JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Xenin augments duodenal anion secretion via activation of afferent neural pathways

Izumi Kaji, Yasutada Akiba, Ikuo Kato, Koji Maruta, Atsukazu Kuwahara, Jonathan D Kaunitz

Greater Los Angeles Veterans Affairs Healthcare System (I.K., Y.A., J.D.K.); Departments of Medicine (I.K., Y.A., K.M.) and Surgery (J.D.K.), David Geffen School of Medicine at UCLA, Los Angeles, California; Department of Medical Biochemistry, Kobe Pharmaceutical University, Kobe (I.K.); and Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Japan (A.K.)

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: xenin stimulates secretion via afferent nerves

Corresponding to: Jonathan D. Kaunitz, MD

Greater LA VAHS, Bldg. 114, Rm. 217, 11301 Wilshire Blvd., Los Angeles, CA 90073 USA

E-mail: jake@ucla.edu

TEL: +1-310-268-3879

FAX: +1-310-268-4811

Number of text pages, 23

Number of tables, 2

Number of figures 7

Number of references, 54

Number of words in the Abstract, 246

Number of words in the Introduction, 645

Number of words in the Discussion, 1372

Nonstandard abbreviations: 4',6-diamidino-2-phenylindole, DAPI; 8-cyclopentyl-1,3-dipropylxanthine, CPX; 8-(3-chlorostyryl)caffeine, 8CC; calcitonin-gene related peptide, CGRP; enteroendocrine cells, EECs; gastric inhibitory peptide, GIP; high performance liquid chromatography, HPLC; matrix assisted laser desorption/ionization time of flight, MALDI-TOF; pituitary adenylate cyclase-activating peptide, PACAP; SP/neurokinin receptor 1, NK1; transient receptor potential vanilloid 1, TRPV1; vasoactive intestinal peptide, VIP; VIP/VPAC receptor 1, VPAC1

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Xenin-25, a neurotensin (NT)-related anorexigenic gut hormone generated mostly in the duodenal mucosa, is believed to increase the rate of duodenal ion secretion, since xenin-induced diarrhea is not present after Roux-en-Y gastric bypass surgery. Since the local effects of xenin on duodenal ion secretion have remained uninvestigated, we thus examined the neural pathways underlying xenin-induced duodenal anion secretion. Intravenous infusion of xenin-8, a bioactive C-terminal fragment of xenin-25, dose-dependently increased the rate of duodenal HCO_3^- secretion in perfused duodenal loops of anesthetized rats. Xenin was immunolocalized to a subset of enteroendocrine cells in the rat duodenum. The mRNA of the xenin/NT receptor 1 (NTS1) was predominantly expressed in the enteric plexus, nodose and dorsal root ganglia, and in the lamina propria rather than in the epithelium. The serosal application of xenin-8 or xenin-25 rapidly and transiently increased I_{sc} in Ussing-chambered mucosa-submucosa preparations in a concentration-dependent manner in the duodenum and jejunum, but less so in the ileum and colon. The selective antagonist for NTS1, substance P (SP) receptor (NK1) or 5-hydroxytryptamine $(HT)_3$, but not NTS2, inhibited the responses to xenin. Xenin-evoked Cl^- secretion was reduced by tetrodotoxin (TTX) or capsaicin-pretreatment, and abolished by the inhibitor of TTX-resistant sodium channel Nav1.8 in combination with TTX, suggesting that peripheral xenin augments duodenal HCO_3^- and Cl^- secretion through NTS1 activation on intrinsic and extrinsic afferent nerves, followed by release of SP and 5-HT. Afferent nerve activation by postprandial, peripherally-released xenin may account for its secretory effects in the duodenum.

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

The duodenal mucosa, due to its strategic location between the stomach and the rest of the small intestine, senses luminal nutrients, affects appetite, and regulates duodenal anion secretion, particularly HCO_3^- secretion, which in turn is important for nutrient absorption and mucosal protection from gastric acid (Kaji, et al., 2013). We have been investigating the contributions of enteroendocrine cells (EECs) and afferent sensory nerves that link the duodenal mucosa with mucosal defense mechanisms (Akiba and Kaunitz, 2011;Said, et al., 2015). As part of this quest, we hypothesized that xenin, a homologue of amphibian xenopsin and mammalian neurotensin (NT) (Feurle, et al., 1992), which is specifically generated in the duodenal mucosa, directly affects duodenal anion secretion.

Xenin as a 25-amino acid peptide is predominantly detected in human gastric and duodenal mucosa in addition to the hypothalamus (Feurle, et al., 1992). Some xenin degradation fragments (e.g. xenin 9-25, 11-25, 14-25, or 18-25) are identified in some animal species and an octapeptide xenin-8 (18-25) retains the signal sequence (Hamscher, et al., 1995;Martin, et al., 2014). The origin of active xenin-25 in the gastric mucosa has been controversial since xenin can be generated by peptic digestion from its precursor (proxenin), a component of the highly-conserved ubiquitous vesicular coat proteins, coatomer protein complex-α (COPA) (Hamscher, et al., 1995). Thus, xenin peptides could be generated *in vitro* from homogenized gastric mucosa (Hamscher, et al., 1995).

A specific xenin antibody identified a subset of duodenal EECs that co-expressed xenin with gastric inhibitory peptide (GIP), but not 5-hydroxytryptamine (5-HT) in canine duodenum (Anlauf, et al., 2000). Since GIP is released in response to luminal nutrients in the regulation of glucose homeostasis, xenin is thought to also be released from EECs by physiological stimuli.

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Indeed, intravenous (i.v.) xenin delays gastric emptying, decreases gastric acid secretion, and potentiates insulin secretion when combined with GIP (Feurle, 1998;Wice, et al., 2010). Nevertheless, the effects of xenin in the duodenal mucosa have not been previously investigated.

NT, produced by central nervous system and enteroendocrine N cells is present predominantly in the lower ileum and released by dietary fat. NT inhibits intestinal transit and food intake (Dumoulin, et al., 1998;Cooke, et al., 2009), and increases electrogenic Cl^- secretion in human colon *in vitro* (Riegler, et al., 2000). The secretory effects of NT were identified in canine duodenum (Konturek, et al., 1985), consistent with NT receptor expression in the duodenum (Tanaka, et al., 1990). Owing to peptide sequence similarity between NT and the C-terminal of xenin, and the observation that the NT receptors (NTS) 1 and 2 are activated by xenin with similar affinity to that of NT (Botto, et al., 1998), NTS are also thought to be xenin receptors. NTS1 and NTS2 are $G_{q/11}$-coupled G protein-coupled receptors abundantly expressed in neural tissues.

Plasma xenin concentrations are increased by food intake and by sham feeding in humans, suggesting that luminal nutrients and vagal activation release xenin from the duodenal mucosa (Feurle, et al., 2003). Since nocturnal workers with high energy intake and high body fat mass have no elevation of plasma xenin after meals (Schiavo-Cardozo, et al., 2013) and obese adolescents have significantly higher concentrations of plasma xenin between meals compared to healthy controls (Arslan, et al., 2014), xenin-induced satiety signals appear to be disrupted in obesity. Peripheral and central injection of xenin decreases food intake in experimental animals (Alexiou, et al., 1998;Cooke, et al., 2009), consistent with a satiety function of xenin. The infusion of low concentrations (4-12 pmol/kg) of xenin to humans

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

increases satiety and causes mild diarrhea by an unknown mechanism (Gault, et al., 2015;Chowdhury, et al., 2014). Since Roux-en-Y gastric bypass surgery diminishes xenin-associated diarrhea (Sterl, et al., 2016), the duodenum may significantly contribute to the fluid and ion secretory response to xenin. We thus aimed to characterize the secretory effects of xenin in the duodenum and to investigate the neural pathways underlying xenin-induced duodenal anion secretion.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fed a pellet diet and water *ad libitum*. All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee. Rats were fasted overnight with free access to water before the experiments. Animals were euthanized by terminal exsanguination under deep isoflurane anesthesia, followed by thoracotomy.

Duodenal loop perfusion

Rat duodenal loops were prepared and perfused under isoflurane anesthesia as described previously (Mizumori, et al., 2006). In brief, prewarmed saline was infused via the right femoral vein at 1.08 ml/h using a Harvard infusion pump (Harvard Apparatus; Holliston, MA). Under isoflurane anesthesia (2%), a polyethylene tube (diameter 5 mm) was inserted through the forestomach and tied at 0.5 cm caudal from the pyloric ring. Another polyethylene tube was inserted through the distal duodenum and sutured at 0.5 cm oral from the ligament of Treitz. To prevent contamination from bile or pancreatic juice, the pancreaticobiliary duct was ligated just proximal to its insertion into the duodenal wall and cannulated with a PE-10 tube to drain

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

the juice. The resultant closed proximal duodenal loop (perfused length ~2 cm) was perfused with prewarmed saline (pH 7.0) by using a peristaltic pump (Fisher Scientific, Pittsburgh, PA, USA) at 1 ml/min. After stabilization with continuous luminal perfusion of O_2-bubbled saline (pH 7.0) for ~30 min, the time was set as t = 0. The duodenal loop was perfused with saline from t = 0 min until t = 45 min. After i.v. infusion of saline for 15 min, xenin-8 in saline was continuously infused at 3 or 10 nmol/kg/hr from t = 15 min to t = 45 min. Duodenal HCO_3^- secretion was measured using flow-through pH and CO_2 electrodes and expressed as total CO_2 output, calculated from the measured pH and [CO_2] in the effluent solution, as reported previously (Mizumori, et al., 2006).

Immunofluorescence staining and real-time RT-PCR

Small pieces of intestine were immersed in Zamboni's fixative containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) overnight for 4°C. The fixed tissues were then submerged in 20% sucrose in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C, and embedded in optimum cutting temperature (OCT) compound. Frozen sections of 8-μm thickness were placed on aminosilane-coated glass slides (Matsunami Glass USA Inc., Bellingham, WA, USA). Sections were pretreated with 5% normal donkey serum in PBS containing 0.3% Triton X-100 for 1 h, followed by incubation with primary antibodies; rabbit anti-xenin-25 (Phoenix Pharmaceuticals, Belmont, CA, USA, 1 μg/ml), mouse anti-5-HT (dilution 1:100, MCA3190Z; AbD Serotec, Kidlington, UK), goat anti-GIP (dilution 1:200, sc-23554; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat anti-cholecystokinin (CCK) (dilution 1:200, sc-21617; Santa Cruz Biotechnology), or goat anti-glucagon-like peptide (GLP)-2 (dilution 1:200, sc-7781; Santa Cruz Biotechnology) overnight at 4°C. In order to confirm antibody specificity, xenin-25 antibody was pre-absorbed with xenin-25

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

(50 μg/ml) overnight prior to application to tissue sections (absorption test). After rinsing in PBS, fluorescence-conjugated secondary antibodies were reacted for 2 h at room temperature. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and covered with EverBrite mounting medium (Biotium, Hayward, CA, USA). Immunofluorescence was imaged and captured using a confocal laser microscope (LSM710; Carl Zeiss GmbH, Jena, Germany).

The duodenum was separated into the epithelium, interstitium-submucosa, and muscle layers under a stereomicroscope. The tissue was immersed in ice-cold pH 7.0 Krebs buffer containing 10 mM EDTA for 10 min, followed by stripping muscle layers by microdissection using fine forceps. The epithelial cells were separated from the interstitium by gentle scraping using a silicone-coated pipette tip. The separated duodenal samples, dorsal root ganglia (DRG) and nodose ganglia (NG) were kept in a RNA stabilization solution (RNA*later*, Quiagen, Valencia, CA, USA) at 4˚C until use. Real-time polymerase chain reaction (RT-PCR) was performed as described previously (Akiba, et al., 2015) with the following sense and antisense primers, respectively; NTS1 (5'-gtcaaggtcgtcatccaggt-3'; 5'-agaccacaaaggcaatgacc-3'), NTS2 (5'-ccatcgtggctgtgtatgtc-3'; 5'-agcgtgttggtcaccatgta-3'), NK1 (5'-tcctcctgccctacatcaac-3'; 5'-tgaccttgtacacgctgctc-3'), NK2 (5'-gtgaaggccatggtactggt-3'; 5'-tccagcctgtcttcctcagt-3'), and NK3 (5'-tactgccgcttccagaactt-3'; 5'-tccaacgatggtgtaggtga-3'). β-actin was used as an internal control. The expression level was presented as the fold induction per 10^3 copies of β-actin by the ΔCt method.

Short-circuit current measurements in Ussing chambered preparations

Mucosa–submucosa preparations were created from the duodenum as described previously (Kaji, et al., 2015b). The same preparations from the mid-jejunum (~20 cm from the pyloric ring), terminal ileum (5 cm from the ileocecal junction), proximal colon (5 cm from the cecum),

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

and distal colon (5 cm from the anus) were used for comparing segmental differences. Each segment was divided longitudinally into two pieces, and each piece was mounted between two hemi-sliders with an aperture = 0.3 cm^2 (Physiologic Instruments, San Diego, CA, USA). The serosal Krebs-Ringer solution contained (in mM) NaCl, 117; KCl, 4.7; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 11; and bubbled with 95% O_2-5% CO_2 to maintain pH at 7.4. For HCO_3^--free conditions, $NaHCO_3$ was replaced with NaCl and acetazolamide (0.2 mM) was added into the serosal bath bubbled with 100% O_2. For Cl^--free solutions, NaCl, KCl, and $CaCl_2$ were replaced with sodium gluconate, potassium gluconate, and 8 mM calcium gluconate, respectively. The luminal bathing solution for small intestine contained NaCl, 136; KCl, 2.6; $CaCl_2$, 1.8; HEPES, 10 (pH 7.4); and mannitol, 11; bubbled with 100% O_2. The luminal bathing solution for colon was Krebs-Ringer solution. Measurements of short-circuit current (I_{sc}) and tissue conductance (G_t) were conducted as described previously (Kaji, et al., 2015b). Positive values for I_{sc} indicate a negative electrical charge flux from the serosal → luminal bath as a result of anion secretion or cation absorption. Indomethacin (10 μM) was added to the serosal bath for the small intestine or to both baths for the colon to eliminate the effects of endogenous prostaglandin production. The tissues were stabilized for 30–45 min before the effects of xenin and other drugs were investigated.

Peptide Synthesis

Xenin-25 (MLTKFETKSARVKGLSFHPKRPWIL) was synthesized using solid-phase methodology according to the Fmoc-strategy using an automated peptide synthesizer (Model Pioneer, Thermo Fisher Scientific, Waltham, MA, USA). The crude peptide was purified using reverse-phase high performance liquid chromatography (HPLC: Delta 600 HPLC System, Waters, MA, USA) on a column of Develosil ODS-HG-5 (2 x 25 cm, Nomura Chemical Co., Ltd,

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Seto, Japan). The purity of each peptide was confirmed by analytical HPLC and matrix assisted laser desorption/ionization time of flight and mass spectrometry (MALDI-TOF MS) analysis.

Chemicals

The vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP) receptor 1 (VPAC1) antagonist [Ac-His^{1}, D-Phe^{2}, Lys^{15}, Arg^{16}, Leu^{27}]-VIP(1-7)-GRF(8-27) was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). Xenin-8, A803467, SR48692 (meclinertant), NTRC824, SB268262, CP96345, CP99994, MEN10376, osanetant, PSB603, MRS3777 and GR113808 were purchased from Tocris Bioscience (Pittsburgh, PA, USA). Aprepitant was purchased from Cayman chemical (Ann Arbor, MI, USA). Levocabastine, ondansetron, suramin, 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-(3-chlorostyryl)caffeine (8CC), atropine, tetrodotoxin (TTX), lidocaine, capsaicin, indomethacin, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), nifedipine, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Values are expressed as the mean ± SEM. The number of animals in each experimental group was ≥5. Statistical analysis was performed using Prism, ver. 6 (GraphPad Software Inc., La Jolla, CA, USA) with Student's *t* test or ANOVA followed by Dunnett's or Tukey's multiple comparison depending on the number of experimental groups. $P < 0.05$ was considered statistically significant.

