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Glial Derived Neurotrophic Factor modulates Enteric Neuronal Survival and Proliferation through Neuropeptide Y

Mallappa Anitha^{1,#}, Bindu Chandrasekharan^{1,#}, Joana R Salgado², Eric Grouzmann², Simon Mwangi¹, Shanthi V. Sitaraman¹, and Shanthi Srinivasan¹

¹Division of Digestive Diseases, Emory University, 615 Michael Street, Atlanta, GA 30322 ²Centre Hospitalier Universitaire Vaudois, Division de Pharmacologie et Toxicologie Lausanne, Switzerland

Abstract

Background and Aims— Glial Derived Neurotrophic factor (GDNF) promotes the survival and proliferation of enteric neurons. Neuropeptide Y (NPY) is an important peptide regulating gastrointestinal motility. The role of NPY on the survival and proliferation of enteric neurons is not known. We examined the effects of GDNF on the expression and release of NPY from enteric neurons and the role of NPY in promoting enteric neuronal proliferation and survival.

Methods— Studies were performed in primary enteric neuronal cultures and NPY knock out mice (*NPY*^{-/-}). GDNF-induced expression of NPY was assessed by RT-PCR, immunocytochemistry and ELISA. Using NPY-siRNA and NPY- Y1 receptor antagonist we examined the role of NPY in mediating the survival and proliferation effects of GDNF. Gastrointestinal motility was assessed by measuring gastric emptying, intestinal transit and isometric muscle recording from intestinal muscle strips.

Results— GDNF induced a significant increase in NPY mRNA and protein expression in primary enteric neurons, and the release of NPY into the culture medium. NPY (1 μM) significantly increased proliferation of neurons and reduced apoptosis. In the presence of NPY-siRNA and NPY-Y1 receptor antagonist or in enteric neurons cultured from *NPY*^{-/-} mice, GDNF-mediated neuronal proliferation and survival was reduced. NPY increased the phosphorylation of Akt, a downstream target of the PI-3-Kinase pathway. In *NPY*^{-/-} mice there were significantly fewer nNOS-containing enteric neurons compared to WT mice. *NPY*^{-/-} mice had accelerated gastric emptying and delayed intestinal transit compared to WT mice.

Conclusion— We demonstrate that NPY acts as an autocrine neurotrophic factor for enteric neurons.

Keywords

Enteric neurons; NPY; apoptosis; GDNF; proliferation; survival

Corresponding Author with complete address and email address: Shanthi Srinivasan, Division of Digestive Diseases, Emory University, Whitehead Research Building, Suite 246, 615 Michael Street, Atlanta, GA 30322, Tel. No. 404-727-5298, Email: ssrini2@emory.edu.

[#]These authors contributed equally to this work

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Introduction

The enteric nervous system (ENS) is a network of neurons that controls the motility and secretion of the gastrointestinal tract. Several growth factors influence the proliferation and survival of the ENS, including glial cell line-derived neurotrophic factor (GDNF) and neurturin^{1,2}. GDNF binds to its receptor Ret, a transmembrane tyrosine kinase receptor, which requires one of the four glycosylphosphatidylinositol (GPI)-linked co-receptors (GFR α 1–4) for its activation. GDNF/GFR α 1/Ret signaling is essential for the migration, proliferation, maturation and survival of ENS precursors during embryonic development^{3–8}. GDNF-deficient mice, GFR α 1-deficient mice or Ret^{-/-} mice have renal agenesis, total intestinal aganglionosis and defects in the sympathetic, parasympathetic and sensory nervous system^{9–14}.

Neuropeptide Y (NPY) is a neurotransmitter consisting of 36 amino acid residues, and is a member of the peptide family that also includes peptide YY (PYY) and pancreatic polypeptide (PP). PYY and PP are primarily synthesized and released from endocrine cells of the gut and pancreas, respectively, whereas NPY is widely distributed in the central and peripheral nervous system during development and adulthood^{15–18}. By embryonic day E-13, the entire length of the fetal murine bowel contains NPY-staining neurons¹⁹. NPY regulates gastrointestinal motility, feeding behavior and stimulation of cellular growth, including hypertrophy of cardiomyocytes and proliferation of vascular smooth muscle cells^{20–26}. In neural tissue, NPY promotes proliferation of neuroblasts in the olfactory epithelium, post-natal and adult dentate gyrus, and is protective against methamphetamine-induced neurotoxicity in the striatum^{27–29}. The biological effects of NPY are mediated by five G-protein coupled receptors (Y1, Y2, Y4, Y5 and Y6)³⁰. The NPY receptor subtypes are restricted to tissue-specific expression and account for the diverse action of NPY in different tissues. NPY/Y1 double labeling showed that NPY and its Y1 receptor subtype are co-localized within ganglion cells of the submucosal and myenteric plexes³¹. In addition, Y1-expressing neurons co-localize with the nNOS-expressing neurons. The Y1 receptor may act as an autoreceptor within the colonic gut wall³¹.

Certain growth factors like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) can modulate NPY expression^{32–35}. The effects of growth factors on the synthesis and release of peptide neurotransmitters such as NPY have not been studied in the ENS. In the present study, isolated embryonic enteric neuronal cultures were used to examine the effect of GDNF on NPY mRNA and protein expression and the release of NPY into the culture medium. The role of NPY in mediating GDNF-induced proliferation and survival of enteric neurons was further confirmed by using NPY-specific siRNA and a NPY-Y1 receptor antagonist, as well as neurons from NPY^{-/-} mice. Using NPY^{-/-} mice, we assessed the effects of NPY on gastric emptying and intestinal transit in whole animals. We also evaluated the expression of neuronal subtypes in the myenteric plexus and enteric neuronal-mediated intestinal muscle relaxation in longitudinal and circular intestinal muscle strips.

Materials and Methods

Reagents

Collagenase and dispase were obtained from Worthington Biochemical. Tyramide signal amplification kits were obtained (TSA-FITC or TSA-Cy3) from NEN Life Science Products). NPY and BIBP3226 (NPY-Y1 receptor antagonist) from BACHEM, Omniscript Reverse Transcription Kit from Qiagen, Oligo (dT)_{12–18} primers and Lipofectamine 2000 from Invitrogen, NPY and GAPDH primers from IDT, Taq DNA polymerase from Promega, SYBR Green Supermix from Bio-Rad, control Cyclophilin B and NPY-specific siRNA from Dharmacon, and methylene blue from Mayne Pharma. All chemicals not included above were purchased from Sigma. GDNF was produced as previously described³⁶.

Antibodies

The antibodies to p75NTR, NPY, peripherin, nNOS (neuronal Nitric Oxide Synthase), ChAT (Choline Acetyl transferase) were obtained from Chemicon International. Antibodies to Cleaved caspase-3, phospho-Akt and Akt from Cell Signaling, antibody to BrdU from Molecular Probes; antibody to NPY monoclonal (NPY02 and NPY05) and monoclonal anti-C flanking peptide of NPY (CPON01) were from Dr. Eric Grouzmann, antibody to PYY was a gift from Dr. Roger Corder, William Harvey Research Institute.; antibody to β -actin from Sigma; Secondary antibodies including donkey anti-rabbit biotin, donkey anti-mouse biotin and peroxidase-conjugated streptavidin were from Jackson ImmunoResearch Laboratories, Inc.

