over time as normally expected (Figure 2B, $P < 0.01$). Similarly, continuous infusion of NPS 8-833 pmol/kg·min IV inhibited duodenal motility ($P <$
0.05-0.01) (Figure 2C) and diminished reduction in permeability (Figure 2D, $P < 0.01$) in a
dose-dependent fashion.

Because NO is known to reduce motility and increase mucosal permeability, both of
which inhibited by the NOS inhibitor L-NAME (7), dependency of the NPS effects on NOS
signaling was studied. Pretreatment with L-NAME, 3 mg/kg loading dose plus 4 $\mu$g/min
continuous infusion, disinhibited duodenal motility (Figure 3A) and inhibited the net
reduction of paracellular permeability induced by NPS (Figure 3B).

To characterize the motility effects of NPS on the colon, we investigated colonic
motility \textit{in vivo} in the rat. Under baseline conditions with saline infusion, no spontaneous
motility was observed. Therefore, a slow continuous IV infusion of bethanechol 2.5
mmol/kg·min was used to induce stable and long-lasting motility. Against this background
addition of NPS 833 pmol/kg·min IV inhibited colonic motility ($P < 0.01$, Figure 4). In all
anesthetized animals, mean arterial blood pressure and body temperature were stable
throughout experiments at ~111 mm Hg and ~37-38 ºC, respectively.

\textbf{Immunostaining for NPS, NPSR1 and nNOS}

Immunostaining of human gastric corpus, jejunum, ileum and colon showed strong
NPS and NPSR1 (C-terminal) immunoreactivity, however with no specific predominance for
any specific organ. In different parts of the gastrointestinal tract, NPSR1 and NPS both
localized at myenteric plexus (Figure 5A and 5B). The strongest NPSR1 staining occurred at
neurons within the myenteric plexus. No staining occurred at smooth muscle cells. Double
staining verified that NPSR1 resides within neurons and co-localize with nNOS (Figure 6A).
Double staining confirmed that NPSR1 and NPS reside in different neurons, speaking in favor
of a neurocrine function (Figure 6B). Neuron-specific nNOS expression (and by inference,
NPSR1) was confirmed using neuron-specific enolase Ab (data not shown). However, a few cells stained differentially for either NPSR1 or nNOS.

**NPS in human plasma**

In both healthy and subjects with active IBD, plasma NPS was at or below the 18 pM assay detection limit with no indication of higher levels in active IBD or increased levels with a mixed meal. Standard samples gave expected results and spike recovery pre-tests in plasma did not reveal significant losses. This placed NPS concentrations under all tested conditions no higher than ~10 times below the 300 pmol/L Kd for binding to the NPSR1 (16, 21).

**Expression of inflammatory markers**

The expression of inflammatory markers was analyzed after NPS infusion 4000 pmol/kg·min for 60 min the rat multiplex analysis showed an increased expression of IL-1β and CXCL1 in NPS-treated tissue compared with saline-treated animals (P = 0.02 and P = 0.04), respectively (Fig. 7).

**NPS effects on gastrointestinal muscle strips**

In the organ bath, NPS 1-1000 nM had no effect on unstimulated gastric corpus muscle strips or pre-contracted with bethanechol 10 µM (n = 6; data not shown). In small intestinal muscle strips, basal spontaneous contractions were modestly reduced by NPS (Fig 8). In order to see clear responses to NPS contractions were stimulated with bethanechol or EFS. NPS 1-1000 nM caused dose-dependent reduction of the bethanechol-induced contractions and reached statistical significance at and above 10 nM of NPS (Figure 9A, n = 6; P < 0.05). In colonic muscle strips, NPS 1-1000 nM also inhibited bethanechol-induced contractions. This effect was however sporadic, so dose-dependency could not be accurately
quantified (n = 6; data not shown). Similar to the in vivo rat experiments, NPS had no effect on small intestine pre-contracted with L-NAME (n = 6) (Figure 9B). Furthermore, the inhibitory effect of NPS on motility was abolished in human small intestine by pre-treatment with TTX (n = 6) (Figure 9C, P < 0.01). Submaximal EFS-induced contractions of colonic muscle strips were dampened to ~61 ± 7% of reference EFS by addition of NPS 1 nM (n = 6) (Figure 9D, P < 0.01), which displayed recovery to 81 ± 9% of control within 10 min after removing NPS.