Results

Secretory effect of xenin-25 and xenin-8 in Ussing-chambered duodenal mucosa

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

After a 30-min incubation in the Ussing chamber, basal I_{sc} and G_t stabilized at 38.9 ± 4.6 μA/cm^2 and 41.0 ± 1.3 mS/cm^2, respectively, in mucosa-submucosa preparations of distal duodenum. Serosal application of xenin-8 immediately increased I_{sc} and G_t in a dose-dependent manner with EC_{50} = 0.3 nM (Fig. 1A-B). A submaximal concentration (1 nM) was subsequently used in experiments to identify the underlying mechanisms. Serosal xenin-25 (1 nM) increased I_{sc} (83.2 ± 4.7 μA/cm^2; N = 5) with a similar time course and peak value to those of xenin-8. Mucosal application of xenin-8, (1 nM) however, did not alter I_{sc} or G_t, suggesting that xenin receptors are present on the submucosal plexus or epithelial cell basolateral membrane rather than on the apical membrane of duodenocytes.

In order to confirm the nature of anion secretion in Ussing-chambered duodenal mucosa, the effects of xenin-8 were measured in the presence or absence of HCO_3^- or Cl^- in the serosal buffer. HCO_3^- depletion of the buffer did not alter basal I_{sc} or G_t (I_{sc}, 49.3 ± 13.3 μA/cm^2; G_t, 49.6 ± 4.1 mS/cm^2), or the xenin-8-induced I_{sc} increase, compared to normal conditions. Cl^- depletion in the serosal bath, however, reduced basal I_{sc} and G_t (I_{sc}, −14.9 ± 28.6 μA/cm^2; G_t, 22.3 ± 1.6 mS/cm^2) and the response to xenin-8 by 80%, suggesting that the xenin-induced I_{sc} increase is predominantly electrogenic Cl^- secretion (Fig. 1C). The NKCC1 inhibitor bumetanide reduced xenin-8-evoked I_{sc}, further supporting the presence of Cl^- secretion, which depends on basolateral NKCC1 activity.

Segmental differences in the response to xenin-8 in the Ussing chamber

In order to compare the extent of secretory response to xenin-8, we measured ion secretion in mucosa-submucosa preparations from proximal duodenum, distal duodenum, mid-jejunum, terminal ileum, proximal colon, and distal colon. Basal I_{sc} and G_t after stabilization differed among the segments tested (Table 1). Serosal application of xenin-8 (1 nM) increased I_{sc}

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

accompanied by G_t increase to the same extent in the proximal and distal duodenum. The hindgut segments had significantly lower or no response compared to the distal duodenum (Fig. 2A). The viability of the preparations was confirmed by the addition of nicotine (0.1 mM) into the serosal bath after the experiment, which increased I_{sc} to a similar extent in all segments tested (Fig 2B).

Effects of NTS antagonists on xenin-8-induced anion secretion in the duodenum

To evaluate the contribution of NTS1 and NTS2 towards xenin-8-induced anion secretion, we tested the effects of the selective antagonist SR48692 for NTS1, and levocabastine and NTRC824 for NTS2. SR48692 pretreatment did not alter basal I_{sc} or G_t, but dose-dependently inhibited the response to xenin-8 (Fig. 3A), with an IC_{50} ~30 nM, comparable to the results from *in vitro* binding assay (Gully, et al., 1993). Neither the partial NTS2 agonist levocabastine nor the selective and potent NTS2 antagonist NTRC824 altered basal or xenin-8-induced I_{sc}, suggesting that xenin-8-induced duodenal anion secretion is mediated by NTS1, but not by NTS2.

Effects of neuronal channel inhibitors on xenin-8-induced anion secretion in the duodenum

In order to identify the neural pathways mediating the secretory response to xenin, the neural cation channel blockers TTX, A803467, lidocaine, and/or capsaicin were used. Serosal pretreatment with TTX decreased basal I_{sc}, indicating that TTX-sensitive submucosal neural activity is involved in basal ion transport under stabilized conditions. Xenin-8-induced anion secretion was inhibited 50% in the presence of TTX (Fig. 3B). Serosal capsaicin (10 µM) transiently increased I_{sc} only with the first application (14.0 ± 2.8 µA/cm^2, N = 6), suggesting that capsaicin-sensitive afferent nerves innervate duodenal mucosa and contribute to ion secretion. The response to xenin-8 was significantly reduced 15 min after capsaicin

pretreatment (Cap-t) due to desensitization. The combination of TTX and capsaicin did not additionally inhibit the secretory response to xenin, suggesting that capsaicin-sensitive afferent nerves interact with submucosal neurons to stimulate secretion. The TTX-resistant sodium channel Nav1.8 is selectively inhibited by A803467 (Jarvis, et al., 2007), which was tested for its effect on xenin-8-induced anion secretion. Pretreatment with A803467 (1 μM) alone had no effect, but the combination of TTX and A803467 abolished xenin-8-induced anion secretion (Fig. 3B). The local anesthetic lidocaine inhibits sodium channels on TTX-resistant nerves and suppresses colonic secretion and inflammation (Yajima, 1988); (McCafferty, et al., 1994). Serosal, but not mucosal treatment with lidocaine (0.5 mM) abolished the secretory response to xenin-8. These results suggest that serosal xenin activates TTX-sensitive and -resistant afferent nerve fibers localized in the submucosal plexus. The L-type Ca^{2+} channel blocker nifedipine (0.1 mM) significantly inhibited the response to xenin-8 (Fig. 3C), but did not affect forskolin- or bethanechol-induced secretion (data not shown), supporting our hypothesis that xenin-8 activates nerves in the submucosal plexus, rather than directly activates epithelial cells.

Neurotransmitters involved in xenin-8-induced anion secretion

Acetylcholine (ACh) and vasoactive intestinal peptide (VIP) are major and potent neurotransmitters present in submucosal secretomotor neurons, activating muscarinic ACh receptors and VPAC1 on enterocytes, respectively (Xue, et al., 2007). Serosal xenin-8-induced secretion was, however, not altered by the muscarinic antagonist atropine (10 μM) or the selective VPAC1 antagonist [Ac-His^{1}, D-Phe^{2}, Lys^{15}, Arg^{16}, Leu^{27}]-VIP(1-7)-GRF(8-27) (1 μM) (Table 2). Calcitonin-gene related peptide (CGRP) is one of neurotransmitters released from capsaicin-sensitive afferent nerves (Maggi, et al., 1986). A selective CGRP receptor 1

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

antagonist SB268262 (10 μM) had no effect on xenin-8-induced I_{sc} increase (Table 2). These results indicated that NTS1-mediated anion secretion was not mediated by ACh, VIP, or CGRP.

Substance P (SP), 5-hydroxytryptamine (5-HT), and nitric oxide (NO) are peptide, monoamine, and gaseous neurotransmitters, respectively, involved in non-cholinergic, non-adrenergic autonomic neurotransmission. In order to determine the contribution of these transmitters to xenin-induced anion secretion, we tested selective antagonists for tachykinin receptors (NK1, NK2 and NK3), or for 5-HT receptors (5-HT_3 and 5-HT_4), or the NO synthase inhibitor L-NAME for their effects on xenin-8-induced I_{sc}. The NK1-selective antagonists CP96345 or CP99994 (10 μM) significantly inhibited xenin-induced I_{sc}, whereas the NK2-selective antagonist MEN10376 (1 μM), or the NK3-preferred antagonist osanetant (1 μM) had no effect (Fig. 3D). A high concentration of osanetant (10 μM) or co-treatment with CP99994 and osanetant inhibited xenin-8-induced I_{sc} as same inhibitory extent as CP99994 alone, suggesting that NK1 is mainly involved in xenin-8-induced anion secretion. Another selective NK1 antagonist, aprepitant, also abolished the response to xenin-8 at 10 μM (Fig. 3E). Ondansetron (10 μM), a 5-HT_3-selective antagonist, but not the 5-HT_4 selective antagonist GR113808 (10 μM), significantly reduced the response to xenin by 65% (Fig. 3F). The response to serosal xenin-8 was not altered by pretreatment with 5-HT (0.1 mM) in the serosal bath, suggesting that 5-HT_3 receptors are not desensitized under these experimental conditions. Although 5-HT mediates neural NO release in guinea pig colon (Kuwahara, et al., 1998), serosal pretreatment with L-NAME (0.1 mM) had no effect on the basal and xenin-8-stimulated I_{sc} change (Table 2).

NT-induced Cl^- secretion is mediated by adenosine A_1 and A_2 receptors in human colonic mucosa (Riegler, et al., 2000), whereas rat duodenal HCO_3^- secretion is stimulated by luminal adenosine through A_{2B} receptors (Ham, et al., 2010). We thus tested the effect of the

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

purinergic receptor antagonists CPX (50 μM) for A_1 receptors, 8CC (10 μM) for A_{2A} receptors, PSB603 (10 μM) for A_{2B} receptors, and MRS3777 (1 μM) for A_3 receptors on xenin-8-induced Cl^- secretion in the Ussing chamber. Using the same concentration of antagonists as in our previous study (Ham, et al., 2010), none of these selective antagonists affected the xenin-evoked I_{sc} increase (Fig. 3G). Serosal application of CPX alone increased basal I_{sc} (ΔI_{sc}: 23.6 ± 4.0 μA/cm^2), suggesting that A_1 receptors may be activated during stabilization, and an A_1 antagonist unmasks the anti-secretory effect of A_1 activation. From these data, we concluded that the adenosine pathway is not involved in xenin-NTS1-mediated Cl^- secretion in rat duodenum.

Duodenal HCO_3^- secretion in response to i.v. infusion of xenin-8

Intravenous infusion of xenin-8 gradually and dose-dependently increased the rate of duodenal HCO_3^- output (Fig. 4A). Intravenous injection of the NTS1 antagonist SR48692 had no effect on basal HCO_3^- output, but inhibited xenin-8-induced HCO_3^- secretion (Fig. 4B), suggesting that NTS1 does not contribute to basal HCO_3^- secretion in fasted rats, whereas xenin enhances protective duodenal HCO_3^- secretion via NTS1 activation *in vivo*.

Expression of NTS and NK in the duodenum and extrinsic afferent neurons

RT-PCR was performed for NTS1, NTS2, NK1, NK2, and NK3 in the isolated duodenal epithelial cells (Ep), in the lamina propria + submucosa including the submucosal plexus (LP-SM), and extrinsic afferent neurons, DRG and NG. NTS1 and NTS2 were detected at very low levels in the epithelial cells, whereas both receptors were expressed in LP-SM containing enteric neural plexuses, and in extrinsic afferent neurons (Fig. 5A and 5B). In contrast to predominant NTS1-mediated secretion in the Ussing chamber studies, NTS2 expression was abundant in duodenal LP-SM, suggesting that NTS2 may be involved in functions other than

anion secretion. All NK receptor subtypes were detected in Ep and LP-SM (Fig. 5C and 5D). NK1 receptor expression was significantly higher than NK2 or NK3 in Ep, whereas lower than NK2 in LP-SM (Fig. 5D).

Localization of xenin in duodenal mucosa

Immunohistochemical study of cryostat intestinal sections revealed that a small subset of epithelial cells expressed xenin-25 in the duodenum, but not in the ileum or colon (Fig. 6). Pre-absorption the antibody with xenin-25 abolished the immunoreactivity (data not shown). Xenin-positive cells in the duodenum possessed long narrow apical processes with storage in the basolateral cytosol, suggesting that xenin-25 is expressed in EECs. Double immunostaining demonstrated that xenin-25-immunoreactive cells co-expressed 5-HT, GLP-2, or CCK (Fig. 7A-C), suggesting that xenin-25 is released from enterochromaffin (EC), L, and/or I cells. Inconsistent with the study in human and canine duodenum (Anlauf, et al., 2000), most GIP-containing K cells had no xenin-25 immunoreactivity and *vice versa* in rat duodenum (Fig. 7D).

Discussion

We demonstrated that the gut peptide hormone xenin induced neurogenic HCO_3^- and Cl^- secretion through activation of afferent neural reflexes via NTS1, 5-HT_3, and NK1 receptors expressed in rat duodenum. As endogenous xenin was present in a subset of duodenal EECs, postprandial xenin release from the duodenal mucosa may contribute not only to mucosal defense mechanisms, but also to satiety signaling via vagal and spinal afferent nerves.

Although ACh and VIP are the major secretagogues for intestinal epithelia in electrically-activated submucosal plexus (Krueger, et al., 2016), our results showed that serosal xenin failed to activate cholinergic or VIP-ergic neurons. Since the secretory response to xenin was

blocked by selective antagonists for NTS1, 5-HT_3, and NK1 receptors and Nav1.8 channels that are generally expressed on the afferent nerves, NTS1 receptors may be specifically expressed on the intrinsic and/or extrinsic primary afferent neurons that release SP. 5-HT-induced duodenal anion secretion is predominantly mediated by epithelial 5-HT_4 in mice (Tuo, et al., 2004). Luminal 5-HT also stimulates HCO_3^- secretion via 5-HT_4 activation in rat proximal colon (Kaji, et al., 2015a). Nevertheless, we here showed that xenin-induced Cl^- secretion was partially mediated by 5-HT_3, but not 5-HT_4 activation. In the xenin-evoked neurogenic secretory pathway, 5-HT may not be released from EC cells into the lumen as a component of 5-HT_4-mediated secretion. Since 5-HT_3 is expressed in extrinsic afferent neurons (Glatzle, et al., 2002;Raybould, et al., 2003), and since the serosally-applied selective 5-HT_3 agonist SR57227 or NK1 agonist Sar-Met-SP increased I_{sc} in a 5-HT_3-dependent manner in the duodenum (Kaji, unpublished observation), endogenous 5-HT may activate afferent axons via 5-HT_3 receptors, potentiating SP release during NTS1 activation by xenin, implying that SP is an important stimulant for duodenocytes through activation of NK1 on epithelial cells. Although CGRP may be co-released with SP from extrinsic afferent nerves, xenin-induced neurogenic secretion was not mediated by CGRP receptors *in vitro*. It is possible that the secretory effect of released CGRP was minimal, compared to the effect of released SP in our experimental preparation.

The NK1 selective antagonists CP96345 and CP99994 may directly interact with L-type Ca^{2+} channels (McLean, et al., 1993). In addition to these compounds, the L-type Ca^{2+} channel blocker nifedipine also reduced the response to xenin-8, suggesting that L-type Ca^{2+} channel activation in neural tissues mediates xenin-8-evoked anion secretion. Since CP99994 has a considerably lower affinity for Ca^{2+} channels than does CP96345 and the effects of these two antagonists on the response to xenin were identical, the contribution of NK1 to xenin-8-induced

secretion could not be excluded. Indeed, another type of NK1 antagonist, aprepitant, dose-dependently inhibited xenin-8-induced secretion, suggesting that SP-NK1 is downstream of NTS1 activation in xenin-evoked duodenal anion secretion. Although SP is a well-characterized intracellular Ca^{2+}-mediated secretagogue present in intrinsic afferent neurons (Mitsui, 2010), the identity of the physiological stimulus that releases SP has not yet been identified. Our study revealed that a physiological concentration of xenin (<1 nM) strongly activated NK1-mediated anion secretion through TTX-sensitive and -resistant pathways, suggesting that NTS1 activation by xenin releases SP from the afferent nerves. In the myenteric plexus, SP is involved in the regulation of motility mediated by cholinergic excitatory motor neurons (Furness, et al., 2015). Nonetheless, xenin-induced secretion was independent of the cholinergic secretomotor pathway. The neural circuits of secretomotor and muscle motor functions may differ even though the same neurotransmitter is involved. NK1, as well as NK2 and NK3 are expressed in enteric neurons (Grady, et al., 1996), although isolated duodenocytes predominantly expressed NK1, consistent with the functional expression of NK1 in isolated colonocytes (Southwell and Furness, 2001;Hosoda, et al., 2002). Though xenin-positive EECs are a tiny subset of all EECs, local SP release may be stimulated by SP itself via NK1 receptors present on the afferent nerves (Mitsui, 2010) and may potentiate the secretory response.