Primary culture and magnetic bead immunoselection of p75 expressing cells

p75-expressing cells were isolated from stomach, small and large bowel of E14.5 Sprague-Dawley rat embryos (10–12 embryos) or E13.5 mouse embryos (WT and *NPY*^{-/-} mice) by magnetic bead immunoselection with a monoclonal antibody to low affinity NGF receptor (p75NTR, 10 μ g/mL) as previously described^{1,37}. Following immunoselection, 12,500 trypan blue-excluding cells were resuspended in 125 μ l of modified N2 medium³⁷ and plated into a single well of an 8-well slide coated with poly-D-lysine and laminin. The N2 medium contains DMEM/F12 with the addition of sodium selenite (30 nM), putrescine dihydrochloride (100 μ M), progesterone (20 nM), insulin (5 μ g/mL), transferrin (10 μ g/mL), fetuin (0.1 mg/mL), and bovine serum albumin (1mg/mL). An additional 125 μ l of medium were added to each well with the desired factors. Cells were grown in this medium at 37°C in a humidified tissue culture incubator containing 5% CO₂. Unless otherwise stated, cells were grown in the presence of GDNF (100 ng/mL). As described by other investigators³⁸, our immunoselection method yielded a population of 80% Ret-positive cells after 24 h of culture. The p75- negative fraction had 2–3% Ret-positive cells. To investigate the effect of NPY on neuronal cell survival and proliferation, cells were grown in the presence of NPY (1 μ M) for 24 or 48 h.

RT-PCR

Immunoselected rat embryonic enteric neurons were cultured for 24 h in N2 medium in the presence or absence of recombinant rat GDNF (100 ng/mL) in poly-D-lysine/laminin-coated 6-well plates. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). 0.92 μ g of total RNA was used to synthesize first strand cDNA using the Omniscript Reverse Transcription Kit and Oligo (dT)_{12–18} primers according to recommended procedure. The NPY and GAPDH primers used for semi-quantitative and real-time PCR quantification of the NPY and GAPDH messages have been described previously³⁹. For semi-quantitative PCR, 25 μ l reactions were set up comprising of 1x Taq DNA polymerase buffer, 1.5 mM MgCl₂, 200 μ M dNTP mix, 0.4 μ M each of the NPY or GAPDH oligonucleotide primers, 1.25 U Taq DNA polymerase and 1 μ l first-strand cDNA. The reactions were heated for 3 min at 95°C, and subjected to 30 cycles of PCR amplification, each cycle consisting of 30 sec at 95°C, 30 sec at 59°C and 45 sec at 72°C. A final extension for 7 min at 72°C was performed before cooling to 4°C. Two thirds of each reaction were analyzed on a 2% agarose gel stained with ethidium bromide and the amplified products visualized by UV transillumination.

Real-time PCR

For real time PCR, amplifications were detected using SYBR Green I in reactions performed with the iCycler iQ real-time PCR Detection system (Bio-Rad). Each 25 μ l PCR reaction contained 1x iQ SYBR Green Supermix, 0.1 μ M of each primer pair and 1 μ l of each cDNA. PCR reactions were started with an initial denaturation at 95°C for 5 min followed by 45 cycles each consisting of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 45 sec. A melting curve analysis was performed after amplification was completed.

NPY mRNA was then normalized to the respective control GAPDH amounts and presented as a ratio under different conditions.

Neuropeptide Y ELISA

Enteric neurons were cultured in 6-well plates for 48 h in N2 medium in the presence or absence of GDNF (100 ng/mL). The medium was aspirated from the cells and stored in minisorp tubes containing 1 μ l of Tween 20 per mL of medium. The medium was stored at -80°C until the measurement of NPY or PYY.

Microplates (Minisorp, Nunc) were coated for 16 h at 4°C with 50 ng of the anti-NPY monoclonal antibody NPY02 or 150 ng of protein A-purified anti-PYY polyclonal antibody diluted in 50 mM Tris pH 7.5. The plates were washed four times with Tris buffer containing 0.08% Tween-20, 5% lactose, and 0.5% casein. NPY/PYY standards were added to the wells. After 16 h incubation at room temperature, the plates were washed with Tris buffer containing 0.1% Tween-20, followed by 100 μ l of alkaline phosphatase-conjugated NPY-05 diluted 1/4000 with Tris buffer containing 0.1% Tween-20 and 5% non-fat dry milk. The plates were then incubated for 7 h at room temperature and washed four times with Tris buffer containing 150 mM NaCl before the addition of 50 μ l of substrate. Forty-five minutes later, 50 μ l of the amplifier were added to each well according to the immunoselect kit (Gibco, MD) recommendations. Optical densities were measured kinetically at 492 nm using a Molecular Devices microplate reader (Sunnyvale, CA). For NPY the limit of quantification of the assay is 25 attomoles/well or 0.25 pM. The assay is specific for amidated NPY and does not cross-react with PYY and PP at concentrations up to 10 nM⁴⁰. For PYY the limit of quantification of the assay is 0.5 fmole/well or 5 pM. The assay is specific for amidated PYY and does not cross-react with PYY (13–36) and NPY at concentrations up to 1 nM.

Immunocytochemistry

Enteric neurons were cultured according to the conditions described in individual experiments. Cells were fixed (4% p-formaldehyde, 30 min), permeabilized (0.1% Triton X-100, 0.1% sodium citrate in PBS, 2 min on ice), blocked (10% NDS or 5% BSA) and incubated with primary antibody in 1% NDS or 1% BSA: NPY, 1:1000; cleaved caspase-3, 1:2500; peripherin, 1:100; nNOS, 1:500; ChAT, 1:100. Secondary detection was performed with either a FITC-conjugated donkey anti-mouse antibody (ChAT) or in conjunction with Cy3-TSA/FITC-TSA tyramide amplification kit in certain cases. As a negative control, the primary antibody was omitted in certain wells. At least four wells (Two hundred cells/well) were scored in a blinded fashion for each condition in each of the experiments, and the experiment was repeated three times.

BrdU staining

Enteric neurons treated with vehicle, NPY or GDNF in the presence or absence of either NPY-Y1 receptor antagonist (BIBP3226, 1 μ M) or LY294002 (15 μ M) were maintained in culture for 48 h. After 24 h of culture, BrdU (10 μ M) was added to the medium. Twenty-four hours later the cells were fixed with ethanol-fixative (3 volumes of 50 mM glycine, pH 2.0, and 7 volumes of absolute ethanol) for 45 min at RT followed by PBS wash. Peripherin staining was performed in-conjunction with TSA-Cy3 kit. Cells were denatured with 4M HCl for 15 min at RT and washed with PBS to neutralize the pH. Blocking with 0.5% BSA and 0.1% Tween 20 in PBS for 30 min at RT was followed by incubation with anti-BrdU (FITC-linked) antibody in blocking buffer for 1 h at 37°C in a humid chamber. Two hundred peripherin⁺ enteric neurons/well were scored in a blinded fashion to determine the percentage of BrdU⁺/peripherin⁺ cells. At least four wells were scored for each condition and the experiment was repeated four times. To determine the total number of cells and neurons, we counted the number of DAPI⁺ and peripherin⁺ cells per well in a blinded fashion.

TUNEL staining

To visualize apoptotic cells, slides were washed in PBS and then incubated with TUNEL reaction mixture (Roche) in a humidified chamber for 1 hr at 37°C in the dark. Two hundred TUNEL⁺ neurons/well were scored in a blinded fashion to determine the percentage of apoptotic cells. At least four wells were scored for each condition and the experiment was repeated three times.

