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

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Neuropeptide S (NPS) inhibits gastrointestinal motility and increases mucosal permeability through nitric oxide. Am J Physiol Gastrointest Liver Physiol 309: G625–G634, 2015. First published July 23, 2015; doi:10.1152/ajpgi.00104.2015.—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 nitricergic 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 100–4,000 pmol·kg⁻¹·min⁻¹ 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 (Kₐ) of NPSR1, with no difference between healthy and IBD subjects. In human intestinal muscle strips precontracted by bethanechol, NPS 1–1,000 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 neuromodulation acting through NO in the myenteric plexus in rats and humans. Aberrant signaling and upregulation of NPSR1 could potentially exacerbate dysmotility and hyperpermeability by local mechanisms in gastrointestinal functional and inflammatory reactions.

inflammation; inflammatory bowel disease; migrating motor complex; NO; peristalsis

NEUROPEPTIDE S (NPS) is localized to the brain stem and gastrointestinal tract. NPS activates the G protein-coupled receptor NPSR1 (also named GPR154) and activates adenylate cyclase to increase cAMP (17). NPS is regarded as an excitatory neurotransmitter and is involved in stress responses with regulation of arousal, wakefulness, and anxiety in mammals (4, 5, 22). 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 (18).

NPS and NPSR1 exist in gastrointestinal mucosal epithelial cells (3, 9, 21). 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 characterize 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 by 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 neuronal nitric oxide synthase (NOS; nNOS) 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 (Sollentuna, Sweden). For studies of small and large intestinal motility and mucosal paracellular permeability 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, Stockholm, Sweden) and Hypnorm (fentanyl citrate, 0.315 mg/kg plus fluanisone 10 mg/kg; Janssen-Cilag, Oxford, UK) injected subcutaneously (SC) in a volume of 1.5–2.0 ml/kg body wt. Buprenorphine (Temgesic 0.05 mg/kg; Schering-Plough, Stockholm, Sweden) was given SC after surgery to alleviate postoperative pain.

The abdomen was opened by midline incision. Animals were then supplied with three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instruments, Reading, UK) implanted into the muscular wall of the small intestine, 5 (D), 15 (J1), and 25 (J2) cm distal to the pylorus. All animals were supplied with an indwelling Silastic catheter (Dow Corning, Midland, MI) 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 (13, 16, 19). The venous port was used for drug and NPS dosing, as well as for infusion of saline and $^{51}$Cr-labeled ethylenediaminetetraacetic acid ($^{51}$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 mosmol/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$^{-1}$·h$^{-1}$ as continuous intravenous (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 Instruments, Quincy, MA). 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 III activity of

![Fig. 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 4 phase IIIs (maximum amplitude) of the MMC under fasting conditions, NPS infusion 2,000 pmol·kg$^{-1}$·min$^{-1}$ intravenously (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 termination of NPS infusion, there was a progressive recovery with recurrence of phase III activity.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00104.2015)
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 and 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 mosmol/kgH2O, Fresenius Kabi, Halden, Norway) was given via a microinjection pump (CMA 100; Carnegie Medicine, Stockholm, Sweden). Because the fifth activity front had vanished at the first electrode site, an IV infusion of NPS (NeoMPS, Strasbourg, France) at 100, 300, 1,000, 2,000, or 4,000 (each dose n = 8) pmol·kg⁻¹·min⁻¹ 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 because of 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 mmHg 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 LabChart (ADInstruments, 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 per 10 min for any specified period.

To study mucosal permeability, 51Cr-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 the start and the second after the end of the experiment. The blood volume loss was compensated for by injection of an 0.3 ml 7% BSA solution. After centrifugation of samples, 50 μl plasma was removed for measurements of 51Cr-EDTA. The duodenal segment was perfused with saline at a rate of ~0.4 ml/min and the perfusate was collected in 10-min samples. The luminal perfusate and plasma were then analyzed for measurements of 51Cr-EDTA. Quantification was done by 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 51Cr-EDTA from blood to lumen was calculated as described previously (19) and expressed as milliliters per minute per 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 mmol/kg and at 70 min with 5.0 mmol/kg (n = 10) after onset of experiment, or as NPS infusions IV at doses 8, 83, and 833 (n = 10) pmol·kg⁻¹·min⁻¹.

In studies of colonic motility, baseline studies during 30 min were carried out after the administration of a bolus dose (2.5 mmol/kg) followed by a continuous infusion of bethanecol 2.5 mmol·kg⁻¹·h⁻¹ 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⁻¹·min⁻¹ IV was also administered (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 0.25 mg/h for a baseline period of 30 min and throughout the experiments. One group was pretreated 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⁻¹·min⁻¹ 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⁻¹·min⁻¹ 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)] by 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 with use of Bradford reagent (Bio-Rad, Hercules, CA). 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 multiplex array via 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 ileocecal valve (n = 24) and midportion of the transverse colon within 40 cm distal of the ileocecal valve (n = 24) from patients undergoing elective surgery for nonobstructive colorectal cancer. The experiments were approved by the Regional Ethics Committee at Uppsala University.