Our immunohistochemical data demonstrated that xenin-25 was present in a part of 5-HT/ CCK- or GLP-2-expressing EECs respectively termed I cells and L cells. Xenin-25 and GIP immunoreactivities were mostly detected in distinct cells; only few GIP-containing K cells co-expressed xenin-25. The concept of EEC classification has recently been reconsidered. Egerod *et al*. reported that CCK, secretin, GIP, GLP-1, PYY, and NT but not somatostatin were

co-expressed in a novel group of EECs, according to the endocrine cell lineage (Egerod, et al., 2012). Cho *et al*. reported that 5-HT and CCK were often coexpressed in EEC of mouse duodenum (Cho, et al., 2014). Therefore, our observation suggests that EECs expressing multiple gut hormones also express xenin-25, particularly in the duodenum. Further studies are required to fully categorize and identify the particular nutrient receptors expressed in these multi-hormone cells. As the flow rate of luminal contents is quite rapid in the duodenum (Quon, et al., 1989), it is reasonable to hypothesize that multiple gut hormones are released at once in response to luminal nutrients in order to promptly activate postprandial physiological responses, including mucosally protective ion secretion, metabolic responses, and satiety signals via hormonal and neural effectors.

Nav1.8 is functionally expressed in extrinsic afferent neurons of the NG and DRG, whereas it is rarely identified in enteric neurons (Miranda-Morales, et al., 2010;Gautron, et al., 2011). Interestingly, although Nav1.8 inhibition alone had no effect, in combination with TTX, Nav1.8 inhibition abolished xenin-8-evoked anion secretion. Furthermore, TTX or capsaicin pretreatment alone similarly inhibited xenin-8-induced secretion, whereas co-treatment with TTX and capsaicin had no additional effect. These results suggest that xenin-8-induced anion secretion is primarily neurogenic, possibly involving the synergistic activation of TTX-sensitive and -resistant afferent nerves. Our study did not distinguish intrinsic afferent activation from extrinsic afferent activation. Since the capsaicin receptor TRPV1 is expressed on extrinsic afferent nerves, and since the secretomotor cholinergic pathway was not involved, our results further suggest that xenin-NTS1-SP pathway mainly involves extrinsic afferent nerve activation. These results suggest that intact extrinsic nerve innervation is required to exert full effect of xenin.

Luminal adenosine induces duodenal HCO_3^- secretion through the activation of A_{2B} receptors expressed on the apical membrane of duodenocytes, whereas A_1 receptors are only expressed in enteric neurons (Ham, et al., 2010). Serosal xenin-8-induced Cl^- secretion was not altered by any serosal antagonist for A_1, A_{2A}, A_{2B}, or A_3 receptors, indicating that adenosine receptors expressed on submucosal neurons were not involved in NTS1-mediated Cl^- secretion. Consistent with the previous reports in the colon (Hancock and Coupar, 1995;Cooke, et al., 1999), A_1 receptor inhibition increased I_{sc}, suggesting that adenosine or its precursor ATP/ADP/AMP is released in the submucosal plexus and suppresses basal electrogenic secretion in the duodenum. Therefore, luminal and basolateral stimuli may separately and locally regulate epithelial secretory function, by the same mediator via different receptor activation.

Although the precise $t_{1/2}$ of xenin-25 in the circulation is unknown, we speculate that xenin activity as a gut hormone may last longer than other gut peptides, since xenin-derived peptide fragments of varying length have similar affinities to its receptor (Martin, et al., 2014). Therefore, xenin is not rapidly de-activated by proteolytic cleavage, implying a much-extended physiological $t_{1/2}$, which in turn may influence its function in other tissues, such as the central nervous system and pancreas, in addition to the duodenum. Despite the presence of NTS1 expression and NT-containing N cells, the secretory response to xenin was significantly reduced in the lower intestine compared with the duodenum or jejunum, suggesting that NTS1 activation is less involved in the secretory response in the lower intestine. NTS1 expression is upregulated in colonocytes obtained from inflamed mucosa (Bossard, et al., 2007); furthermore, the expression of NK1 and 5-HT_3 are increased in inflamed mucosal afferent nerves (Utsumi,

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 2016). Segmental differences in NTS1-mediated secretory responses suggest varying function of xenin and NT in the upper and lower intestine, respectively.

Consistent with xenin localization in the upper intestine, the presence of xenin signaling in the duodenum for stimulating mucosal protective HCO_3^- secretion further suggests that the xenin-NTS1 pathway, independent of the cholinergic or VIP-ergic pathways, is the alternative pathway that enhances duodenal mucosal defenses. NTS1 activation is implicated in the suppression of tonic pain, consistent with the abundance of NTS1 expression in DRG neurons (Roussy, et al., 2008). Long-lasting xenin fragments may be useful for duodenal mucosal protection, appetite control, and anti-nociceptive therapeutics as a potent, selective agonist for NTS1 receptors on intrinsic/extrinsic afferent neurons.

Acknowledgements

The authors thank Ms. Stacey Jung for manuscript preparation, and Drs. Paul H Guth and Eli Engel for their consultancy.

Authorship Contributions

Participated in research design: Kaji, Akiba, and Kaunitz

Conducted experiments and analyzed data: Kaji, Akiba, and Maruta

Contributed new reagents: Kato and Kuwahara

Wrote or contributed to the writing of the manuscript: Kaji, Akiba, Kato, Kuwahara, Maruta, and Kaunitz

Reference

Akiba Y, Inoue T, Kaji I, Higashiyama M, Narimatsu K, Iwamoto K, Watanabe M, Guth PH, Engel E, Kuwahara A and Kaunitz JD (2015) Short-chain fatty acid sensing in rat duodenum. ***J Physiol*** **593:585-599.**

Akiba Y and Kaunitz JD (2011) Luminal chemosensing in the duodenal mucosa. ***Acta Physiol (Oxf)*** **201:77-84.**

Alexiou C, Zimmermann JP, Schick RR and Schusdziarra V (1998) Xenin--a novel suppressor of food intake in rats. ***Brain Res*** **800:294-299.**

Anlauf M, Weihe E, Hartschuh W, Hamscher G and Feurle GE (2000) Localization of xenin-immunoreactive cells in the duodenal mucosa of humans and various mammals. ***J Histochem Cytochem*** **48:1617-1626.**

Arslan N, Sayin O and Tokgoz Y (2014) Evaluation of serum xenin and ghrelin levels and their relationship with nonalcoholic fatty liver disease and insulin resistance in obese adolescents. ***J Endocrinol Invest*** **37:1091-1097.**

Bossard C, Souaze F, Jarry A, Bezieau S, Mosnier JF, Forgez P and Laboisse CL (2007) Over-expression of neurotensin high-affinity receptor 1 (NTS1) in relation with its ligand neurotensin (NT) and nuclear beta-catenin in inflammatory bowel disease-related oncogenesis. ***Peptides*** **28:2030-2035.**

Botto JM, Chabry J, Sarret P, Vincent JP and Mazella J (1998) Stable expression of the mouse levocabastine-sensitive neurotensin receptor in HEK 293 cell line: binding properties, photoaffinity labeling, and internalization mechanism. ***Biochem Biophys Res Commun*** **243:585-590.**

Cho HJ, Callaghan B, Bron R, Bravo DM and Furness JB (2014) Identification of enteroendocrine cells that express TRPA1 channels in the mouse intestine. ***Cell Tissue Res*** **356:77-82.**

Chowdhury S, Reeds DN, Crimmins DL, Patterson BW, Laciny E, Wang S, Tran HD, Griest TA, Rometo DA, Dunai J, Wallendorf MJ, Ladenson JH, Polonsky KS and Wice BM (2014) Xenin-25 delays gastric emptying and reduces postprandial glucose levels in humans with and without type 2 diabetes. ***Am J Physiol Gastrointest Liver Physiol*** **306:G301-G309.**

Cooke HJ, Wang Y, Liu CY, Zhang H and Christofi FL (1999) Activation of neuronal adenosine A1 receptors suppresses secretory reflexes in the guinea pig colon. *Am J Physiol* 276:G451-G462.

Cooke JH, Patterson M, Patel SR, Smith KL, Ghatei MA, Bloom SR and Murphy KG (2009) Peripheral and central administration of xenin and neurotensin suppress food intake in rodents. *Obesity (Silver Spring)* 17:1135-1143.

Dumoulin V, Moro F, Barcelo A, Dakka T and Cuber JC (1998) Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. *Endocrinol* 139:3780-3786.

Egerod KL, Engelstoft MS, Grunddal KV, Nohr MK, Secher A, Sakata I, Pedersen J, Windelov JA, Fuchtbauer EM, Olsen J, Sundler F, Christensen JP, Wierup N, Olsen JV, Holst JJ, Zigman JM, Poulsen SS and Schwartz TW (2012) A Major Lineage of Enteroendocrine Cells Coexpress CCK, Secretin, GIP, GLP-1, PYY, and Neurotensin but Not Somatostatin. *Endocrinol* 153:5782-5795.

Feurle GE (1998) Xenin--a review. *Peptides* 19:609-615.

Feurle GE, Hamscher G, Kusiek R, Meyer HE and Metzger JW (1992) Identification of xenin, a xenopsin-related peptide, in the human gastric mucosa and its effect on exocrine pancreatic secretion. *J Biol Chem* 267:22305-22309.

Feurle GE, Ikonomu S, Partoulas G, Stoschus B and Hamscher G (2003) Xenin plasma concentrations during modified sham feeding and during meals of different composition demonstrated by radioimmunoassay and chromatography. *Regul Pept* 111:153-159.

Furness JB, Poole DP, Cho HJ, Callaghan BP and Rivera LR (2015) The innervation of the gastrointestinal tract, in *Yamada's Textbook of Gastroenterology* pp 239-258, John Wiley & Sons, Ltd.

Gault VA, Martin CM, Flatt PR, Parthsarathy V and Irwin N (2015) Xenin-25[Lys13PAL]: a novel long-acting acylated analogue of xenin-25 with promising antidiabetic potential. *Acta Diabetol* 52:461-471.

Gautron L, Sakata I, Udit S, Zigman JM, Wood JN and Elmquist JK (2011) Genetic tracing of Nav1.8-expressing vagal afferents in the mouse. *J Comp Neurol* 519:3085-3101.

Glatzle J, Sternini C, Robin C, Zittel TT, Wong H, Reeve JR, Jr. and Raybould HE (2002) Expression of 5-HT3 receptors in the rat gastrointestinal tract. *Gastroenterology* 123:217-226.

Grady EF, Baluk P, Bohm S, Gamp PD, Wong H, Payan DG, Ansel J, Portbury AL, Furness JB, McDonald DM and Bunnett NW (1996) Characterization of antisera specific to NK1, NK2, and NK3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. *J Neurosci* 16:6975-6986.

Gully D, Canton M, Boigegrain R, Jeanjean F, Molimard JC, Poncelet M, Gueudet C, Heaulme M, Leyris R, Brouard A and . (1993) Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proc Natl Acad Sci U S A* 90:65-69.

Ham M, Mizumori M, Watanabe C, Wang JH, Inoue T, Nakano T, Guth PH, Engel E, Kaunitz JD and Akiba Y (2010) Endogenous luminal surface adenosine signaling regulates duodenal bicarbonate secretion in rats. *J Pharmacol Exp Ther* 335:607-613.

Hamscher G, Meyer HE, Metzger JW and Feurle GE (1995) Distribution, formation, and molecular forms of the peptide xenin in various mammals. *Peptides* 16:791-797.

Hancock DL and Coupar IM (1995) Functional characterization of the adenosine receptor mediating inhibition of intestinal secretion. *Br J Pharmacol* 114:152-156.

Hosoda Y, Karaki S, Shimoda Y and Kuwahara A (2002) Substance P-evoked Cl(-) secretion in guinea pig distal colonic epithelia: interaction with PGE(2). *Am J Physiol Gastrointest Liver Physiol* 283:G347-G356.

Jarvis MF, Honore P, Shieh CC, Chapman M, Joshi S, Zhang XF, Kort M, Carroll W, Marron B, Atkinson R, Thomas J, Liu D, Krambis M, Liu Y, McGaraughty S, Chu K, Roeloffs R, Zhong C, Mikusa JP, Hernandez G, Gauvin D, Wade C, Zhu C, Pai M, Scanio M, Shi L, Drizin I, Gregg R, Matulenko M, Hakeem A, Gross M, Johnson M, Marsh K, Wagoner PK, Sullivan JP, Faltynek CR and Krafte DS (2007) A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuates neuropathic and inflammatory pain in the rat. *Proc Natl Acad Sci U S A* 104:8520-8525.

Kaji I, Akiba Y and Kaunitz JD (2013) Involvement of gut chemosensing in the regulation of mucosal barrier function and defense mechanisms. *J Anim Sci*. 91:1957-1962.

Kaji I, Akiba Y, Said H, Narimatsu K and Kaunitz JD (2015a) Luminal 5-HT stimulates colonic bicarbonate secretion in rats. *Br J Pharmacol* 172:4655-4670.

Kaji I, Iwanaga T, Watanabe M, Guth PH, Engel E, Kaunitz JD and Akiba Y (2015b) SCFA transport in rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 308:G188-G197.

Konturek SJ, Bilski J, Tasler J and Laskiewicz J (1985) Gut hormones in stimulation of gastroduodenal alkaline secretion in conscious dogs. *Am J Physiol* 248:G687-G691.

Krueger D, Michel K, Zeller F, Demir IE, Ceyhan GO, Slotta-Huspenina J and Schemann M (2016) Neural influences on human intestinal epithelium in vitro. *J Physiol* 594:357-372.

Kuwahara A, Kuramoto H and Kadowaki M (1998) 5-HT activates nitric oxide-generating neurons to stimulate chloride secretion in guinea pig distal colon. *Am J Physiol* 275:G829-G834.

Maggi CA, Manzini S, Giuliani S, Santicioli P and Meli A (1986) Extrinsic origin of the capsaicin-sensitive innervation of rat duodenum: possible involvement of calcitonin gene-related peptide (CGRP) in the capsaicin-induced activation of intramural non-adrenergic non-cholinergic neurons. *Naunyn Schmiedebergs Arch Pharmacol* 334:172-180.

Martin CM, Parthsarathy V, Pathak V, Gault VA, Flatt PR and Irwin N (2014) Characterisation of the biological activity of xenin-25 degradation fragment peptides. *J Endocrinol* 221:193-200.

McCafferty DM, Sharkey KA and Wallace JL (1994) Beneficial effects of local or systemic lidocaine in experimental colitis. *Am J Physiol* 266:G560-G567.

McLean S, Ganong A, Seymour PA, Snider RM, Desai MC, Rosen T, Bryce DK, Longo KP, Reynolds LS, Robinson G and . (1993) Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J Pharmacol Exp Ther* 267:472-479.

Miranda-Morales M, Ochoa-Cortes F, Stern E, Lomax AE and Vanner S (2010) Axon reflexes evoked by transient receptor potential vanilloid 1 activation are mediated by tetrodotoxin-resistant voltage-gated Na+ channels in intestinal afferent nerves. *J Pharmacol Exp Ther* 334:566-575.

Mitsui R (2010) Immunohistochemical characteristics of submucosal Dogiel type II neurons in rat colon. *Cell Tissue Res* 340:257-265.

Mizumori M, Meyerowitz J, Takeuchi T, Lim S, Lee P, Supuran CT, Guth PH, Engel E, Kaunitz JD and Akiba Y (2006) Epithelial carbonic anhydrases facilitate PCO_2 and pH regulation in rat duodenal mucosa. *J Physiol* 573:827-842.

Quon MG, Mena I and Valenzuela JE (1989) Abnormalities in the duodenal transit and motility in duodenal ulcer patients: studies with a new isotopic technique. *Gut* 30:579-585.

Raybould HE, Glatzle J, Robin C, Meyer JH, Phan T, Wong H and Sternini C (2003) Expression of 5-HT3 receptors by extrinsic duodenal afferents contribute to intestinal inhibition of gastric emptying. *Am J Physiol Gastrointest Liver Physiol* 284:G367-G372.

Riegler M, Castagliuolo I, Wang C, Wlk M, Sogukoglu T, Wenzl E, Matthews JB and Pothoulakis C (2000) Neurotensin stimulates Cl(-) secretion in human colonic mucosa In vitro: role of adenosine. *Gastroenterology* 119:348-357.