Western Blotting

Western blotting was performed according to standard methods as previously described⁴¹. A semi-quantitative measurement of the band intensity was performed using the computer software *Scion Image* (Maryland, USA) and expressed as a ratio of band intensity with respect to the loading control.

Cleaved caspase-3

Cell lysates were obtained from vehicle and NPY-treated cells and approximately 15 µg of protein/lane were loaded onto a 12% SDS-PAGE gel. β-actin was used as a loading control. Western blotting was performed using an antibody specific for cleaved caspase-3.

pAkt

Isolated enteric neurons cultured for 24 h in the presence of vehicle and washed with Krebs buffer for 1 h were treated with vehicle, NPY (1 µM) or GDNF (100 ng/ml) in the presence or absence of LY294002 (15 µM) for 30 min in Krebs buffer. Cell lysates were obtained and 15 µg of protein/lane were loaded onto a 12% SDS-PAGE gel. Phospho-Akt was probed by using phospho-Akt (Ser473) polyclonal antibody. As a measure of equal protein loading, total Akt was probed using Akt polyclonal antibody.

nNOS

Tissue homogenates were obtained from the stomach and colon of WT and *NPY*^{-/-} mice using lysis buffer containing protease inhibitors. Twenty micrograms of total protein were loaded onto a 4–20% SDS-PAGE gel. Western blot was performed using an nNOS specific antibody. β-actin was used as a loading control.

siRNA Transfection

Enteric neurons were transfected with control Cyclophilin B/NPY-specific siRNA (5 pmol/well) (Dharmacon) and cultured for 24 h in the presence of vehicle, followed by 24 h in the presence of vehicle or GDNF. Transfection was performed as per manufacturer's recommendation. Cells were fixed and assessed for apoptosis by the TUNEL/Peripherin double-labeling method. For the assessment of proliferation BrdU (10 µM) was added to the medium 24 h prior to fixation of the cells. Proliferation was quantitated by the Peripherin/BrdU double-labeling method. To assess the percentage of nNOS and ChAT neurons we stained the transfected slides for peripherin/nNOS or peripherin/ChAT and determined the number of nNOS⁺ or ChAT⁺ neurons in two hundred peripherin⁺ neurons per well. Knock down of NPY by NPY siRNA was verified by staining the cells for NPY/Peripherin. Cells were scored for the percentage of NPY⁺ neurons as a semi-quantitative measure of siRNA knock down.

NPY knock out mice (*NPY*^{-/-})

All animal studies were approved by the Emory University Institutional Review Board. WT and *NPY*^{-/-} mice were obtained from Charles River. Experiments were performed in four week old male mice. The WT and *NPY*^{-/-} mice were maintained on a 129S1/SvImJ background. The WT mice were litter and age matched. In the *NPY*^{-/-} mice, the NPY gene was disrupted

in embryonic stem cells by homologous recombination using a targeting vector in which exon 2, encoding the signal peptide and 35 amino acids of NPY, was replaced by the lacZ reporter gene and a neomycin-resistance cassette⁴². Mutant mice are born at approximately the expected frequency and are viable through adulthood. The mice do not have gross anomalies in the brain or other organs. Growth of mutant mice occurs at a normal rate, and both male and female mutants are fertile. A subset of young adult NPY-deficient mice had mild seizures (usually at 8 weeks of age), and these seizures do not occur as the mice age. No other behavioral modifications were obvious in NPY-deficient mice⁴².

Whole Mount staining

For assessment of neuronal and fiber density, proximal colons (1 cm) were obtained from four week old WT and *NPY*^{-/-} mice. The colon was cut along the mesentery and pinned down in a silicone dish. Care was taken that the segment was not stretched to more than 1.1 cm. Fixation and careful peeling of myenteric plexus and the longitudinal muscle layer and NADPH Diaphorase was done as described⁴³. For ChAT staining the sections were incubated with goat anti ChAT (1:100) overnight. The positive cells were visualized using the ABC kit (Vector labs) in conjunction with the DAB stain. Peripherin staining was performed using a rabbit anti-peripherin polyclonal antibody (1:500) and donkey anti-rabbit IgG-Alexa Fluor 594 (1:500). Myenteric fiber counts were performed on both NADPH-diaphorase and ChAT-stained intestines by counting fibers crossing a 0.1 mm² grid with ten horizontal and ten vertical lines⁴⁴. Ten randomly selected fields per mouse were evaluated from six animals of each genotype. The neurons were counted within the 0.1 mm² grid and the total number of neurons with each stain was calculated. Data were analyzed using the Prism Software in conjunction with ANOVA.

Measurement of gastric emptying and intestinal transit

Wild type and *NPY*^{-/-} mice received 0.1 mL methylene blue-labeled 10% dextrose solution using oral gavage and sacrificed at the indicated times (5 or 15 min). Gastric emptying and intestinal transit were determined as described previously⁴³. The intestinal transit was measured from the pylorus to the most distal point of migration of methylene blue.

Isometric muscle recording with electrical field stimulation

Longitudinal muscle strips (with myenteric plexus) and circular muscle strips were obtained from the proximal colon of WT and *NPY*^{-/-} mice by careful dissection using a dissecting microscope. Settings for recording were done as described previously⁴³. Colonic relaxation was measured in response to transmural stimulation and was recorded by electrical field stimulation (EFS) (100 V/48 V, 20 Hz, 5 msec pulse for a duration of 30 s) in the presence or absence of atropine and guanethidine⁴⁵. We chose 20 Hz as we get our most reproducible relaxations at this frequency. For quantitative determination of relaxation longitudinal and circular intestinal strips from at least three WT and *NPY*^{-/-} mice were used and the average of relaxations was obtained from EFS. The NO dependence of the relaxation was confirmed by incubation with L-NAME prior to EFS.

Statistical Analysis

Neuronal staining and motility measurements between the WT and *NPY*^{-/-} mice were compared with the one-way analysis of variance (ANOVA) (1999 Graph Pad software Inc). Repeated-measures analyses were used to compare animals under each of the 3 treatment conditions for apoptosis, proliferation and differences in specific neuronal staining in cultured enteric neurons. Multiple comparisons between treatment conditions were made with Tukey's Honestly Significant Difference (HSD) procedure. Repeated-measures analyses of apoptosis and proliferation were done with a means model with SAS Proc Mixed (version 8) providing

separate estimates of the means by age (E12.5, E14.5 and E16.5) and treatment condition. A compound symmetry variance-covariance form in the repeated measurements was assumed for each outcome, and robust estimates of the standard errors of parameters were used to do statistical tests and construct confidence intervals. Statistical tests were 2-sided. A P value 0.05 was considered statistically significant.

Results

GDNF increased NPY mRNA and protein expression

The effect of growth factors on the synthesis and release of peptide neurotransmitters such as NPY has not been studied in the enteric nervous system. GDNF is known to promote enteric neuronal proliferation and suppress apoptosis^{2,37}. We examined the effect of GDNF on the expression of NPY. Enteric neurons obtained from E14.5 rat embryos were cultured in the presence of GDNF or vehicle for 24 h and assessed for NPY mRNA and protein expression. The presence of GDNF resulted in a significant increase in NPY mRNA expression assessed by semi-quantitative PCR (Fig. 1a), and real-time PCR compared to vehicle (Fig. 1b). GDNF induced an increase in the number of NPY-expressing neurons (Fig. 1c, d), assessed using an antibody specific for NPY. Using a sensitive immunoassay for NPY, we determined the effects of GDNF on the release of NPY and a closely related peptide, PYY, into the culture medium. GDNF (48 h) induced a significant release of NPY into the culture medium compared to vehicle and had no effect on PYY release (Fig. 1 e).