**DISCUSSION**

Disturbed neuroendocrine regulation of gastrointestinal functions is hypothesized to be a factor in the manifestation of functional gastrointestinal disorders and inflammatory bowel disease. In this study, we demonstrated that NPS inhibits small intestinal and colonic motility along with an early inflammatory response. In rats, small and large intestinal motility as well as the pattern and frequency of the MMC were inhibited by NPS in tandem with higher mucosal paracellular permeability. Furthermore, mechanistic studies showed the NPS effects on motility and permeability to be abolished by L-NAME pretreatment, suggesting NO dependency. To this end TTX also inhibited the NPS-induced effects, which is in line with these findings and with a neuronal mechanism of NPS. Taken together with our histological findings of NPS being localized to myenteric neuronal structures in the gut and co-existence with nNOS further support this data. NPS has in many instances been referred to as an excitatory transmitter. The fact that NPS has inhibitory actions in the gut may be explained by an excitatory effect on prejunctional inhibitory neurons in the myenteric plexus.

NPS increases cAMP as second messenger. The potency of NPS to raise cAMP is tenfold higher for the IBD-associated 107<sup>Ile</sup> variant of the NPSR1 (EC<sub>50</sub> 107<sup>Asn</sup> ~32 nM vs 107<sup>Ile</sup> ~3.5 nM) (16). This suggests that individuals expressing the 107<sup>Ile</sup> variant could be
more prone to dysmotility and increased mucosal permeability.

The relevance of our animal studies to human conditions was examined using human gastrointestinal muscle strips. In small intestine muscle strips pre-contracted with bethanechol, NPS at concentrations close to the nanomolar range of receptor binding (21) elicited a relaxatory response. Colon muscle strips also responded to nanomolar concentrations of NPS, albeit with less clear dose-dependence. For comparison, most gene expression modulations induced by NPS occur with an EC$_{50}$ ~100 nM (4). There are several mechanisms that can explain such discrepancies, such as involvement of endogenous functional antagonists or variations in NPSR1 expression (6).

Within the intestinal muscularis layer, the NPSR1 immunostaining was confined to myenteric neurons. Consistent with this, in control experiments using fura-2, NPS did not affect intracellular $[\text{Ca}^{2+}]$ in cultured human intestinal smooth muscle (unpublished). Hence, NPS-induced muscle relaxation is apparently secondary to neuronal signaling. The smooth muscle relaxing effect seen when NPS was given prior to EFS indicates a modulatory function of NPS on neuro-muscular transmission effects. Based on presented immunostaining and lack of $\text{Ca}^{2+}$ changes, it seems that gastrointestinal smooth muscle does not have NPSR1 and therefore cannot respond directly to NPS. A plausible explanation for the effect of NPS seems to be a prejunctional action where NPS acts on NPSR1 at nerve cells that results in muscle relaxation. That TTX abolished the inhibitory motility effects of NPS further indicates a coupling of the NPSR1 to neuronal action potentials and synaptic signaling. The inhibitory actions of NPS were abolished by L-NAME, suggesting that NPSR1 relies on NOS for its actions on motility as well as mucosal permeability. Double staining revealed that NPSR1 and nNOS coexist in the same myenteric neurons, albeit some neurons expressing NPSR1 were not immunoreactive for nNOS. Since NPS is able to induce cellular accumulation of cAMP (21), findings by others showing that cAMP signaling can increase NO production apart from
relaxation of intestinal and vascular smooth muscle (10, 22) are in line with our results. Current literature therefore permits a model of NPS signaling through neural release of NO. NPS can then act on adjacent NPSR1 expressing neurons, some also possessing nNOS, others not. Those neurons expressing nNOS can then release NO, which might dampen contractions along the muscular layer of the gut.

Our report demonstrates increased duodenal paracellular permeability by NPS. This would mean that NPS has a common feature with other neuropeptides thought to play a role in the pathogenesis of IBD and includes substance P, corticotropin-releasing hormone, neurotensin, and vasoactive intestinal peptide (14). The assumption that NPS has a role in IBD speaks in favour of a role also in functional intestinal disorders as previously suggested. One pathophysiological implication is that enhanced signaling through gain-of-function NPSR1 mutants, such as variant 107Ile that are associated with IBD (3), could represent an early step in the inflammatory process or aggravate it. Since the permeability tests in our study were performed in vivo, this could have been driven directly within the mucosal epithelium, known to possess NPS as well as NPSR1 (3, 19). The gastrointestinal mucosa is also generally known to express NOS (7), so blockade of the NPS effect by L-NAME might even occur within these same cells. Neuronal involvement such as innervation from the submucous nerve plexus is also possible.