Roussy G, Dansereau MA, Dore-Savard L, Belleville K, Beaudet N, Richelson E and Sarret P (2008) Spinal NTS1 receptors regulate nociceptive signaling in a rat formalin tonic pain model. *J Neurochem* 105:1100-1114.

Said H, Kaji I and Kaunitz JD (2015) Gastroduodenal mucosal defense mechanisms. *Curr Opin Gastroenterol* 31:486-491.

Schiavo-Cardozo D, Lima MM, Pareja JC and Geloneze B (2013) Appetite-regulating hormones from the upper gut: disrupted control of xenin and ghrelin in night workers. *Clin Endocrinol (Oxf)* 79:807-811.

Southwell BR and Furness JB (2001) Immunohistochemical demonstration of the NK_1 tachykinin receptor on muscle and epithelia in guinea pig intestine. *Gastroenterology* 120:1140-1151.

Sterl K, Wang S, Oestricker L, Wallendorf MJ, Patterson BW, Reeds DN and Wice BM (2016) Metabolic responses to xenin-25 are altered in humans with Roux-en-Y gastric bypass surgery. *Peptides* 82:76-84.

Tanaka K, Masu M and Nakanishi S (1990) Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* 4:847-854.

Tuo BG, Sellers Z, Paulus P, Barrett KE and Isenberg JI (2004) 5-HT induces duodenal mucosal bicarbonate secretion via cAMP- and Ca^{2+}-dependent signaling pathways and 5-HT4 receptors in mice. *Am J Physiol Gastrointest Liver Physiol* 286:G444-G451.

Utsumi D, Matsumoto K, Amagase K, Horie S and Kato S (2016) 5-HT3 receptors promote colonic inflammation via activation of substance P/neurokinin-1 receptors in dextran sulphate sodium-induced murine colitis. *Br J Pharmacol* 173:1835-1849.

Wice BM, Wang S, Crimmins DL, Diggs-Andrews KA, Althage MC, Ford EL, Tran H, Ohlendorf M, Griest TA, Wang Q, Fisher SJ, Ladenson JH and Polonsky KS (2010) Xenin-25 potentiates glucose-dependent insulinotropic polypeptide action via a novel cholinergic relay mechanism. *J Biol Chem* 285:19842-19853.

Xue J, Askwith C, Javed NH and Cooke HJ (2007) Autonomic nervous system and secretion across the intestinal mucosal surface. *Auton Neurosci* 133:55-63.

Yajima T (1988) Luminal propionate-induced secretory response in the rat distal colon in vitro. *J Physiol* 403:559-575.

Footnotes

This study was supported by VA Merit Review (JDK); the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [R01-DK54221] (JDK); and American Gastroenterology Association-Rome Foundation Functional Gastroenterology and Motility Disorders Pilot Research Award (IK).

No conflicts of interest are declared.

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1 Effect of xenin on short-circuit current (I_{sc}) in mucosa-submucosa preparation of rat duodenum mounted in Ussing chambers. **A:** Representative traces of I_{sc}. Serosal (s) application of xenin-8 (dark blue line) or xenin-25 (red line) at 1 nM immediately increased I_{sc}, whereas mucosal (m) application of xenin-8 (green line) had no effect. **B:** Concentration-response curve of serosal xenin-8-induced I_{sc} increases. Effect of xenin-25 at 1 nM (unfilled diamond) was similar extent with that of xenin-8. **C:** Xenin-8-induced I_{sc} increases in the presence or absence of Cl^-, HCO_3^-, or bumetanide in the serosal bathing solution. The serosal bath was replaced with each anion-free buffer, or bumetanide (0.1 mM) was added 15 min before the application of xenin-8 (1 nM). Each datum represents the mean ± SEM (n = 5). *P < 0.05 vs. Control group by ANOVA followed by Dunnett's test.

Fig. 2 Segmental heterogeneity in response to xenin-8 (A) and nicotine (B) in Ussing chambered rat intestinal segments. The I_{sc} peak values in response to serosal xenin-8 (1 nM) or nicotine (0.1 mM) were compared among mucosa-submucosa preparations from proximal duodenum (pD), distal duodenum (dD), mid-jejunum (J), terminal ileum (tI), proximal colon (pC), and distal colon (dC). Each datum represents the mean ± SEM (n > 5). *P < 0.05 vs. dD, n.s. (not significant) by ANOVA followed by Dunnett's test.

Fig. 3 Effect of selective receptor antagonists and neural sodium channel inhibitors on xenin-8-induced I_{sc} changes. **A:** Neurotensin receptor (NTS) 1 antagonist SR48692, or NTS2 antagonists levocabastine (Levo) or NTRC824 was applied to the serosal bath. **B:** Tetrodotoxin (TTX, 1 μM), capsaicin (Cap-t, 10 μM), and/or A803467 (1 μM) were applied to the serosal bath. Lidocaine (Lidoc, 0.5 mM) was applied to mucosal (m) or serosal (s) bath. **C:** The L-type Ca^{2+} channel blocker nifedipine (0.1 mM) was applied to serosal bath. **D:**

Substance P/tachykinin receptor (NK) 1 antagonist CP96345 or CP99994, NK2 antagonist MEN10376 (MEN), and/or osanetant (Osanet) were applied to the serosal bath. **E:** Selective NK1 antagonist aprepitant was applied to the serosal bath. **F:** The 5-HT_3 receptor antagonist ondansetron, 5-HT_4 antagonist GR113808, or 5-HT was applied to the serosal bath. **G:** The adenosine receptor A_1 antagonist (CPX), A_{2A} antagonist (8CC), A_{2B} antagonist PSB603 (PSB), or A_3 antagonist MRS3777 (MRS) was applied to the serosal bath. Each datum represents the mean ± SEM (n > 5). *P < 0.05 vs. vehicle by paired t-test (C) or by ANOVA followed by Dunnett's test (A, B, D-G).

Fig. 4 Effect of intravenous (iv) infusion of xenin-8 and SR48692 on luminal HCO_3^- secretion in the duodenum. A duodenal loop was perfused with pH 7 saline and total CO_2 output was measured in the perfusate with flow-through pH and CO_2 electrodes. A: Saline with or without xenin-8 was infused into the femoral vein from time = 15 min. Intravenous infusion of xenin-8 dose-dependently increased total CO_2 output. B: SR48692 was injected at time = 5 min. Bolus injection of SR48692 had no effect on basal HCO_3^- secretion, but significantly inhibited the response to xenin-8. Each data point represents the mean ± SEM (n = 6 rats). *P < 0.05 vs. saline, $^{\dagger}P$ < 0.05 vs. another group by two-way ANOVA followed by Tukey's test.

Fig. 5 Expression of mRNA for NTS1, NTS2, and NK receptors in rat tissues determined by real-time RT-PCR. NTS1 (A) and NTS2 (B) expression were compared in isolated duodenal epithelial cells (Ep), lamina propria + submucosa with submucosal plexus from the duodenum (LP-SM), dorsal root ganglia (DRG) and nodose ganglia (NG). *P < 0.05 vs. Ep, $^{\dagger}P$ < 0.05 vs. LP-SM by ANOVA followed by Tukey's test. NK1, NK2, and NK3 expressions were compared

in Ep (C) and LP-SM (D). *$P < 0.05$ vs. NK1, $^{\dagger}P < 0.05$ vs. NK2 by ANOVA followed by Tukey's test. Each data represents the mean ± SEM (n = 4).

Fig. 6 Immunoreactivity for xenin-25 in frozen sections of rat duodenum, ileum, and proximal colon. Xenin-25 positive cells in duodenal mucosa (arrow and inset, higher magnification: bar, 5 μm) had enteroendocrine-like morphology. Nuclei were counterstained with DAPI (blue). Bar, 50 μm (duodenum) or 100 μm (ileum and colon).

Fig. 7 Double immunostaining for xenin-25 and enteroendocrine cell (EEC) markers in frozen sections of rat duodenum. A part of glucagon-like peptide 2 (GLP-2)-, cholecystokinin (CCK)-, or serotonin (5-HT)-containing cells co-expressed xenin-25 (arrows). Most of gastric inhibitory peptide (GIP)-containing cells were xenin-25-negative. Arrowheads indicate EECs, which are stained with a single marker. Nuclei were counterstained with DAPI (blue). Bar, 20 μm.

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Segmental differences of basal I_{sc} and G_t in-Ussing chambered intestinal segments

	Basal I_{sc} [μA/cm^2]	**Basal G_t [mS/cm^2]**
Proximal duodenum	39.6 ± 4.5	41.0 ± 1.9
Distal duodenum	37.2 ± 2.0	41.2 ± 0.8
Jejunum	48.2 ± 12.3	52.8 ± 2.8
Terminal ileum	42.2 ± 10.1	28.5 ± 3.4
Proximal colon	55.1 ± 4.65	18.4 ± 0.8
Distal colon	31.7 ± 5.06	11.5 ± 1.6

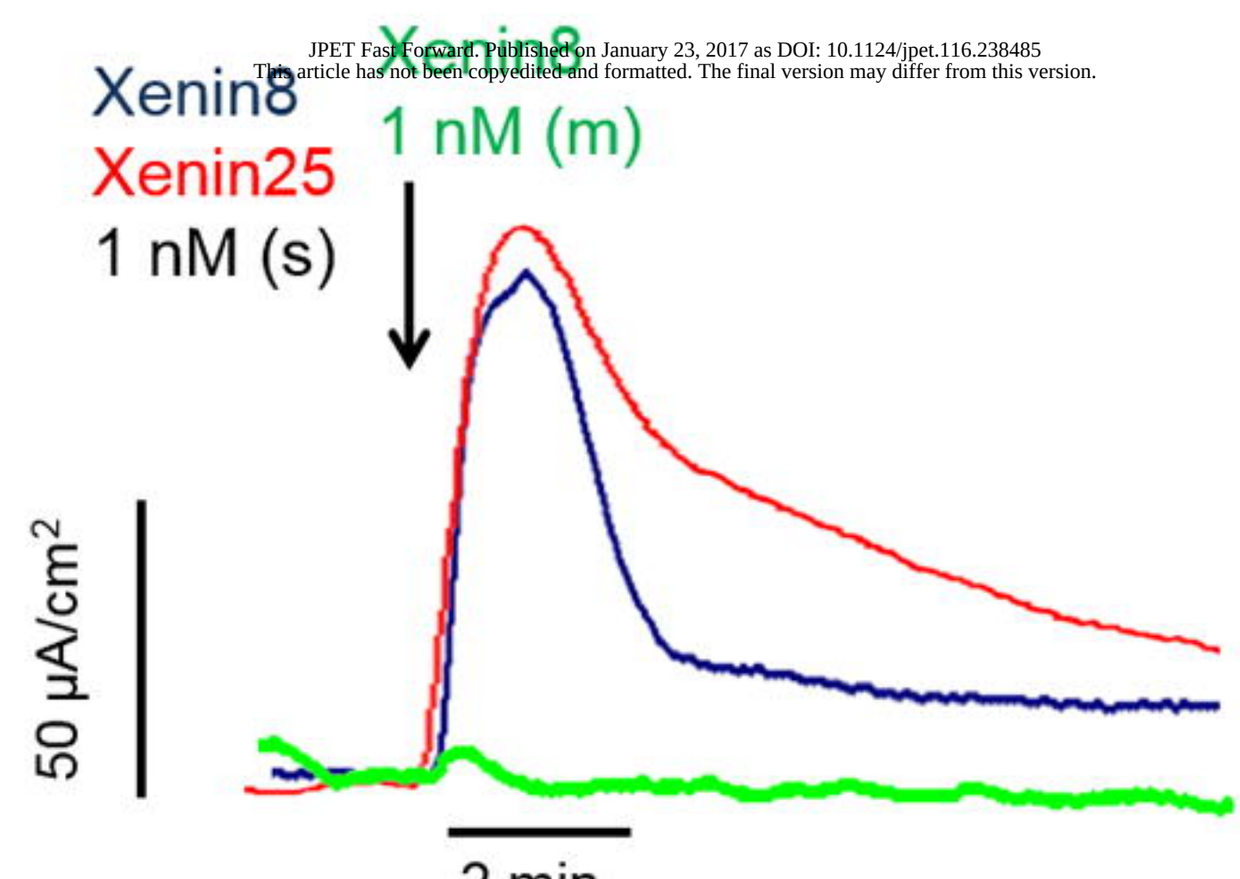
JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

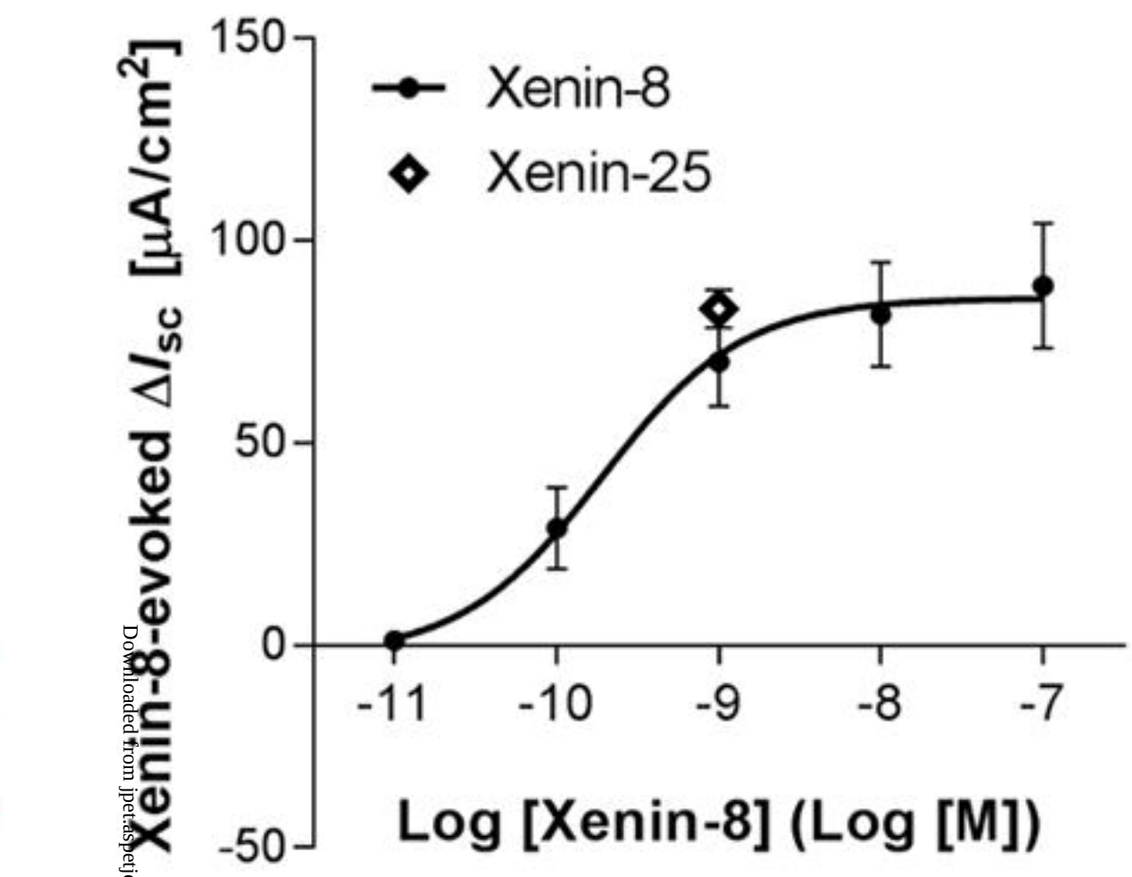
Table 2. Xenin-8-evoked I_{sc} increases in the presence or absence of neurotransmitter receptor antagonists or nitric oxide synthase inhibitor

	Vehicle [μA/cm^2]	**Treatment [μA/cm^2]**
Atropine	69.5 ± 7.1	58.7 ± 7.3
SB268262, 1 μM	88.1 ± 6.1	111.9 ± 26.3
SB268262, 10 μM		95.7 ± 26.6
VPAC1 antagonist	71.4 ± 15.1	92.1 ± 22.1
L-NAME	85.0 ± 20.6	111.2 ± 24.1

Fig 1

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.