GDNF-induced enteric neuronal proliferation and survival is mediated through NPY

We next examined the effect of knock down of NPY on GDNF mediated enteric neuronal survival and proliferation. Enteric neurons were transfected with control Cyclophilin B or NPY-specific siRNA for 24 h in the presence of vehicle. Neurons were subsequently cultured for 24 h in the presence or absence of GDNF and fixed to assess proliferation and apoptosis. The neurons were then stained for either Peripherin/BrdU or Peripherin/TUNEL. Treatment of enteric neurons with NPY siRNA, but not control Cyclophilin B siRNA, resulted in a reduction in proliferation and an increase in apoptosis in vehicle-treated and GDNF-treated enteric neurons. (Fig. 2a, b). Knock down of NPY with NPY siRNA was confirmed by staining for NPY using the NPY/Peripherin double-labeling method (Fig. 2c, d). These data suggest that NPY plays a significant role in GDNF-mediated survival and proliferation of enteric neurons.

GDNF-induced proliferation and survival of enteric neurons is reduced in the presence of NPY-Y1 receptor antagonist and the PI-3-kinase inhibitor, LY294002

Since the GDNF-induced proliferation and survival of enteric neurons was mediated through NPY, we assessed the effects of NPY on enteric neuronal proliferation and survival by directly adding NPY to the culture medium. Embryonic enteric neurons treated with vehicle, NPY or GDNF in the presence or absence of either NPY-Y1 receptor antagonist (BIBP3226, 1 μ M) or the PI-3-kinase inhibitor LY294002 (15 μ M) were maintained in culture for 24 h, then fixed and assessed for apoptosis by the Peripherin/TUNEL method. Total cell number was determined through DAPI staining, and enteric neurons were specifically identified by peripherin staining. For the cell proliferation assessment, BrdU was added to the medium 24 h prior to fixation and was assessed by the BrdU/Peripherin method. Exposure of enteric neurons to NPY significantly increased proliferation, and this was inhibited in the presence of BIBP3226 and LY294002 (Fig. 3a, b). GDNF and NPY increased the total number of neurons per well (peripherin staining), and this was reflected in the total number of cells per well (DAPI staining) (Fig. 3c, d). NPY suppressed apoptosis similar to GDNF. This suppression of apoptosis was inhibited in the presence of BIBP3226 and LY294002 (Fig. 4 a, b). In the presence of the antagonist there is an increase in apoptosis in the vehicle treated conditions as

the autocrine effects of NPY in the culture medium are inhibited. Addition of GDNF and NPY together did not have an additive effect on increasing the total number of neurons (number of neurons: Vehicle: 1013 + 53; GDNF: 1663 + 116; NPY: 1466 + 62; GDNF + NPY: 1797 + 33, $P > 0.05$ for GDNF + NPY vs. GDNF or NPY alone) or suppressing apoptosis over GDNF or NPY alone (% apoptosis: Vehicle: 29.5 + 0.3; GDNF: 19.3 + 0.7%; NPY: 20.35 + 0.9%; GDNF + NPY: 18.88 + 0.7%, $P > 0.05$ for GDNF + NPY vs. GDNF or NPY alone).

As an additional method to measure apoptosis, caspase-3 cleavage was assessed. Enteric neurons were cultured in the presence or absence of NPY (1 μ M) for 24 h and probed for cleaved caspase-3 by Western blotting. Western blot analysis showed less cleaved caspase-3 in NPY-treated cells compared to vehicle (Fig. 4c, d). These data suggest that GDNF induces the release of NPY, which mediates PI-3-kinase dependent survival and proliferation of enteric neurons, by acting in an autocrine manner through the Y1 receptor.

NPY increases pAkt levels in enteric neurons

The PI-3-kinase pathway is an important signal transduction pathway in GDNF-mediated enteric neuronal survival³⁷. GDNF induces phosphorylation of Akt, one of the major downstream targets of PI-3-kinase. We examined if this phosphorylation can be induced also by NPY. Western blotting was performed using an antibody specific for pAkt. Figure 5 illustrates that NPY and GDNF significantly increased the phosphorylation of Akt compared to vehicle. This increased phosphorylation was lost when the cells were treated with the PI-3-Kinase inhibitor LY294002. These results suggest that NPY activates signal transduction pathways in a manner similar to GDNF.

Characterization of neurons induced by NPY

We next assessed the type of neurons that were induced by the addition of NPY to primary culture neurons. Primary neurons treated with vehicle, GDNF or NPY for 24 h were stained for nNOS or ChAT and co-stained with peripherin. The total number of ChAT⁺ and nNOS⁺ neurons per well were determined. We found that both GDNF and NPY induced a significant increase in the number of nNOS⁺ enteric neurons compared to vehicle-treated cells in a PI-3-kinase-dependent manner (Fig. 6a, b). No changes in ChAT⁺ cells were noted following either NPY or GDNF exposure for 24 h (Fig. 6c, d). In the presence of NPY siRNA there was a reduction in the percentage of nNOS⁺ neurons but not ChAT⁺ neurons (Fig. 6e, f). GDNF increases the peripherin⁺ neurons by approximately 70%. Approximately 40% of the neurons are nNOS⁺ and 45% are ChAT⁺. Thus we would predict that there is co-localization between the nNOS⁺ and ChAT⁺ neurons. We found that 17 + 3% of the nNOS⁺ neurons co-localize with ChAT⁺ neurons in the presence of GDNF.

NPY expressing neurons co-localize with nNOS staining neurons—We examined the co-localization of NPY with nNOS⁺ and ChAT⁺ neurons. We found that NPY co-localizes with about 77% of nNOS⁺ neurons and 36% of ChAT⁺ neurons (Fig. 7). These findings are consistent with our observations that NPY induces an increase in nNOS⁺ neurons.

NPY has differential effects on proliferation and apoptosis at different developmental stages—The developmental expression of NPY was assessed in rat embryonic stages E12.5 and E16.5. As seen in Fig. 8a we observed the expression of NPY in enteric neurons of the proximal intestine as early as E12.5. We next assessed the effect of GDNF and NPY on apoptosis and proliferation from enteric neurons obtained at different developmental stages. In our culture system, we found that NPY affects proliferation in the early stages (E12.5) and apoptosis in the later embryonic stages (E16.5) (Fig 8 b, c). This effect of NPY is similar to the effect of GDNF noted by Chalazonitis *et al*⁴⁶ who demonstrated an effect of GDNF on proliferation at the earlier developmental stages and on apoptosis at the

later developmental stages⁴⁶. We found that both GDNF and NPY caused an increase in the number of nNOS neurons beginning at developmental stage E12.5 (Fig 8d).

GDNF suppression of apoptosis is lost in enteric neurons isolated from *NPY*^{-/-} mice—We compared the effects of GDNF on enteric neurons isolated from embryonic (E13.5), WT and *NPY*^{-/-} mice. Lack of NPY expression in *NPY*^{-/-} mice was demonstrated by immunohistochemistry using an antibody to NPY (Fig. 8e). We assessed the effect of GDNF and NPY on apoptosis in WT and *NPY*^{-/-} mice enteric neurons. GDNF suppressed apoptosis in enteric neurons from WT mice, but not *NPY*^{-/-} mice (Fig. 8 f).