In conclusion, NPS displays gastrointestinal effects that might be related to function and an inflammation. NPS inhibits motility in rats and humans and is apparently coupled to NOS activity within the myenteric nerve plexus. NPS also increases gastrointestinal mucosal permeability in vivo, the mechanism of which is unknown, albeit NO-dependent. Clarification of the mechanisms of action of NPS on immunological and inflammatory reactions will likely yield new aspects on the importance of neuropeptides in functional disorders and inflammatory reactions in the gastrointestinal tract.
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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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AUTHOR CONTRIBUTIONS

W.S.W. Saudi and E. Rosenqvist performed in vivo study in anesthetized rat for motility and permeability, W.S.W. Saudi acquired and analyzed data, did statistics and co-wrote manuscript. M.A. Halim performed organ bath study with human tissue samples, intracellular [Ca$^{2+}$] measurement, ELISA and immunohistochemistry, acquired and interpreted data, did statistics, wrote manuscript and did critical revision. T. Rudholm-Feldreich and L. Gillberg performed the in vivo study in conscious rats, acquired and interpreted data and co-wrote manuscript. A. Tengholm supervised intracellular [Ca$^{2+}$] study and did critical review of
the manuscript. M. Sundbom and U. Karlbom supported the study by tissue materials and did
critical review. D.-L. Webb and P.M. Hellström conceived and designed the study. D.-L.
Webb, P.M. Hellström, M. Sjöblom and E. Näslund supervised the study and did critical
revision. D.-L. Webb and P.M. Hellström drafted and finalized the manuscript. D.-L. Webb,
M. Sjöblom, P.M. Hellström funded the study. D.-L. Webb critically checked all the data,
figures and statistics.
REFERENCES


FIGURE LEGENDS

Figure 1. Electromyographic recordings of neuropeptide S (NPS) inhibition of the migrating myoelectric complex (MMC) of rat small intestine. Representative recording (n = 8) where D indicates electrode site 5 cm distal to the pylorus, J1 located 15 cm distal and J2 located 25 cm distal to the pylorus. Upper trace: After four phase IIIss (maximum amplitude) of the MMC under fasting conditions, NPS infusion 2000 pmol/kg·min IV was started (downward arrows with lines) and continued for 60 min. Middle trace: During infusion, NPS induced irregular spiking with prolongation of MMC cycle length. Lower trace: After terminating NPS infusion, there was a progressive recovery with recurrence of phase III activity.

Figure 2. Effects of neuropeptide S (NPS) on duodenal motility and mucosal paracellular permeability in the rat in vivo. Panel A) Bolus injection of NPS IV (n = 10) decreased duodenal motility relative to saline injected controls (n = 9). Panel B) The net permeability decrease was reduced by bolus NPS injection (n = 10) compared to controls (n = 9). Panel C) Continuous NPS infusion IV (n = 10) also induced marked and dose-dependent decrease in duodenal motility compared to saline controls (n = 9). Panel D) The net permeability decrease was also reduced by continuous NPS infusion (n = 10) compared to controls (n = 10). # and ## indicate significant ($P<0.05$ and $P<0.01$) decreases compared with baseline (0-30 min) in the same group. * and ** indicate significant ($P<0.05$ and $P<0.01$) differences between groups.

Figure 3. $N^\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME) abolishes neuropeptide S (NPS) effects on duodenal motility and mucosa paracellular permeability in rats. Panel A) Pre-treatment with IV L-NAME infusion (3 mg/kg bolus + 0.25 mg/h, L-NAME; n = 6)
Figure 4. Neuropeptide S (NPS) inhibits bethanechol induced colonic motility in rats. Continuous NPS infusion IV (n = 5) inhibited colonic motility relative to control rats receiving saline (n = 5). \(^{##}\) indicates a significant \((P < 0.01)\) decrease compared with baseline (0-30 min) within the same group.

Figure 5. Immunostaining of neuropeptide S receptor 1 (NPSR1) (panel A) and neuropeptide S (NPS) (panel B) at the myenteric nerve plexus. Left and middle panels are 10X magnification, indicating low background throughout. Rightmost panels are 40X magnification centered at myenteric plexus. N = myenteric neuron; LM = longitudinal muscle; CM = circular muscle. Brown color is HRP-DAB staining and pink-red color is AP-Fast Red staining.