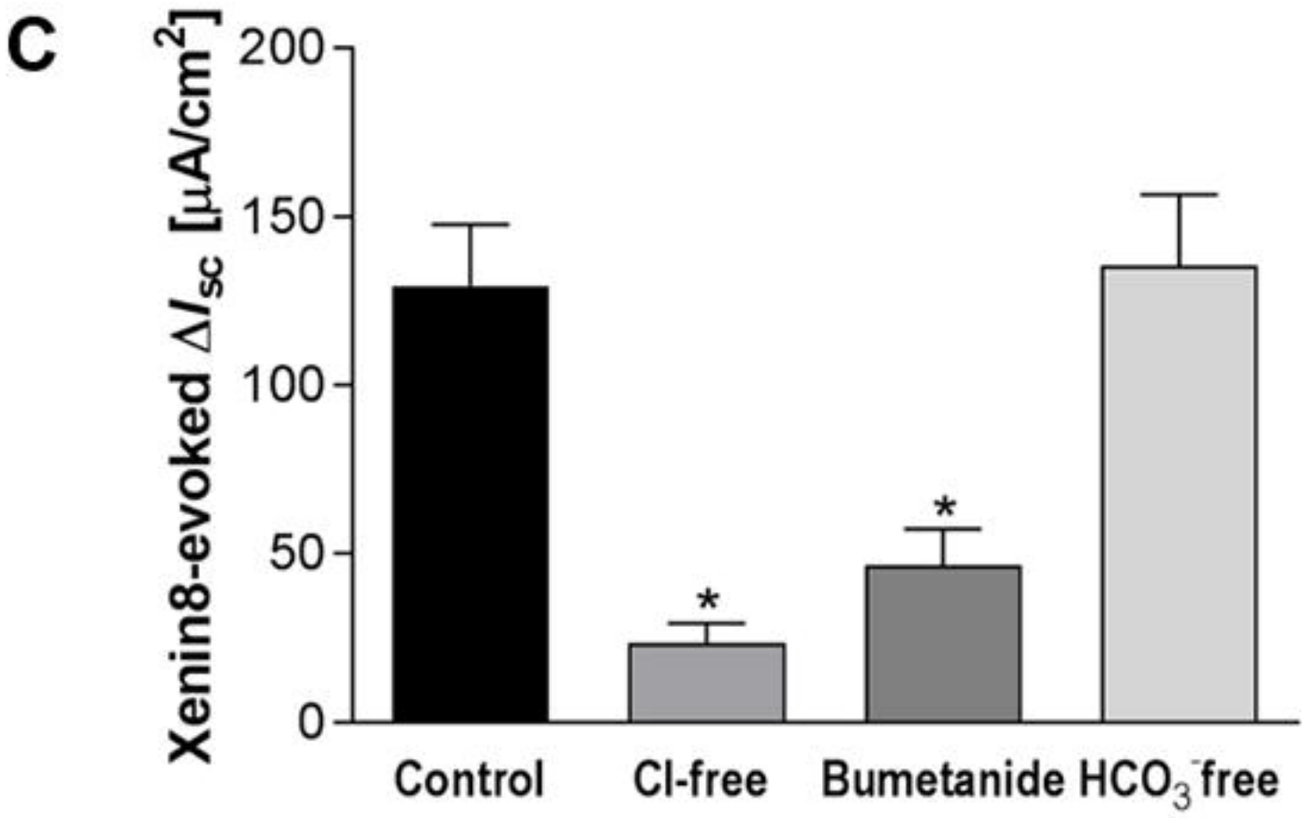


Fig 2

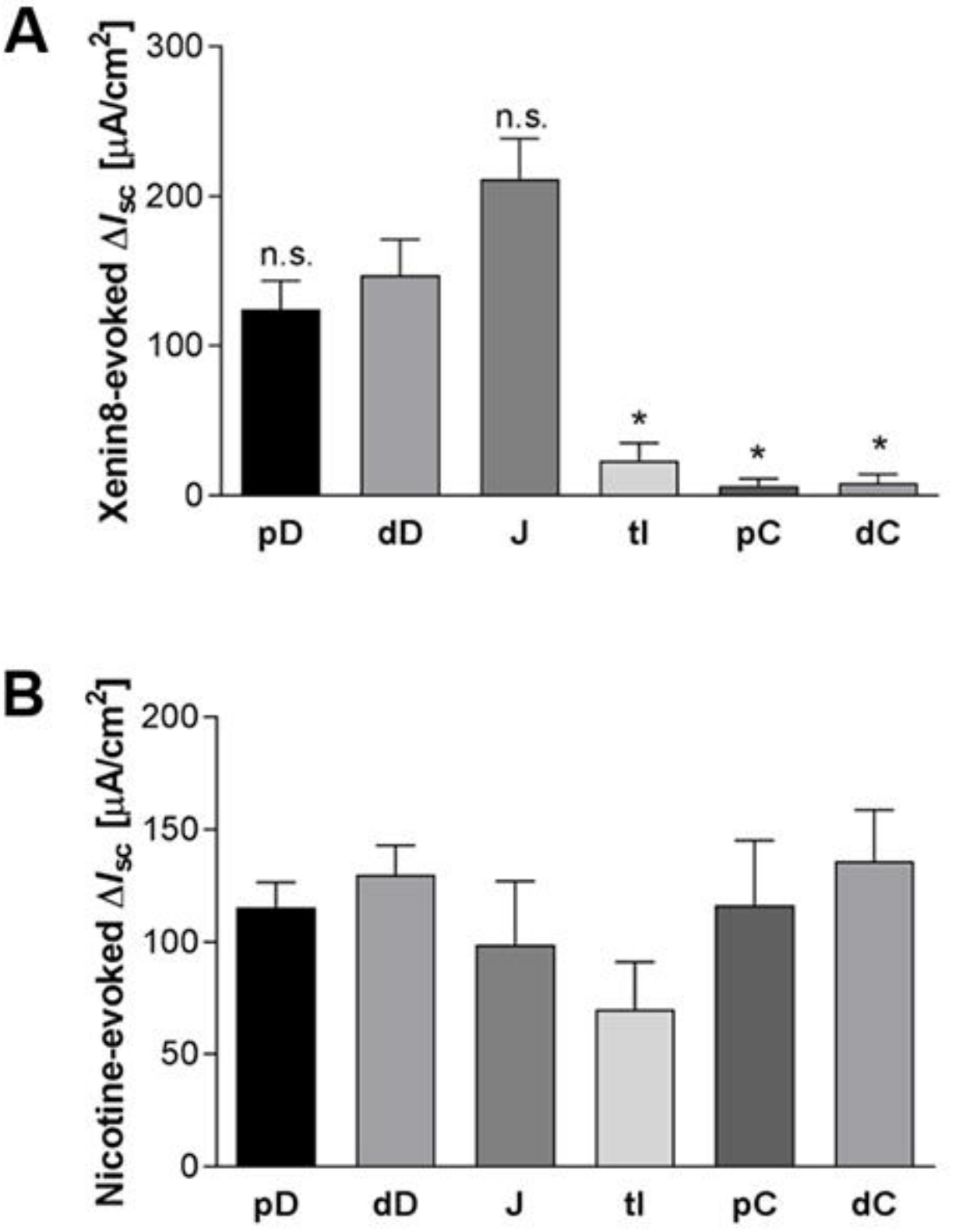

A
Xenin8-evoked ΔIsc [μA/cm²]
300
200
100
0
n.s.
n.s.
*
*
*
pD
dD
J
tl
pC
dC
B
Nicotine-evoked ΔIsc [μA/cm²]
200
150
100
50
0
pD
dD
J
tl
pC
dC

Fig 3

JPET Fast Forward. Published on January 23, 2017 as DOI: 10.1124/jpet.116.238485
This article has not been copyedited and formatted. The final version may differ from this version.

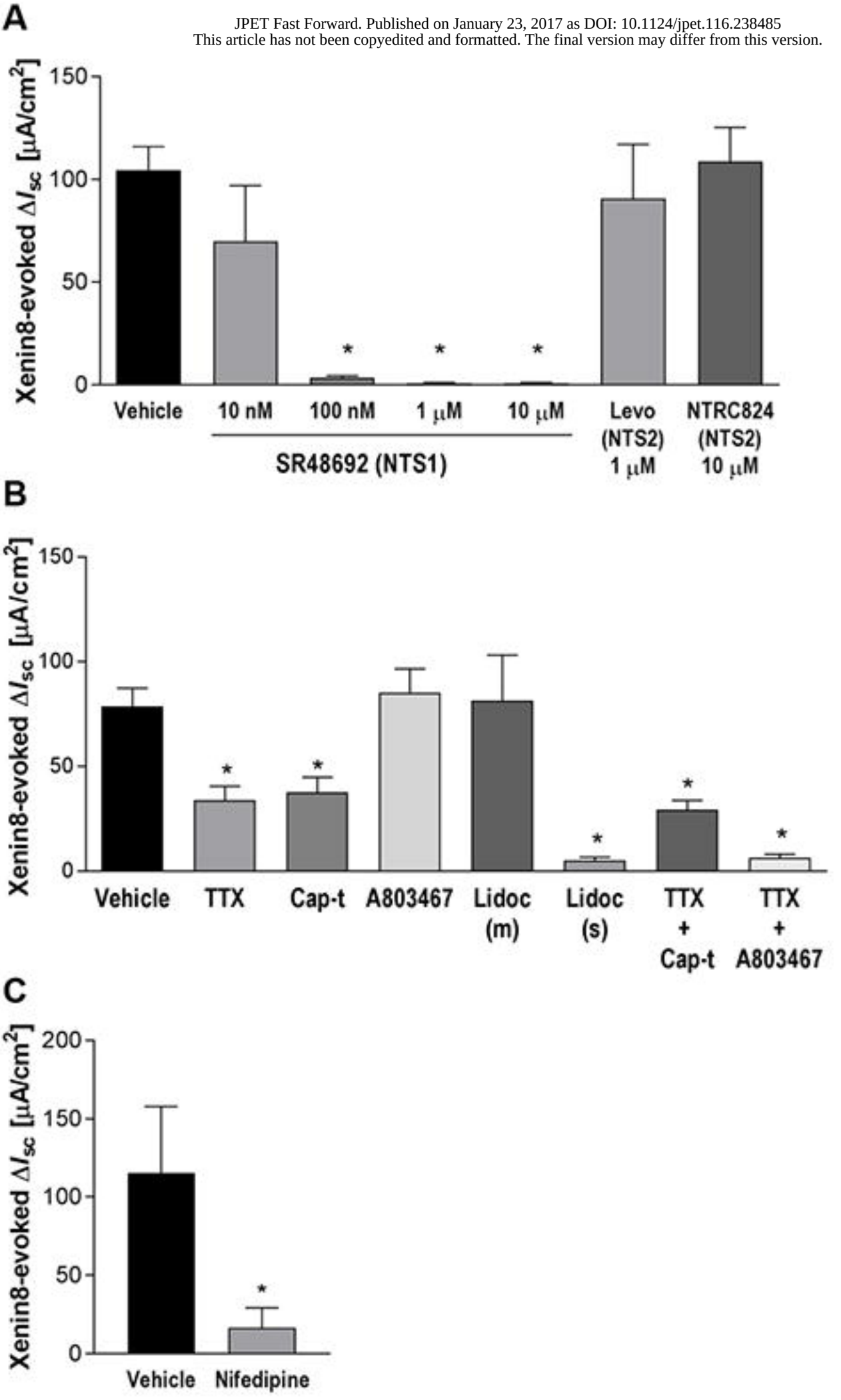
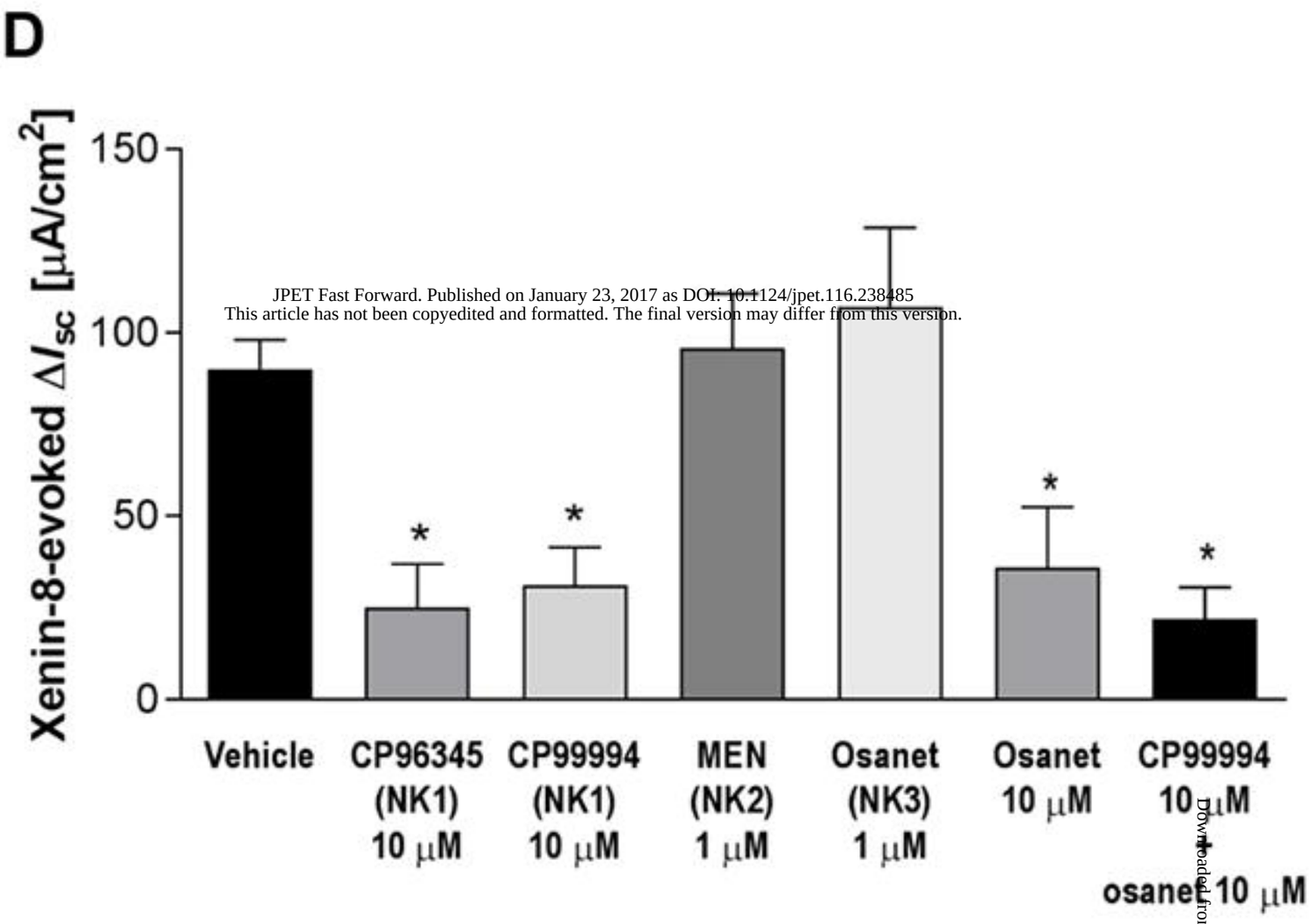
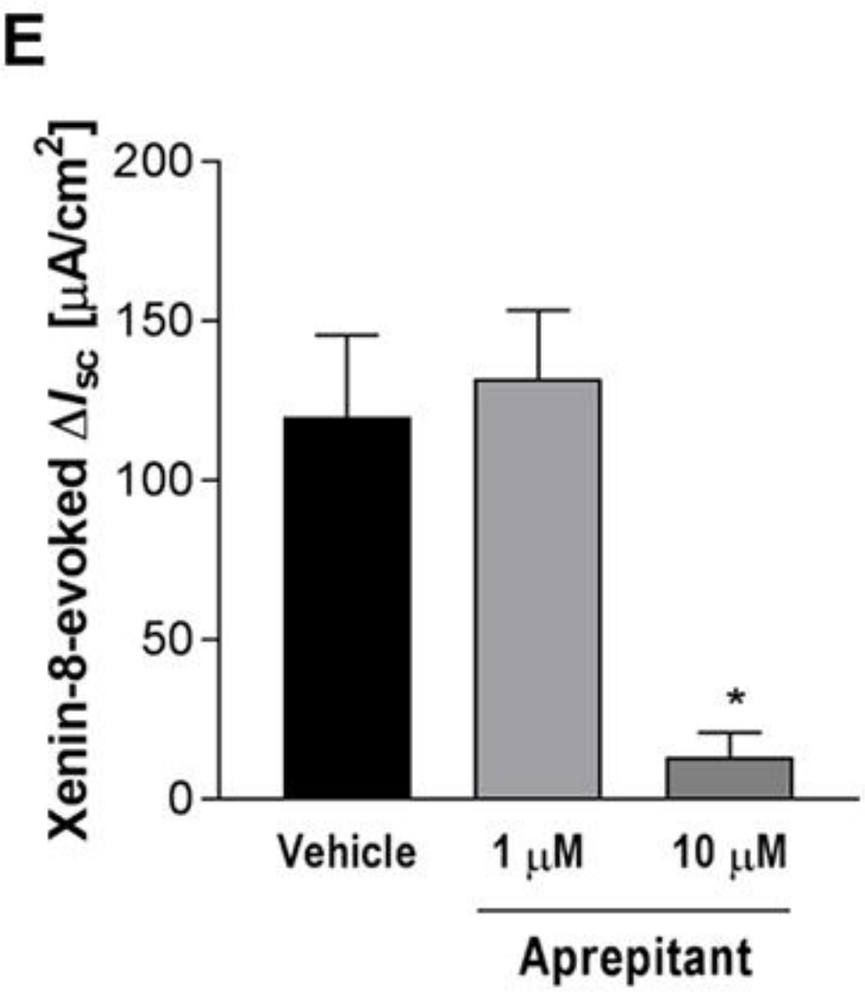
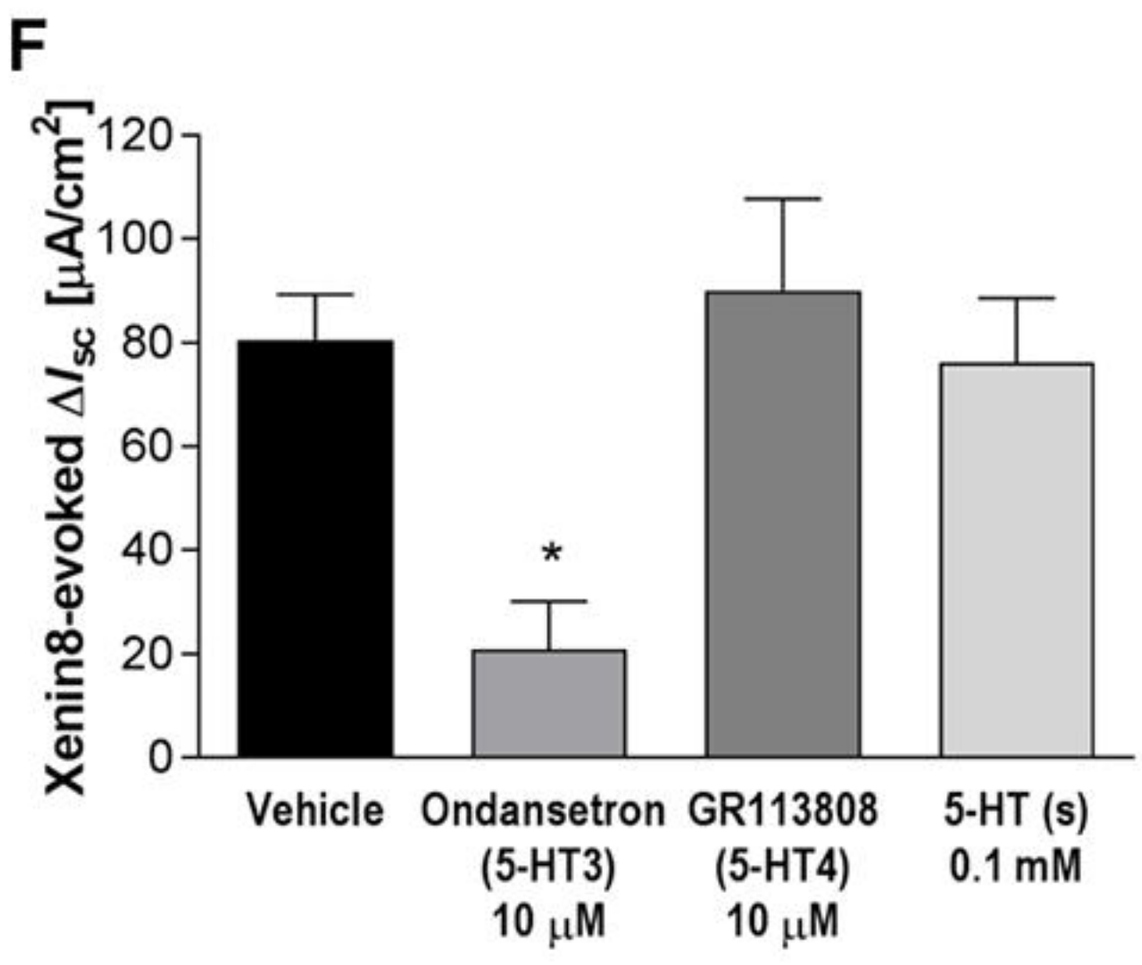