***NPY*^{-/-} mice have reduced inhibitory myenteric neurons in the small bowel and colon compared to WT mice**

Having demonstrated the effects of NPY in primary enteric neuronal culture, we next assessed the effect of the lack of NPY expression on enteric neuronal numbers using the *NPY*^{-/-} mouse model. Proximal colon from 4 week old mice was obtained from WT and *NPY*^{-/-} mice, fixed and stained. The number of NADPH-diaphorase⁺ inhibitory neurons and large fibers were significantly decreased in *NPY*^{-/-} mice compared to WT (Fig. 9a, b, c). No change was noted in the number of ChAT⁺ excitatory neurons and large fibers in the *NPY*^{-/-} versus WT mice colon (Fig. 9d, e, f). Total number of neurons was assessed by peripherin staining. The number of peripherin⁺ neurons was decreased in the colon of *NPY*^{-/-} mice compared to WT (Fig. 9g, h).

We also assessed the expression of nNOS in the stomach and colon obtained from WT and *NPY*^{-/-} mice by Western blot analysis. We found a reduction in nNOS expression in the colon and stomach of *NPY*^{-/-} compared to the WT mice (Fig. 9i).

Gastric emptying, intestinal transit and colonic muscle relaxation in response to electrical field stimulation in WT and *NPY*^{-/-} mice

Liquid gastric emptying and intestinal transit were assessed in WT and *NPY*^{-/-} mice using methylene blue. Gastric emptying at 15 minutes after dye administration was significantly higher while intestinal transit was lower in *NPY*^{-/-} mice compared to WT mice (Fig. 10 a, b). To assess the effects of lack of NPY expression in enteric neurons independent of the central effect of NPY, we performed isometric muscle relaxation in conjunction with electrical field stimulation. This technique allowed assessment of the number and function of inhibitory neurons in the myenteric plexus. Percent relaxation was calculated by determining the difference between the maximal force generated in response to acetylcholine and the minimum force following electrical stimulation. Value was expressed as a percentage of the force generated in response to acetylcholine. Transmural stimulation (100 V/48 V, 20 Hz, 5 msec, 60 s) evoked relaxation of longitudinal muscle, and circular muscle (data not shown) strips of the proximal colon were significantly impaired in *NPY*^{-/-} mice compared to WT (P<0.05, Fig. 10c, d). Pretreatment with L-NAME (10⁻⁴ mol/L, Fig. 10e) significantly reduced muscle relaxation (90.8 + 0.82 % reduction), indicating that muscle relaxation in our recording conditions were primarily mediated by nitric oxide production from the myenteric plexus. Relaxation induced by electrical field stimulation was significantly reduced (92 + 1.2 % reduction) in the presence of tetrodotoxin (10⁻⁷mol/L), indicating mediation through a neuronal pathway. These data support the presence of fewer numbers of inhibitory neurons in the *NPY*^{-/-} mice.

Discussion

Growth factors like BDNF and NGF can modulate NPY expression^{32–35}. BDNF-induces NPY production and secretion from neuronal cortical cells into the medium³⁴. GDNF is an

important growth factor for enteric neuronal survival and proliferation. The role of GDNF on modulating NPY expression has not been studied. In the present study, we have examined the effects of GDNF on NPY expression in enteric neurons. We characterized the effects of NPY on apoptosis and proliferation of enteric neurons. We observed that GDNF induced a significant increase in NPY mRNA expression and the release of NPY into the culture medium. Interestingly, PYY, a neuropeptide closely related to NPY, was unaffected by GDNF, suggesting a distinct regulation of NPY by GDNF. NPY is present both in the central and peripheral nervous system compared to PYY, which is mainly localized to the gastrointestinal tract^{47, 48}. This may explain the higher basal levels of PYY in our cultured enteric neurons.

Using NPY siRNA, as well as an antagonist to NPY-Y1 receptor, we show a role for NPY in mediating GDNF-induced enteric neuronal survival and proliferation. GDNF and NPY increase nNOS⁺ neurons in culture, but do not affect ChAT⁺ neurons. In *NPY*^{-/-} mice we found a loss of nNOS⁺ myenteric neurons. Thus, we demonstrate a NPY-mediated autocrine mechanism to regulate enteric neuronal survival.

NPY exerts its action through several different receptor subtypes (Y1, Y2, Y4, Y5 and Y6). These are G-protein coupled receptors whose activation results in inhibition of adenylate cyclase activity. In order to delineate the involvement of a specific receptor, the Y1 receptor antagonist BIBP3226 was used in enteric neuronal cultures supplemented with NPY. The effects of NPY were abolished in the presence of the antagonist, confirming that the Y1 receptor plays an important role in mediating the effects of NPY on enteric neurons. NPY exerts hypertrophic effects on cardiomyocytes by the activating PI-3-kinase pathway²⁵. The neuroproliferative effect of NPY in the dentate gyrus is Y1 receptor-mediated and involves extracellular signal-regulated kinase (ERK)1/2 activation²⁸. The growth factor-induced expression of NPY can be dependent on either the PI-3-kinase or ERK-dependent pathways. GDNF is known to promote the survival of enteric neurons through the PI-3-kinase pathway³⁷. The PI-3 kinase/Akt/FOXO signaling pathway plays a critical role in ENS precursor survival and neurite extension³⁷. We now demonstrate that similar to GDNF, NPY also activates the PI-3-kinase pathway in enteric neurons. Although both GDNF and NPY were observed to act via the PI-3-kinase pathway, the addition of both compounds into the medium had little additive effect on survival/proliferation of enteric neurons in culture. The percent survival or proliferation rates for enteric neurons in the presence of GDNF or NPY alone were comparable to that in presence of both these growth factors. The percent survival rates for enteric neurons under our culture conditions were higher than what has been previously reported⁸ and this is due to different plating density as well as different culture medium supplements.

Our findings are similar to those obtained with GDNF^{+/-} mice studies², suggesting that GDNF mediates the proliferation and survival of enteric neurons in part through NPY and the PI-3-kinase pathway, but other signaling pathways also contribute to enteric nervous system development. The reduction in nNOS⁺ neurons is modest and reflects the fact that other compensatory pathways might prevent a more dramatic reduction in nNOS⁺ neurons. Unlike the *GDNF*^{+/-} mice, the *NPY*^{-/-} mice do not have an obvious macroscopic bowel phenotype. A number of studies have identified increased NPY innervation in the aganglionic bowel of patients with Hirschsprung's disease⁴⁹⁻⁵¹. We believe this may be a compensatory response in the aganglionic segment, and this increased NPY may promote the extrinsic hyperinnervation that has been noted in Hirschsprung's disease⁵². The *GDNF*^{-/-} mice die at birth and these mice completely lack the enteric nervous system¹⁰. The *NPY*^{-/-} mice have specific loss of the nNOS containing neurons.