**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</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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⁻¹·min⁻¹</td>
<td>12.3 ± 0.6</td>
<td>3.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Control period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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⁻¹·min⁻¹</td>
<td>10.9 ± 1.0</td>
<td>3.7 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Control period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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 1,000 pmol·kg⁻¹·min⁻¹</td>
<td>12.4 ± 1.4</td>
<td>5.6 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Control period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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 2,000 pmol·kg⁻¹·min⁻¹</td>
<td>15.8 ± 1.4</td>
<td>11.2 ± 0.4†</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Control period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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 4,000 pmol·kg⁻¹·min⁻¹</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 means ± SE for all migrating myoelectric complex cycles during the respective period as measured from the J2 electrode.

*P < 0.05, †P < 0.005.*
(2010/157 and 2010/184). Written, 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/H11005 3) were immunostained by horseradish peroxidase-di-aminobenzidine (HRP-DAB) (mouse primary Abs) or alkaline phosphatase (AP)-Fast red (rabbit primary Abs). Primary Abs were mouse monoclonal clone 7C5 against NPSR1 (GPRA-A, cat. no. 501-100, COOH-terminal selective, antigen: CREQRSQDSRMTFRERTER from accession number Q6W5P4-1, the canonical isoform 1 sequence, 1:1,000) from Icosagen (Tartu, Estonia), rabbit polyclonal against NPS from Abcam (1:1,000, cat. no. ab92424, Cambridge, UK), and rabbit polyclonal against nNOS from Santa Cruz Biotechnology (1:400, NOS1, cat. no. sc-648, Dallas, TX). Neuron-specific staining with this nNOS primary Ab was confirmed by using rabbit monoclonal primary Ab against neuron-specific enolase from Cell Signaling Technology (Beverly, MA) (1:1,000, clone D20H2, cat. no. 8171). Double staining was done by using HRP-DAB and AP-Fast red simultaneously on the 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/H11005 5). This was compared with plasma from a biobank of active IBD cases (n/H11005 14) of which lipopolysaccharide and C-reactive protein were elevated relative to healthy controls. Active IBD was defined as partial Mayo score /H11350 6 for ulcerative colitis or Harvey-Bradshaw index /H11350 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. no. EZHFGF21-19K, Millipore, Billerica, MA) according to the 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 CaCl2, 1.2 KH2PO4, 4.7 KCl, 1.2 MgSO4, 25 NaHCO3, 5.6 D-glucose, equilibrated with 5% CO2 and 95% O2). 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% CO₂ and 95% O₂, maintained at 37°C and pH 7.4. Tension was monitored via isometric force transducers (MLT0201, Panlab). Data acquisition was performed with 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 (EC₅₀, 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 1–1,000 nM (Bachem) 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) by using biphasic square wave pulses (10 Hz, 50 V, 0.6 ms) with a Grass S88 stimulator (Grass Technologies, Astro-Med, West Warwick, RI) (n = 6). NPS was added to the muscle strip preparations ~30 s 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 1-NASE M 1 μM, a nonselective NOS inhibitor (n = 6).

**Chemicals and Drugs**

Pentobarbital sodium was obtained from Apoteksbolaget (Solna, Sweden). Parecoxib (Dynastat, Pfizer, New York, NY) was obtained through Apoteket (Uppsala, Sweden).51Cr-EDTA was purchased from AstraZeneca (Södertälje, Sweden). NPS was purchased from Bachem (Bubendorf, Switzerland). (Xylocaine spray) was purchased from AstraZeneca (Södertälje, Sweden), and Pentobarbital sodium was obtained from Apoteksbolaget (Solna, Sweden). NPSR1 and NPS both localized at myenteric plexus (Fig. 5, A and B). Double staining verified that NPSR1 resides within neurons and colocalize with nNOS (Fig. 6A). Double staining confirmed that NPSR1 and nNOS (Fig. 6A).

**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 100-1,000 pmol·kg⁻¹·min⁻¹ caused irregular spiking (P < 0.05), whereas higher doses exceeding 1,000 pmol·kg⁻¹·min⁻¹ 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 2,000 pmol·kg⁻¹·min⁻¹ 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 ~407 ± 27 AUC/10 min (Fig. 2, A and C) and mucosal paracellular permeability (blood-to-lumen clearance of 51Cr-EDTA) gradually decreased from start to end of experiments (Fig. 2, B and D, P < 0.01). Compared with control rats, injection of NPS 5 nmol/kg IV significantly reduced duodenal motility (Fig. 2A, P < 0.05) and net paracellular permeability failed to decrease over time as normally expected (Fig. 2B, P < 0.01). Similarly, continuous infusion of NPS 8-833 pmol·kg⁻¹·min⁻¹ IV inhibited duodenal motility (P < 0.05-0.01) (Fig. 2C) and diminished reduction in permeability (Fig. 2D, P < 0.01) in a dose-dependent fashion.