Figure 6. Double staining of neuropeptide S receptor 1 (NPSR1) and neuronal nitric oxide synthase (nNOS) (panel A), and NPSR1 and NPS (panel B). Left and middle panels are 10X magnification, indicating low background throughout. Rightmost panels are 40X magnification centered at myenteric plexus. N = myenteric neuron; LM = longitudinal muscle; CM = circular muscle. Brown color is HRP-DAB staining and pink-red color is AP-Fast Red staining. Deep red color is double staining.

Figure 7. Relative expression of interleukin-1\(\beta\) and CXCL1 in response to challenge with saline and neuropeptide S (NPS). * \(P < 0.04\) and \(P < 0.02\), respectively, \(n = 6\).
Figure 8. Effect of neuropeptide S (NPS) on basal spontaneous contractions of the small intestine (P = 0.087) and recovery after wash-out.

Figure 9. Neuropeptide S (NPS) reversibly induces relaxation of human intestinal smooth muscle strips. Panel A) NPS inhibited contractions in bethanechol-stimulated small intestine (n = 6). Panel B) Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 1 µM) abolished NPS-induced relaxation (n = 6). In A and B, control is bethanechol in absence of NPS or L-NAME. Panel C) Tetrodotoxin (TTX; 1 µM) abolished the inhibitory effects induced by NPS in small intestine (n = 6). As shown in the leftmost bar, TTX alone induced a ~20% increase in tone, most likely due to blockade of nitrergic mechanisms. Panel D) Submaximal contractions stimulated by EFS (defined as a 100% reference) in colon smooth muscle were reduced to 61 ± 7% by addition of NPS at 1 nM (n = 6), which recovered to 81 ± 9% within 10 min after removal of NPS. In all experiments, recovery was seen after chamber washout. # and ## indicate significant (P < 0.05 and P < 0.01) differences from baseline. * and ** indicate significant (P < 0.05 and P < 0.01) differences between groups.
Table 1. Characteristics of phase III of the migrating myoelectric complex in the rat during infusion of neuropeptide S.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cycle length (min)</th>
<th>Duration (min)</th>
<th>Velocity (cm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control period (saline alone)</td>
<td>11.4 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>NPS infusion 100 pmol/kg·min</td>
<td>12.3 ± 0.6</td>
<td>3.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Control period (saline alone)</td>
<td>10.3 ± 0.6</td>
<td>4.0 ± 0.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>NPS infusion 300 pmol/kg·min</td>
<td>10.9 ± 1.0</td>
<td>3.7 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Control period (saline alone)</td>
<td>11.9 ± 1.1</td>
<td>4.1 ± 0.5</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>NPS infusion 1000 pmol/kg·min</td>
<td>12.4 ± 1.4</td>
<td>5.6 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Control period (saline alone)</td>
<td>10.5 ± 0.8</td>
<td>4.4 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>NPS infusion 2000 pmol/kg·min</td>
<td>15.8 ± 1.4*</td>
<td>11.2 ± 0.4**</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Control period (saline alone)</td>
<td>11.3 ± 0.9</td>
<td>3.6 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>NPS infusion 4000 pmol/kg·min</td>
<td>23.9 ± 2.2*</td>
<td>12.4 ± 0.7**</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

NPS, neuropeptide S. Values are mean ± SEM for all MMC cycles during the respective period as measured from the J2 electrode. *P < 0.05, **P < 0.005).
Baseline

D

J1

J2

0.5 mV

10 min

Neuropeptide S  2000 pmol kg\(^{-1}\)min\(^{-1}\) IV

D

J1

J2

Recovery

D

J1

J2
**A**

Motility (% of baseline) over time (min) with NPS 0.5 nmol/kg and 5.0 nmol/kg in control and NPS groups.

**B**

Net $\text{Cr-EDTA}$ clearance (ml/min·100 g$^{-1}$) over time (min) with NPS 0.5 nmol/kg and 5.0 nmol/kg in control and NPS groups.

**C**

Motility (% of baseline) over time (min) with NPS i.v. infusion (pmol/kg/min) 8, 83, and 833 in control and NPS groups.

**D**

Net $\text{Cr-EDTA}$ clearance (ml/min·100 g$^{-1}$) over time (min) with NPS i.v. infusion (pmol/kg/min) 8, 83, and 833 in control and NPS groups.
Relative protein expression

Saline
NPS 4000 pmol/kg min

IL-1β

CXCL1

*