NPY is broadly expressed in the developing nervous system and is maintained at moderately high levels in the adult, although its actions vary according to the target cell. The inhibitory

influence of NPY neurons is consistent with the established ability of NPY to inhibit acetylcholine release in isolated nerve/muscle preparations⁵³. Our *in vivo* study shows faster gastric emptying and impaired intestinal transit in *NPY*^{-/-} mice compared to WT. These data reflect the role of NPY in the central nervous system. The peripheral effects of lack of NPY expression were the loss of inhibitory nNOS⁺ myenteric neurons. Even though the nNOS neurons were reduced in the *NPY*^{-/-} mice, and the *nNOS* knockout mouse has delayed gastric emptying⁵⁴⁻⁵⁶, gastric emptying was accelerated in *NPY*^{-/-} mice. We cannot fully explain this discrepancy but centrally administered NPY delays gastric emptying⁵⁷ and so the phenotype could reflect a dominant effect of loss of central control by NPY compared with the effect of NPY on nNOS neurons.

The decrease in nNOS⁺ neurons in *NPY*^{-/-} mice was further validated at the physiological level through isometric muscle recording experiments. Longitudinal and circular muscle strips from proximal colon of *NPY*^{-/-} mice showed impaired /decreased relaxation on electrical field stimulation when compared to WT counterparts confirming the immunohistochemical studies. NPY co-localizes predominantly with nNOS neurons compared to ChAT neurons. Similar observations have been previously demonstrated^{31,58}. The immunostaining data was further confirmed by siRNA studies. We observed that NPY siRNA treatment resulted in significant decrease of nNOS neurons only. Further we have previously demonstrated that GDNF increases the number of nNOS⁺ neurons but not the ChAT⁺ neurons⁴³.

Analysis of NPY expression at various rat developmental stages showed NPY-expressing neurons as early as E12.5. NPY was present in the stomach and the proximal and distal gut. The effects of NPY were similar to the effects of GDNF as demonstrated by Chalazonitis *et al*⁴⁶. We found that NPY had profound effects on proliferation at early developmental stages and on survival at later developmental stages. Our findings demonstrate that GDNF, through NPY expression, can modulate enteric neuronal survival and proliferation and the specific neuronal subtypes in postnatal animals. However, the addition of both these trophic factors into the cell culture media had little additive effect, probably indicating a saturation of the common signaling pathway which is activated.

Recently, it was reported that *NPY*^{-/-} mice were more sensitive than WT mice to methamphetamine-induced neuronal apoptosis in both enkephalin- and nitric oxide synthase-containing neurons, suggesting that NPY plays a general neuroprotective role within the striatum²⁹. Reduced NPY content in the intestine has been demonstrated with aging, and this may contribute to age-associated gastrointestinal motility disorders. The ability of NPY to stimulate neuroproliferation in the ENS may be useful in fields such as stem cell therapy²⁷. NPY analogs might be useful therapeutic agents against the neuronal losses²⁹ that occur in the enteric nervous system, in disease conditions such as diabetes.

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Abbreviations

GDNF	Glial cell line-derived neurotrophic factor
WT	Wild type
<i>NPY</i>^{-/-}	Neuropeptide Y knock-out

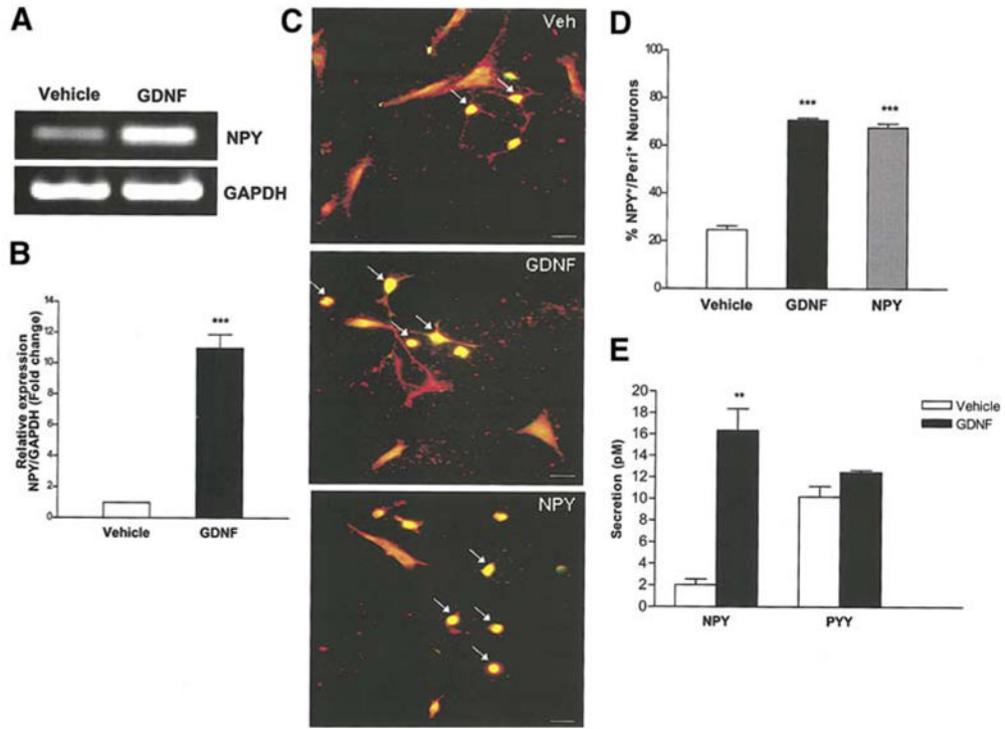


Figure 1. GDNF increases NPY mRNA and protein expression. (a) RNA was isolated from enteric neurons cultured in the presence or absence of GDNF for 24 h and amplified for GAPDH (loading control) and NPY. The presence of GDNF causes an increase in NPY mRNA. (b) Quantitative real-time PCR showing the relative level of expression of NPY (GDNF vs. vehicle) after normalization to GAPDH expression. Results are mean + S.E., n=9, *** = p<0.001. (c, d) Enteric neurons cultured in the presence of vehicle, GDNF or NPY for 24 h were assessed for peripherin (red)/NPY (green) and the results expressed as % NPY positive neurons. GDNF induced an increase in the number of NPY⁺ neurons (arrows pointing at yellow neurons). Results are mean + S.E., n=3, *** = p<0.001. Scale bar: 20 μm. (e) Enteric neurons were cultured in the presence or absence of GDNF (48 h) and medium was assayed for NPY and PYY by ELISA. GDNF induced a significant release of NPY but not PYY into the culture medium compared to vehicle. Results are mean + S.E., n=3, ** = p<0.01. P value is with respect to neurons cultured in the presence of vehicle.