Because NO is known to reduce motility and increase mucosal permeability, we wanted to elucidate the effects of NPS during NOS inhibition using l-NAME (7). Pretreatment with l-NAME, 3 mg/kg loading dose plus 0.25 mg/h continuous infusion, disinhibited duodenal motility (Fig. 3A) and inhibited the net increment of paracellular permeability induced by NPS (Fig. 3B).

To characterize the motility effects of NPS on the colon, we investigated colonic motility in vivo in the rat. Under baseline conditions with saline infusion, no spontaneous motility was observed. Therefore, a bolus dose of 2.5 mmol/kg followed by a slow continuous IV infusion of bethanechol 2.5 mmol·kg⁻¹·h⁻¹ was used to induce stable and long-lasting motility. Against this background, addition of NPS 833 pmol·kg⁻¹·min⁻¹ IV inhibited colonic motility (P < 0.01, Fig. 4). In all anesthetized animals, mean arterial blood pressure and body temperature were stable throughout experiments at ~111 mmHg and ~37-38°C, respectively.

**Immunostaining for NPS, NPSR1, and nNOS**

Immunostaining of human gastric corpus, jejenum, ileum, and colon showed strong NPS and NPSR1 (COOH-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 (Fig. 5, A and B). 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 colocalize with nNOS (Fig. 6A). Double staining confirmed that NPSR1 and...
NPS reside in different neurons, speaking in favor of a neurocrine function (Fig. 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 subjects 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 pretests 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 $K_d$ for binding to the NPSR1 (17, 22).

Expression of Inflammatory Markers

The expression of inflammatory markers was analyzed after NPS infusion 4,000 pmol·kg$^{-1}$·min$^{-1}$ for 60 min in 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 1-1,000 nM had no effect on unstimulated gastric corpus muscle strips or precontracted with bethanechol 10 µM ($n = 6$; data not shown). In small intestinal muscle strips, basal spontaneous contractions were modestly reduced by NPS (Fig. 8). To see clear responses to NPS contractions were stimulated with bethanechol or EFS. NPS
1-1,000 nM caused dose-dependent reduction of the bethanechol-induced contractions and reached statistical significance at and above 10 nM of NPS (Fig. 9A, n = 6; P < 0.05). In colonic muscle strips, NPS 1-1,000 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 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 with TTX (n = 6) (Fig. 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) (Fig. 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 IBD. 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 coexistence 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 presynaptic 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 107Ile variant of the NPSR1 (EC50 107Asn ~32 nM vs 107Ile ~3.5 nM) (17). This suggests that individuals expressing the...
Ile variant could be more prone to dysmotility and increased mucosal permeability.

The relevance of our animal studies to human conditions was examined in human gastrointestinal muscle strips. In small intestine muscle strips precontracted with bethanechol, NPS at concentrations close to the nanomolar range of receptor binding (17) 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 EC₅₀ $\pm$ 100 nM (15). 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 Ca²⁺ concentration ([Ca²⁺]ᵢ) in cultured human intestinal smooth muscle (M. A. Halim, unpublished observations). 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 neuromuscular transmission effects. Based on presented immunostaining and lack of Ca²⁺ 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 in which 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 (17), findings by others showing that cAMP signaling can increase NO production apart from relaxation of intestinal and vascular smooth muscle (10, 23) 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 (12).

This would mean that NPS has a common feature with other neuropeptides, including substance P, corticotropin-...
releasing hormone, neurotransin, and vasoactive intestinal peptide, proposed to play a role in the pathogenesis of IBD (11). The assumption that NPS has a role in IBD speaks in favor 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, 20). 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 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 author(s).

AUTHOR CONTRIBUTIONS

W.S.W.S. and E.R. performed in vivo study in anesthetized rat for motility and permeability, W.S.W.S. acquired and analyzed data, did statistics, and cowrote manuscript. M.A.H. performed organ bath study with human tissue samples, intracellular [Ca²⁺] measurement, ELISA, and immunohistochemistry; acquired and interpreted data; did statistics; wrote manuscript; and did critical revision. T.R.-F. and L.G. performed the in vivo study in conscious rats, acquired and interpreted data, and cowrote manuscript. A.T. supervised intracellular [Ca²⁺] study and did critical review of the manuscript. M. Sundbom and U.K. supported the study by tissue materials and did critical review. D.-L.W. and P.M.H. conceived and designed the study. D.-L.W., P.M.H., M. Sjöblom, and E.N. supervised the study and did critical revision. D.-L.W. and P.M.H. drafted and finalized the manuscript. D.-L.W., M. Sjöblom, P.M.H. funded the study. D.-L.W. critically checked all the data, figures, and statistics.

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