Fig 3







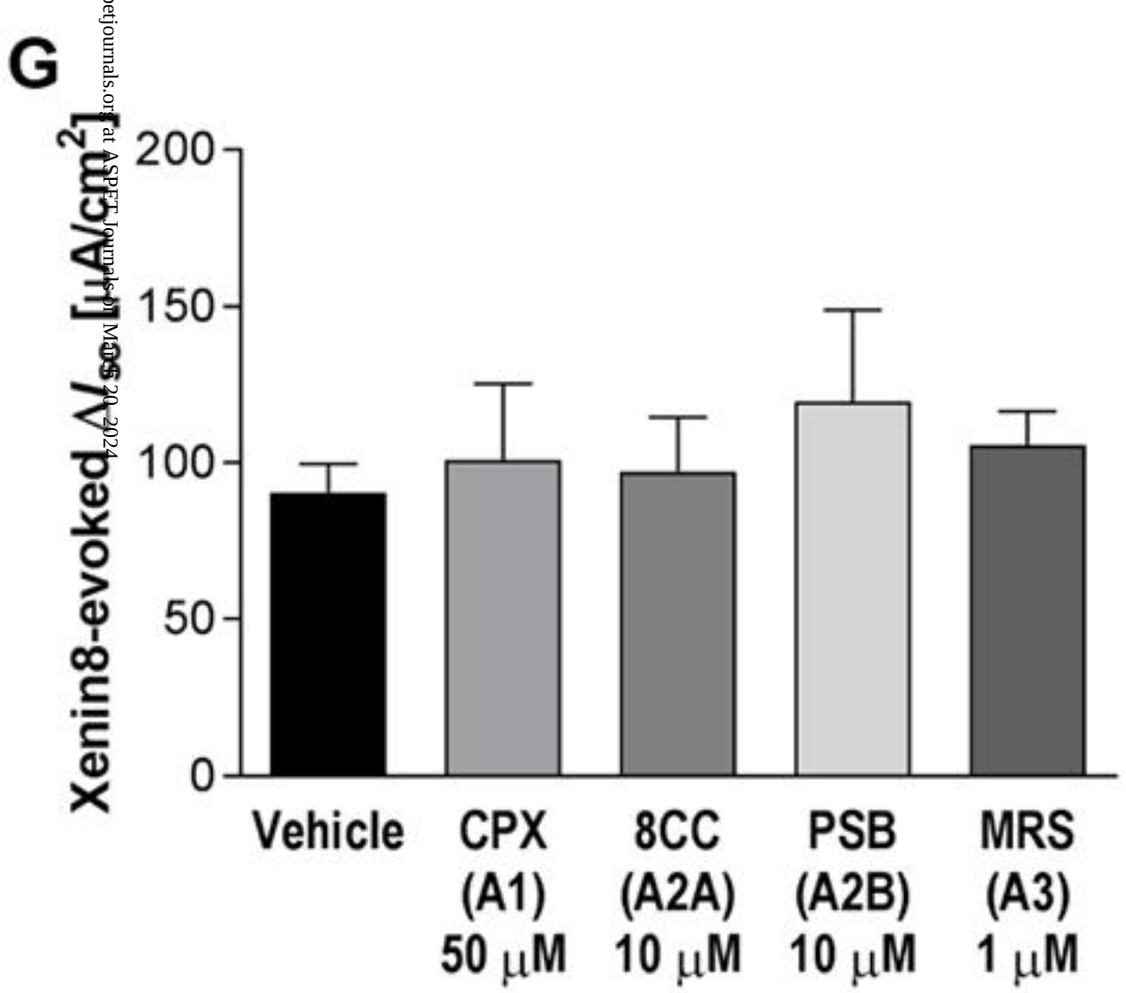


Fig 4

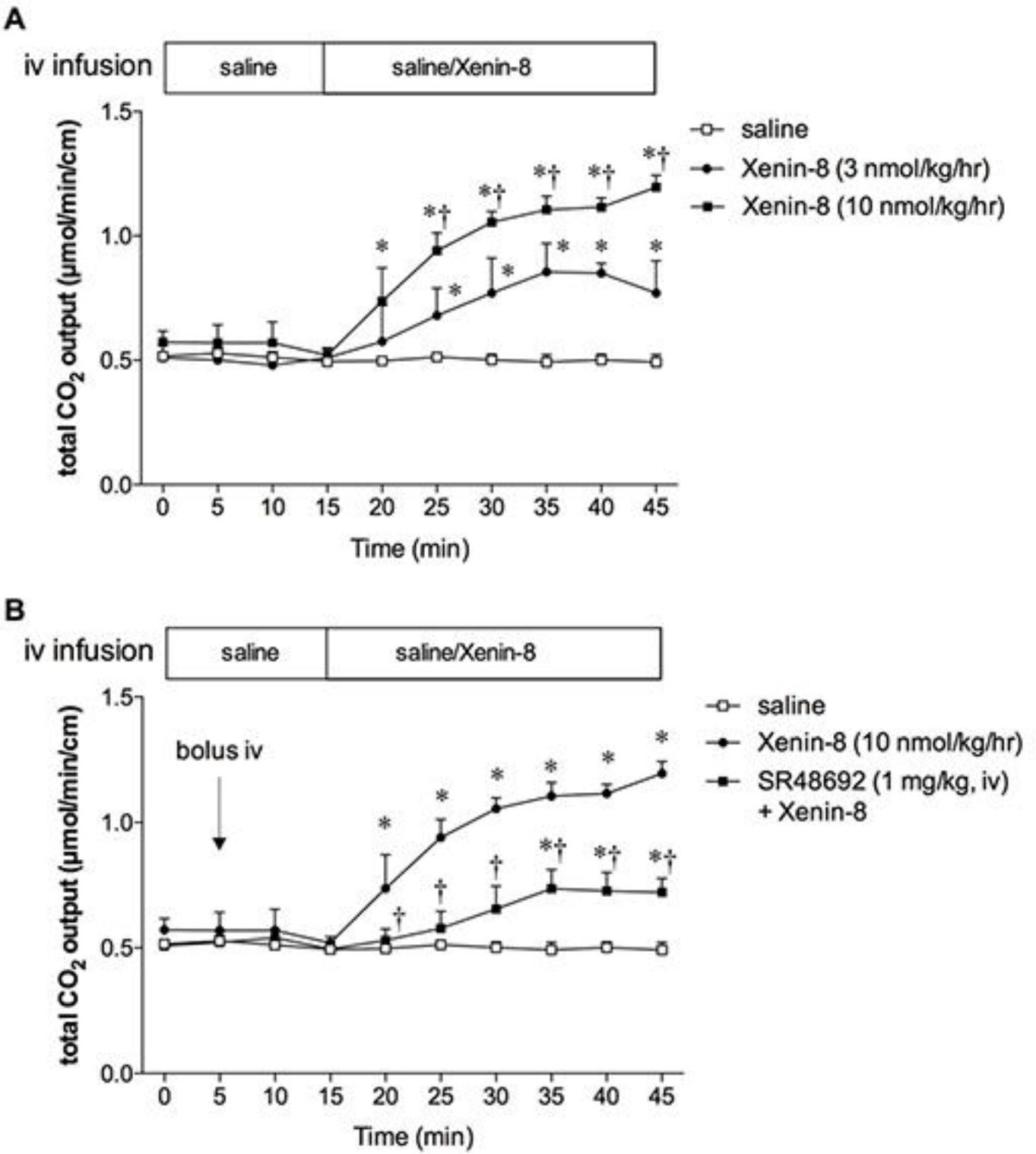

A
iv infusion
saline
saline/Xenin-8
saline
Xenin-8 (3 nmol/kg/hr)
Xenin-8 (10 nmol/kg/hr)
total CO2 output (µmol/min/cm)
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
Time (min)
B
iv infusion
saline
saline/Xenin-8
bolus iv
saline
Xenin-8 (10 nmol/kg/hr)
SR48692 (1 mg/kg, iv)
+ Xenin-8
total CO2 output (µmol/min/cm)
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
Time (min)