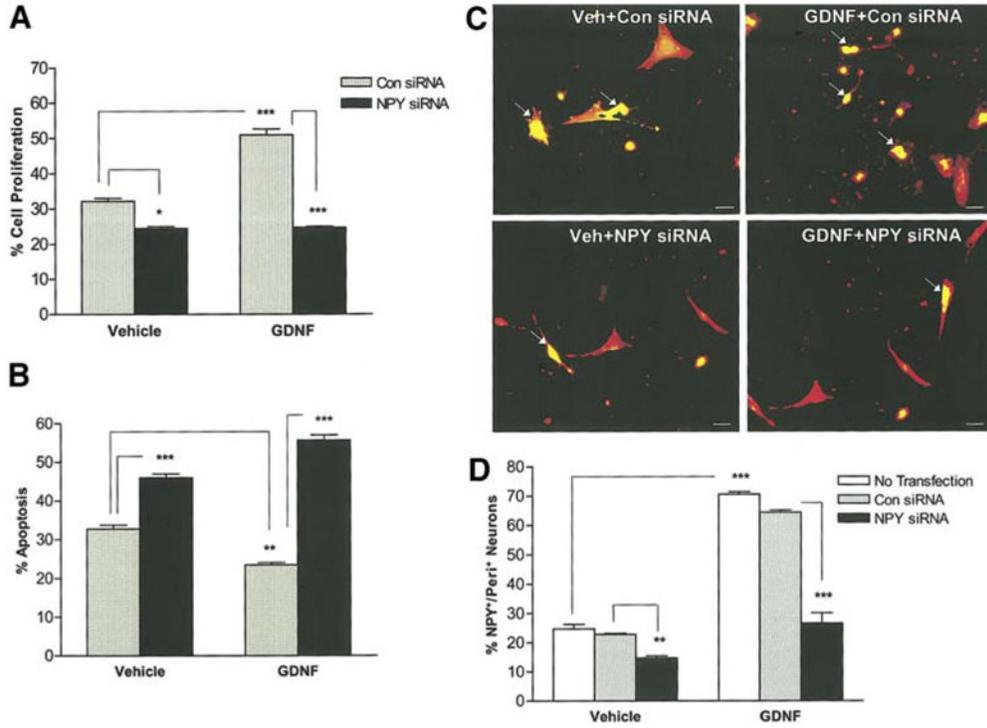


Figure 2. GDNF-induced proliferation and survival of enteric neurons is reduced in the presence of NPY siRNA. **(a)** Enteric neurons in the N2 medium were transfected with control Cyclophilin B or NPY-specific siRNA for 24 h and were subsequently cultured for 24 h in the presence or absence of GDNF. BrdU was added to the medium 24 h prior to the fixation of neurons and the % of BrdU⁺/peripherin⁺ neurons was determined to assess the proliferation, n=4. **(b)** Transfected enteric neurons were assessed for apoptosis using the double-labeling of TUNEL and peripherin, n=3. **(c, d)** To study the NPY siRNA specificity, transfected enteric neurons were stained for NPY (green)/Peripherin (red), n=3. Results are mean + S.E., *** = p<0.001, ** = p<0.01, * = p<0.05. Scale bar: 20 μm.

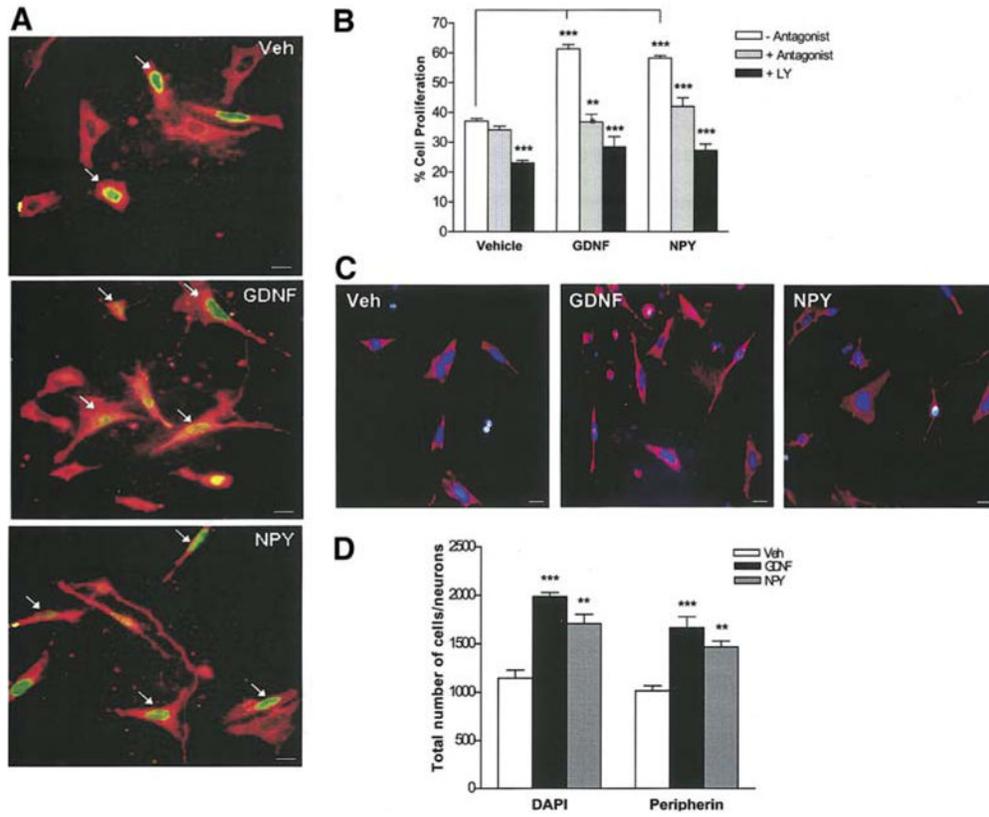


Figure 3.

GDNF induces proliferation of enteric neurons through NPY. **(a, b)** Enteric neurons treated with vehicle, NPY or GDNF in the presence or absence of either the NPY-Y1 receptor antagonist (BIBP3226) or the PI-3-kinase inhibitor (LY294002) were maintained in culture for 48 h and fixed. BrdU was added to the medium 24 h prior to fixation. The % of BrdU⁺ (green)/peripherin⁺ (red) neurons was determined to assess proliferation. Arrows point to the BrdU⁺ neurons. Exposure of enteric neurons to NPY (48 h) significantly increased proliferation similar to GDNF. GDNF- and NPY- induced proliferation of enteric neurons was inhibited by both BIBP3226 and LY294002, n=4. Scale bar: 20 μm. **(c, d)** The total number of cells (DAPI-blue) and neurons (peripherin-red) per well was assessed under the stated culture conditions, n=2. Results are mean + S.E., *** = p<0.001, ** = p<0.01. P value is with respect to the neurons cultured in the absence of antagonist **(b)** or vehicle **(d)** in each respective condition.