Fig 5

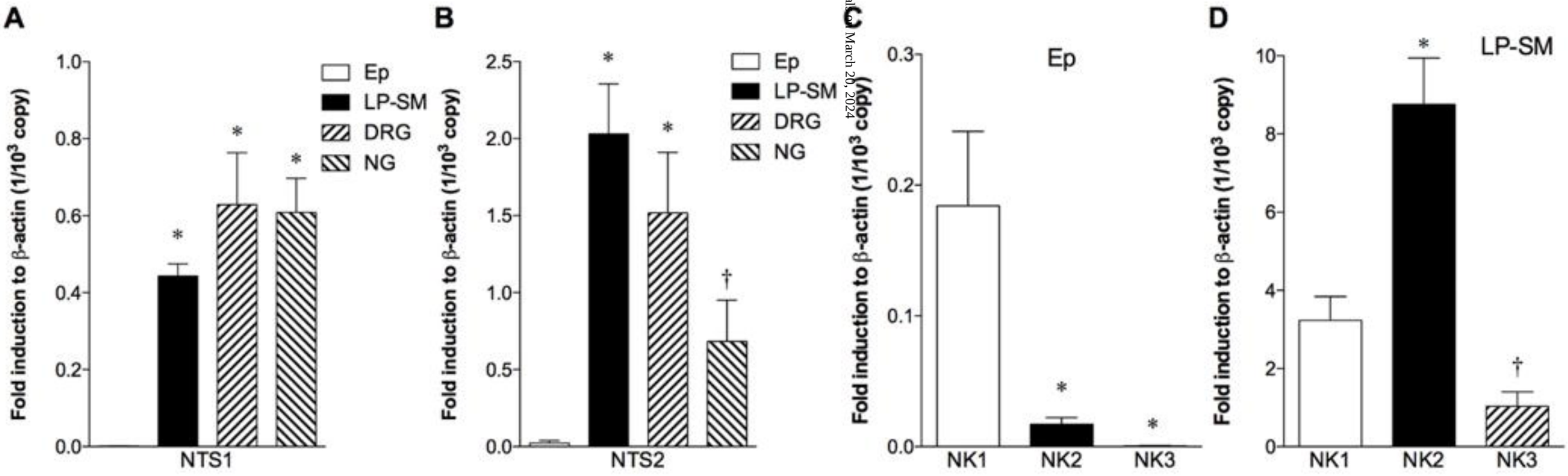
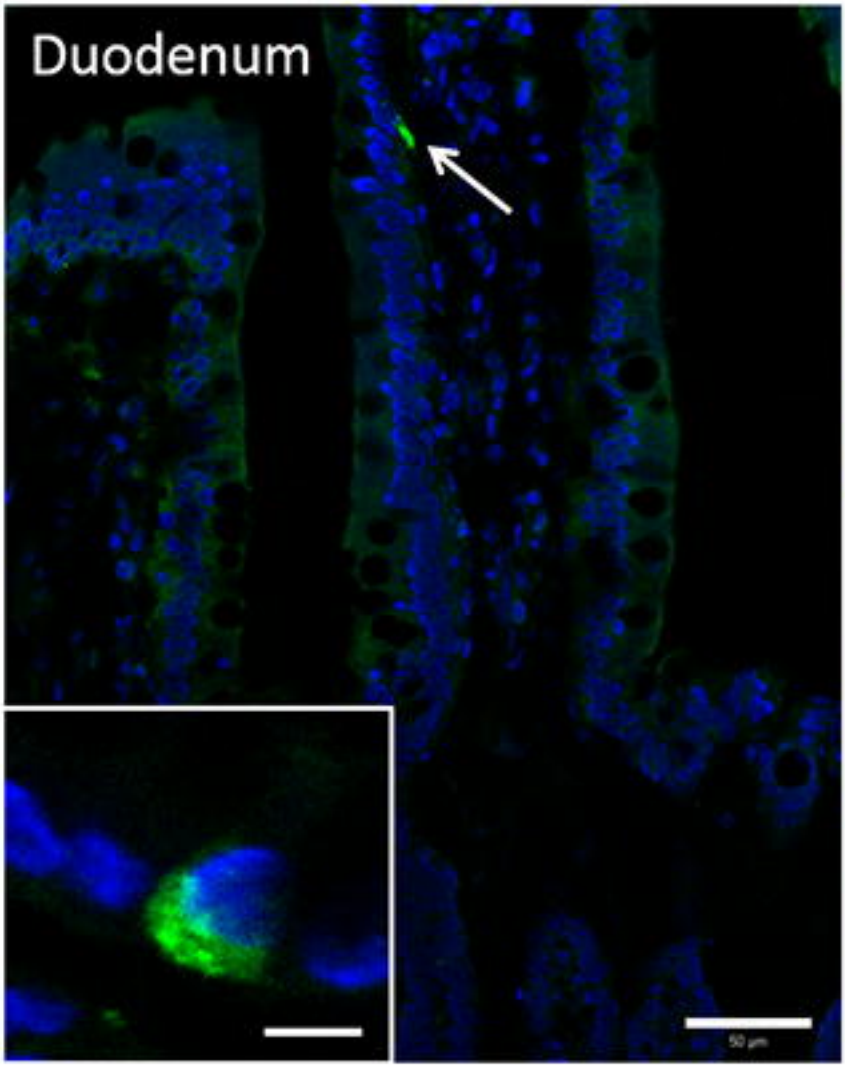
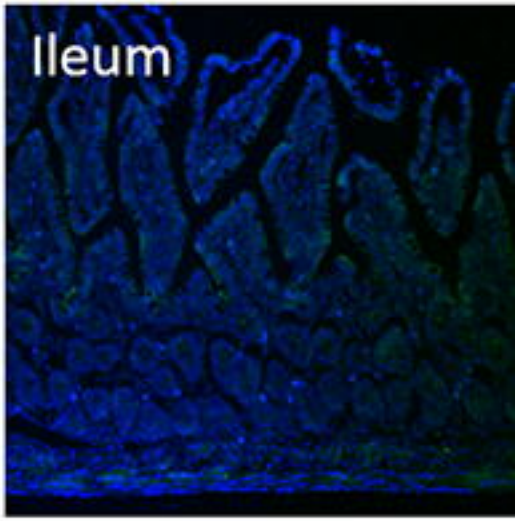


Fig 6





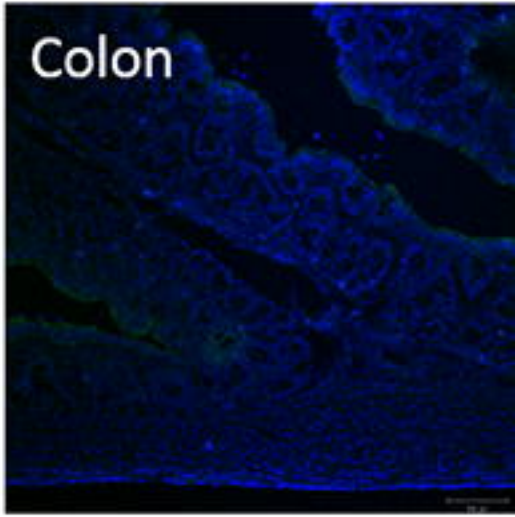
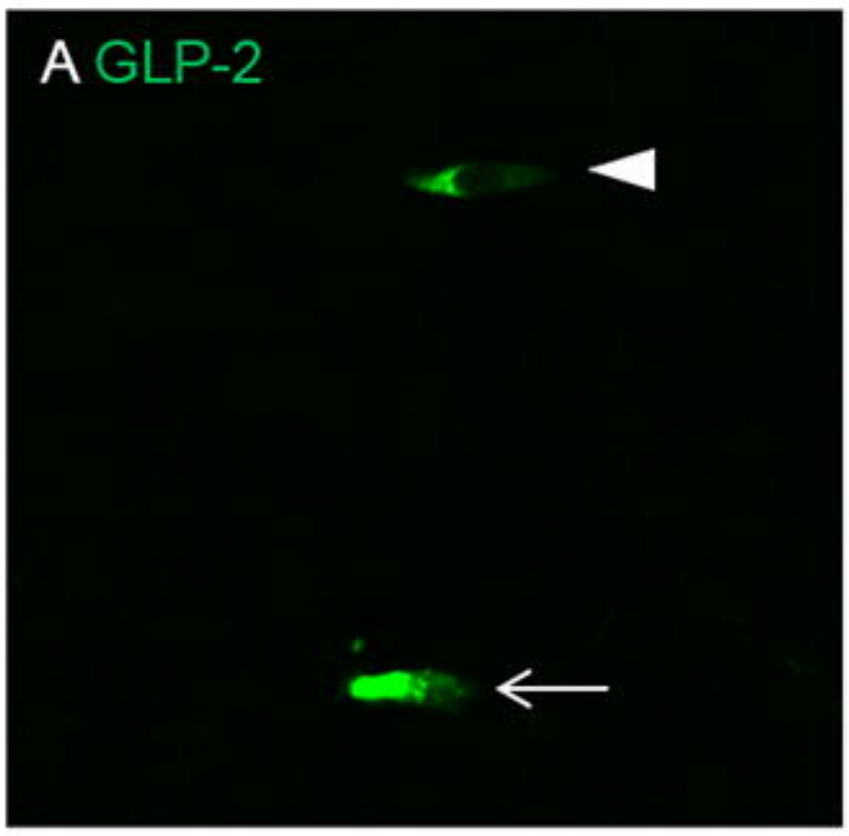
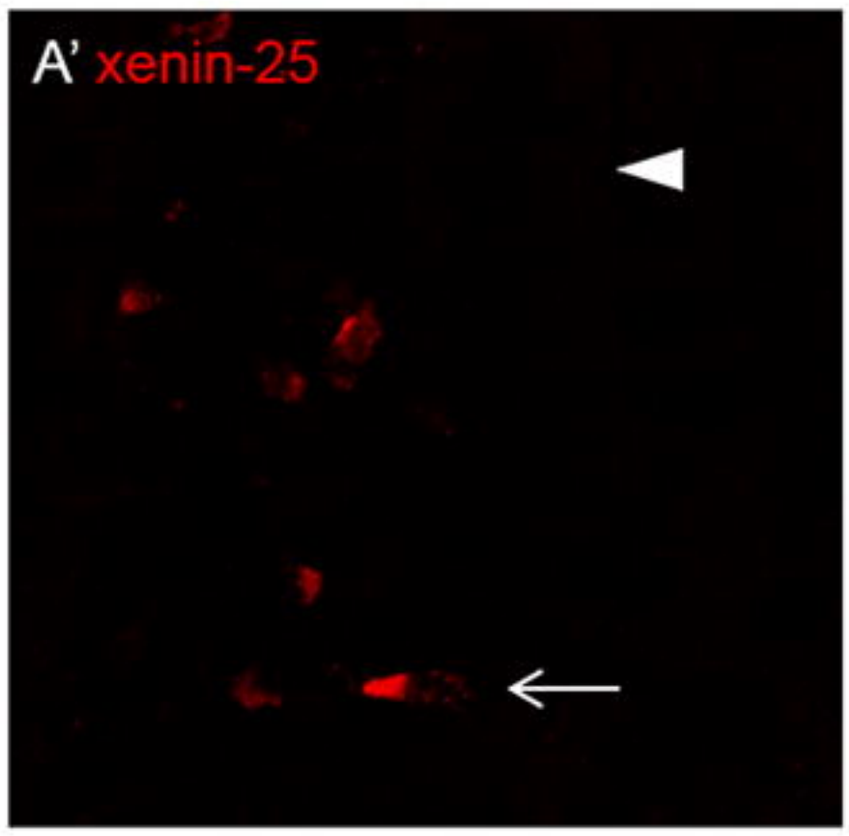
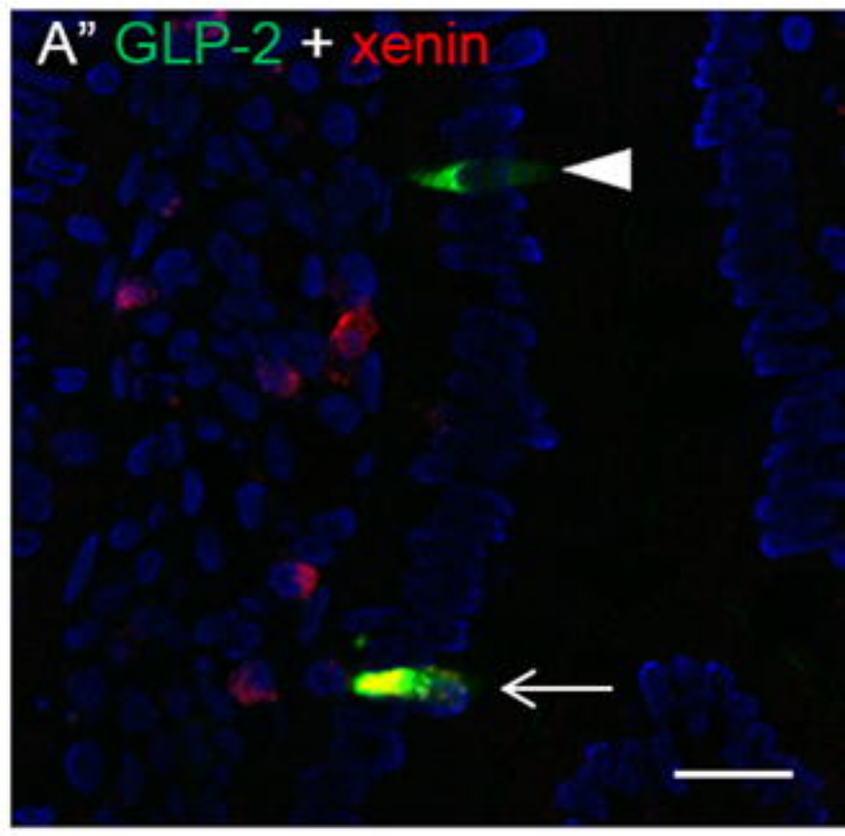
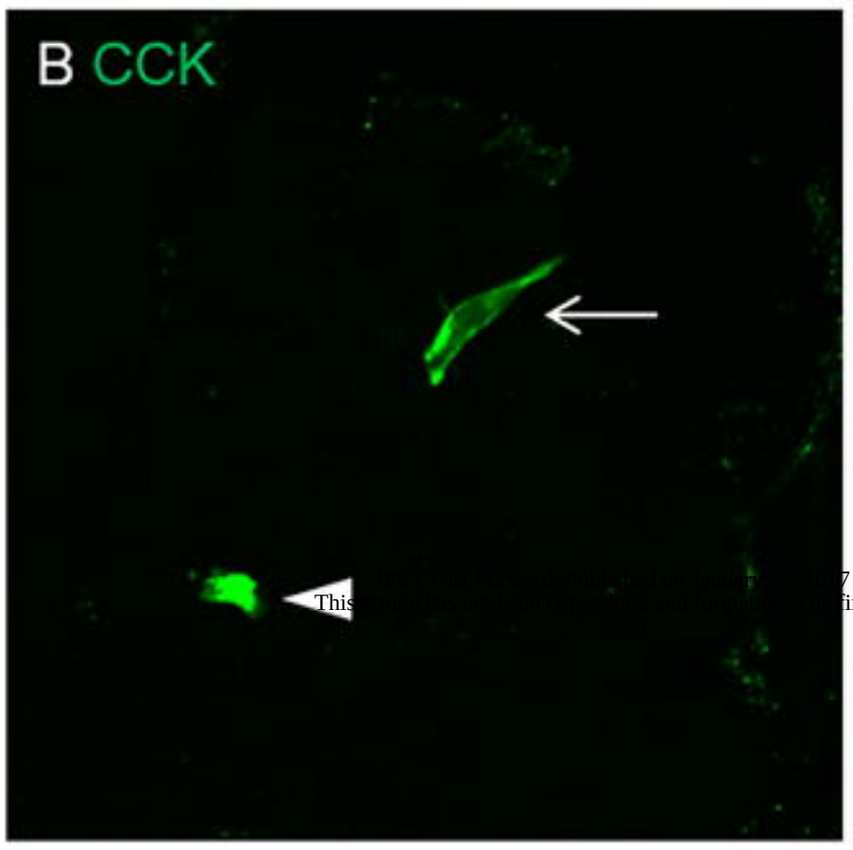


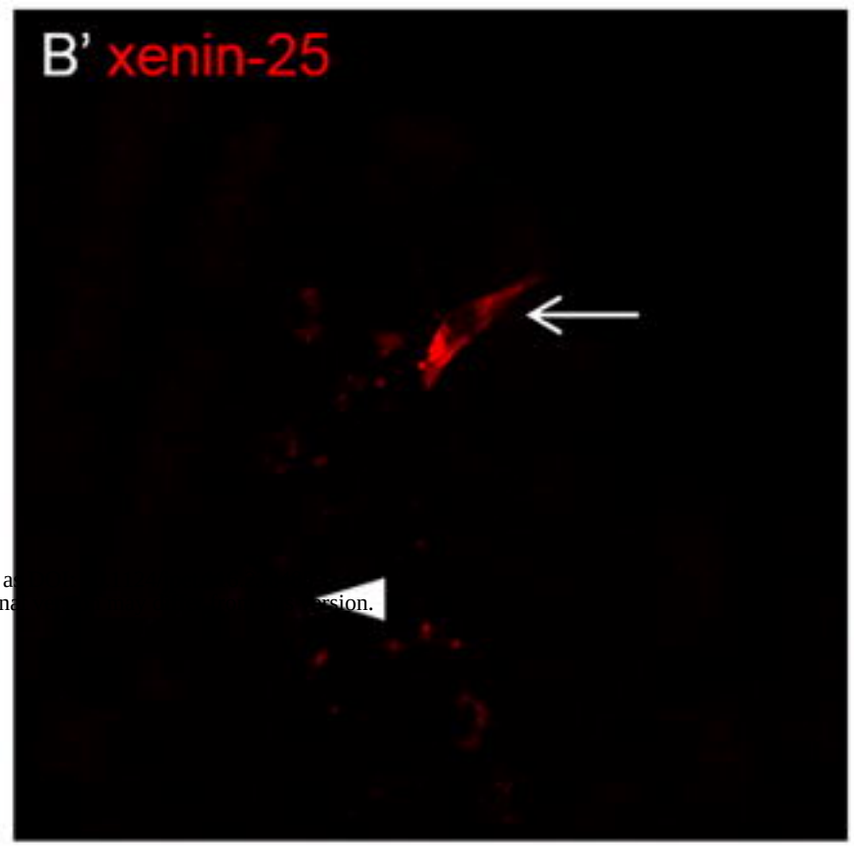
Fig 7

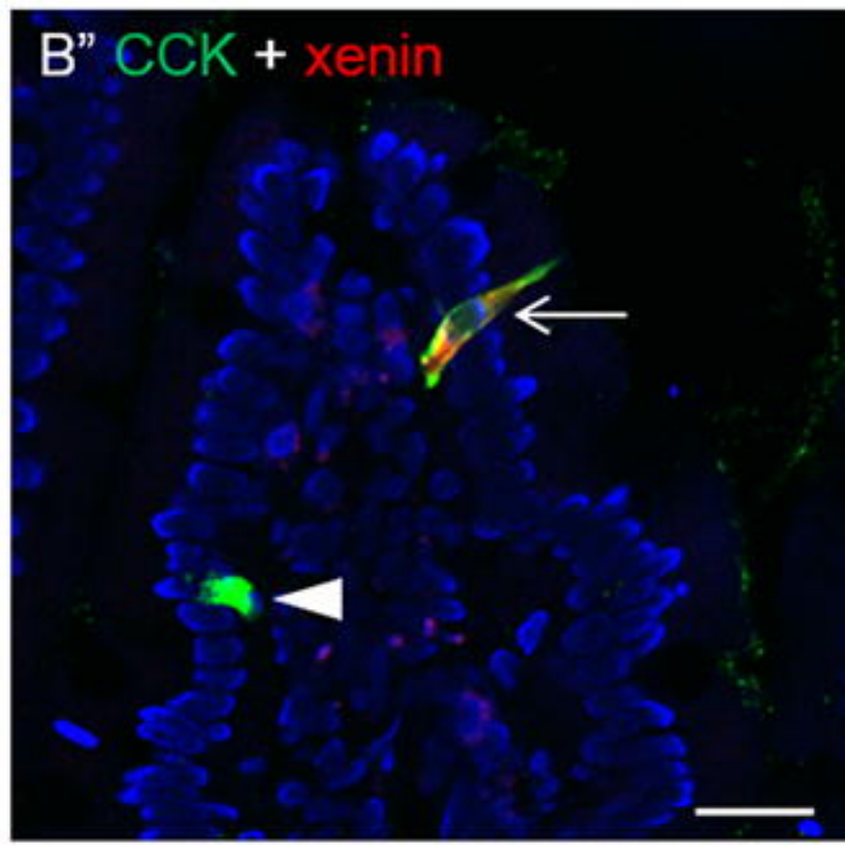

A GLP-2

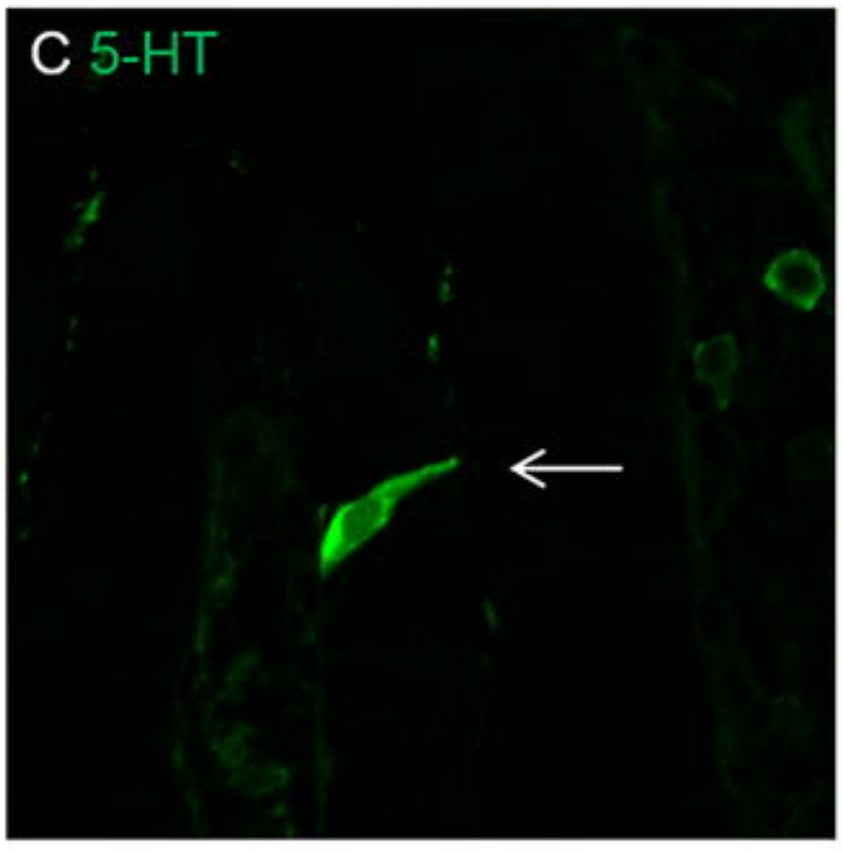

A' xenin-25

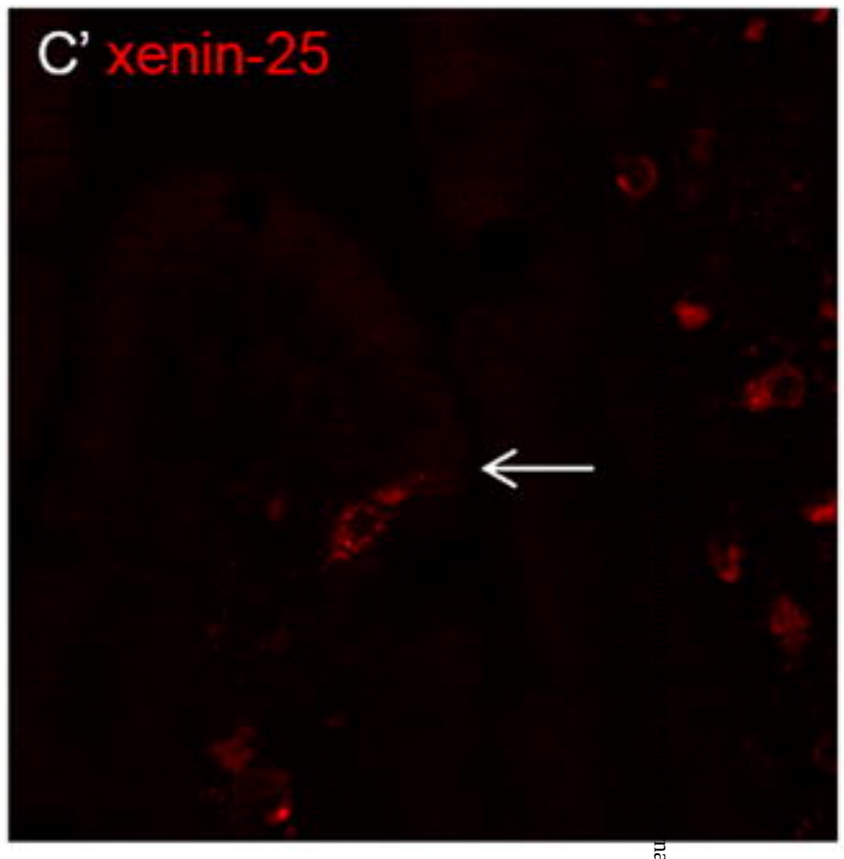

A" GLP-2 + xenin

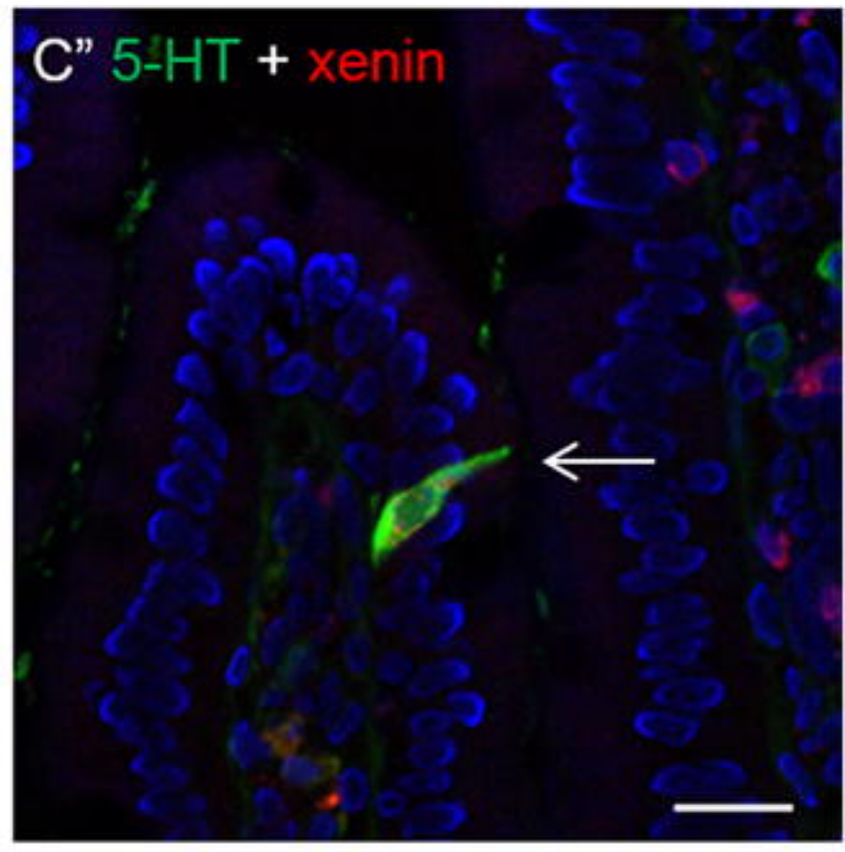

B CCK

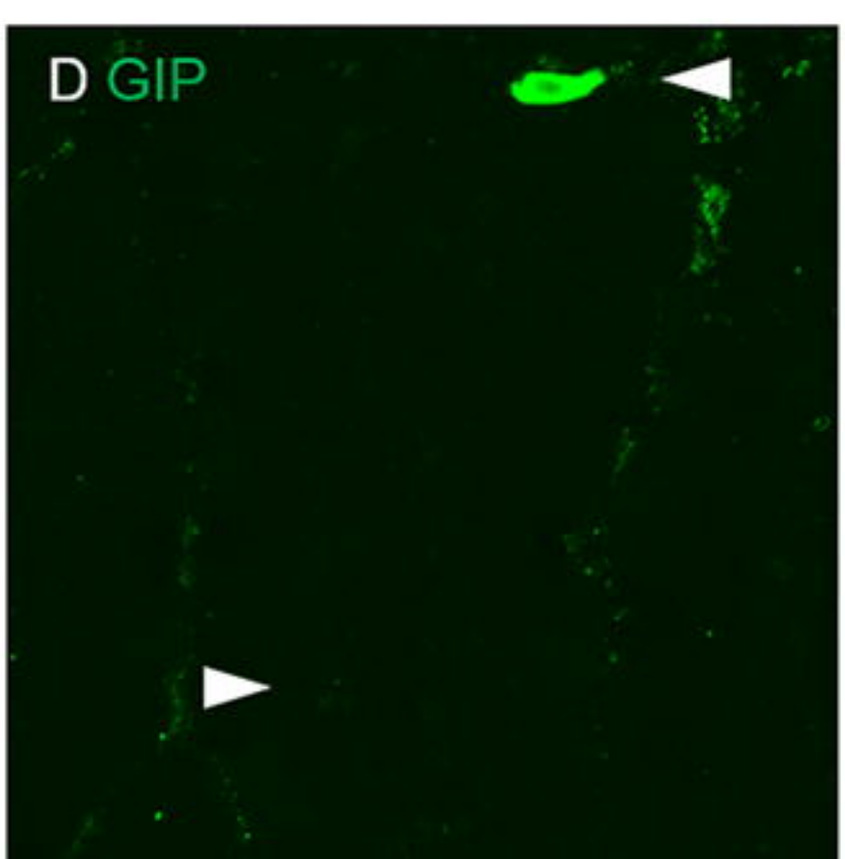

B' xenin-25

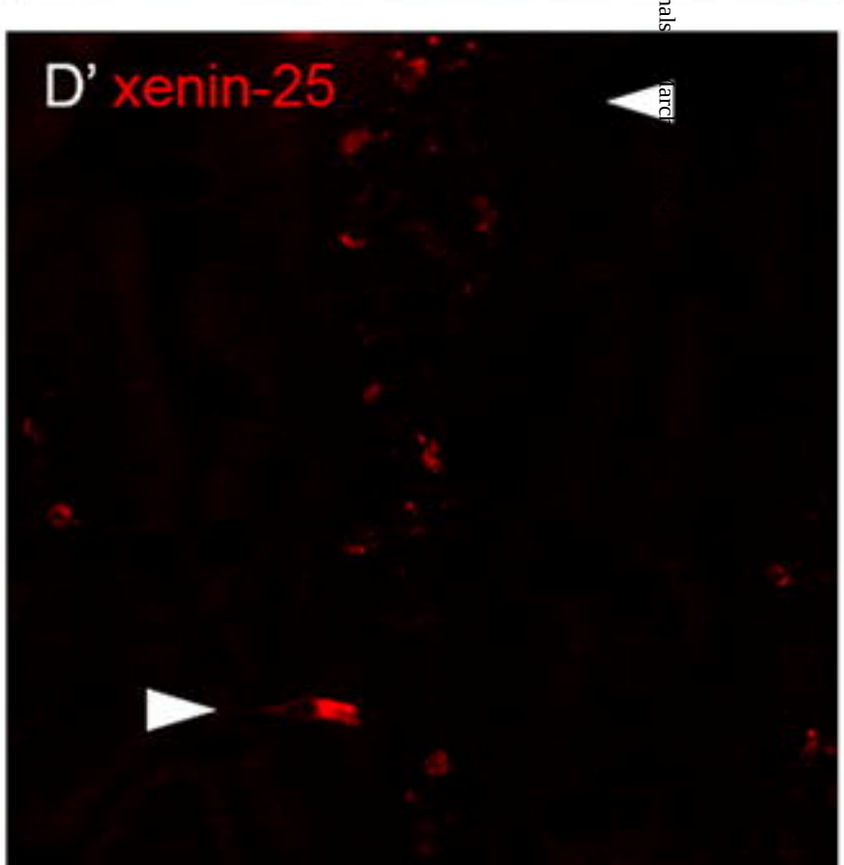

B" CCK + xenin

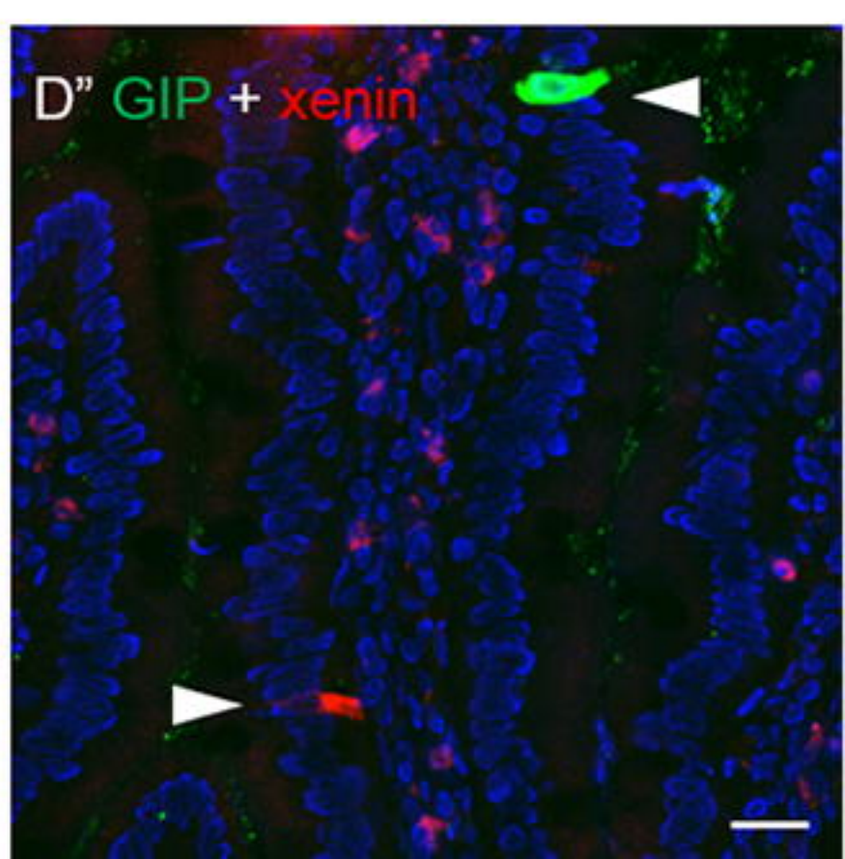

C 5-HT


C' xenin-25


C" 5-HT + xenin


D GIP


D' xenin-25


D" GIP + xenin