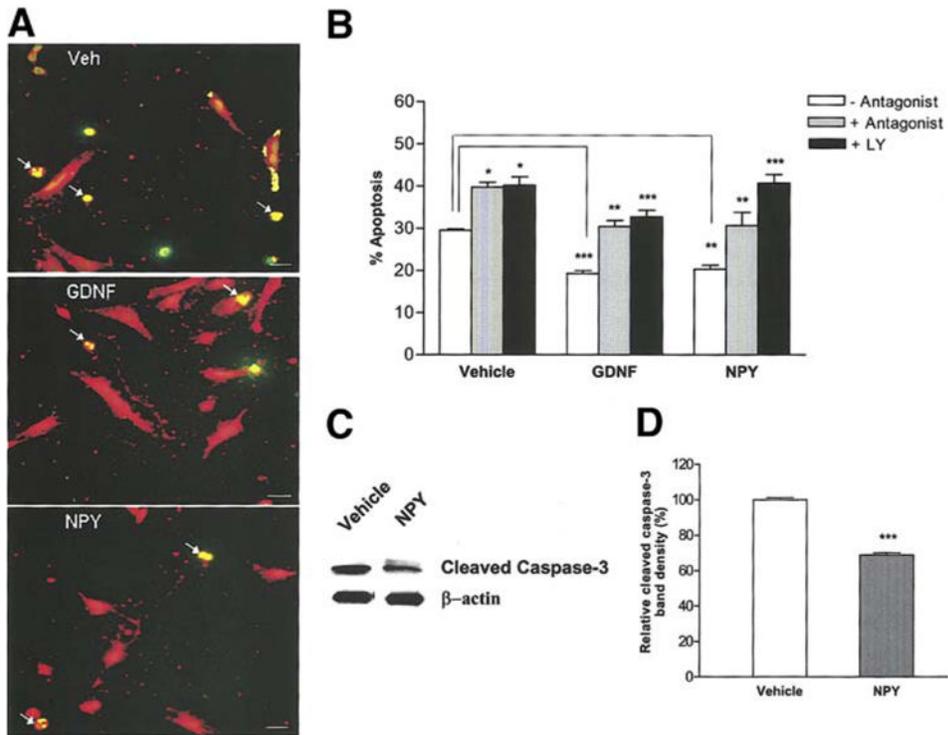


Figure 4. GDNF suppresses apoptosis of enteric neurons through NPY. **(a, b)** Enteric neurons treated with vehicle, NPY or GDNF in the presence or absence of BIBP3226 or LY 294002 for 24 h were assessed for apoptosis by the TUNEL (green)/peripherin (red) method. Exposure of enteric neurons to NPY or GDNF significantly reduced apoptosis compared to vehicle. This effect was inhibited by BIBP3226 and LY294002. Arrows show the apoptotic cells with condensed nuclei. Results are mean + S.E., n=3, *** = p<0.001, ** = p<0.01, * = p<0.05. Scale bar: 20 μ m. P value is with respect to the neurons cultured in the absence of antagonist in each respective condition. **(c, d)** Western blot analysis of cleaved caspase-3 in enteric neurons cultured in the presence or absence of NPY for 24 h. β -actin was used as a loading control. There was a significant reduction in the expression of cleaved caspase-3 in NPY treated cells compared to vehicle. Results are ratio + S.E., n=3, *** = p<0.001.

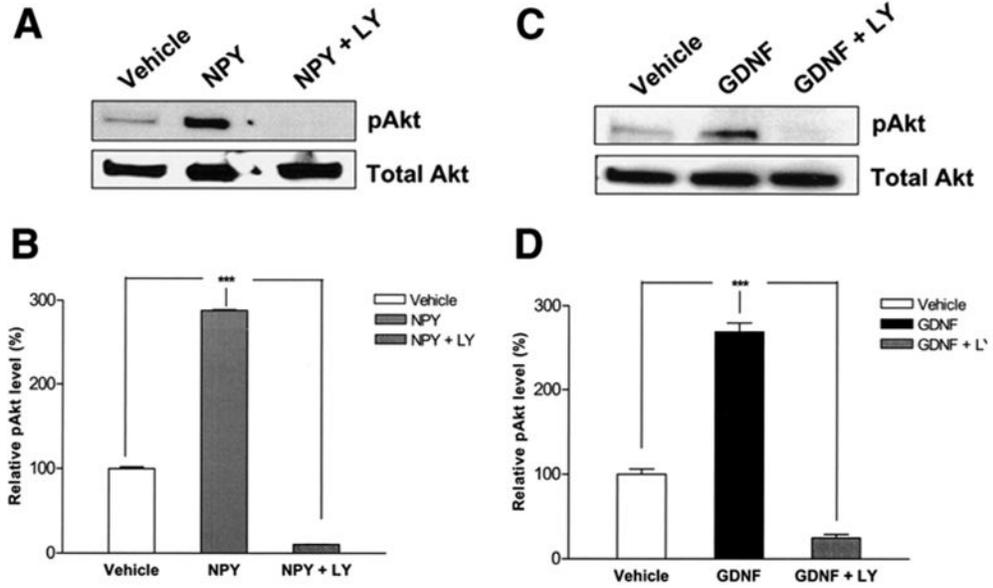


Figure 5. NPY activates the PI-3-kinase pathway. Cell lysates were obtained from enteric neurons treated with vehicle, NPY (a, b) or GDNF (c, d) with or without LY294002 for 30 min and Western blot was performed. GDNF and NPY significantly increased the phosphorylation of Akt compared to vehicle. This was inhibited in the presence of the PI-3-kinase inhibitor LY294002. Results are ratio (pAkt/Akt) \pm S.E., n=3, *** = p<0.001.

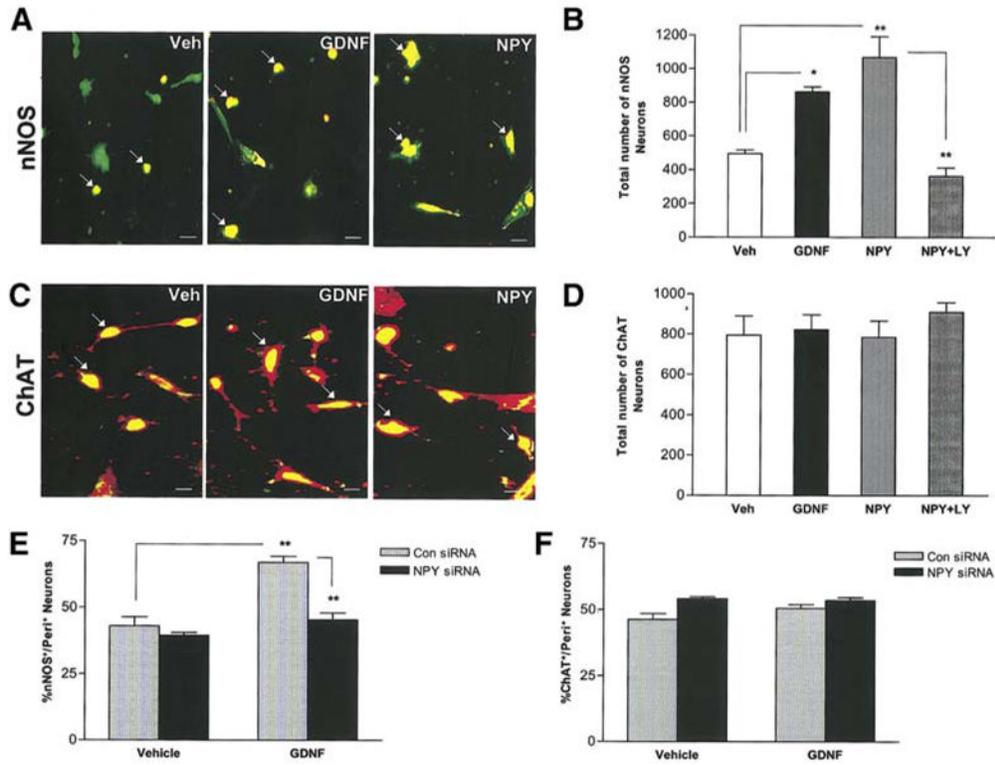


Figure 6. GDNF and NPY increase nNOS⁺ enteric neurons but not ChAT⁺ neurons. Primary enteric neurons were treated with vehicle, NPY, GDNF or NPY+LY294002 for 24 h, fixed and assessed for nNOS (red) and peripherin (green) (a, b) or ChAT (green) and peripherin (red) (c, d), n=4. Scale bar: 20 μ m. (e, f) Enteric neurons were transfected with control Cyclophilin B siRNA or NPY specific siRNA for 24 h and then cultured for an additional 24 h in the presence or absence of GDNF, fixed and assessed for nNOS and ChAT, n=3. Results are mean \pm S.E., ** = p<0.01, * = p<0.05.

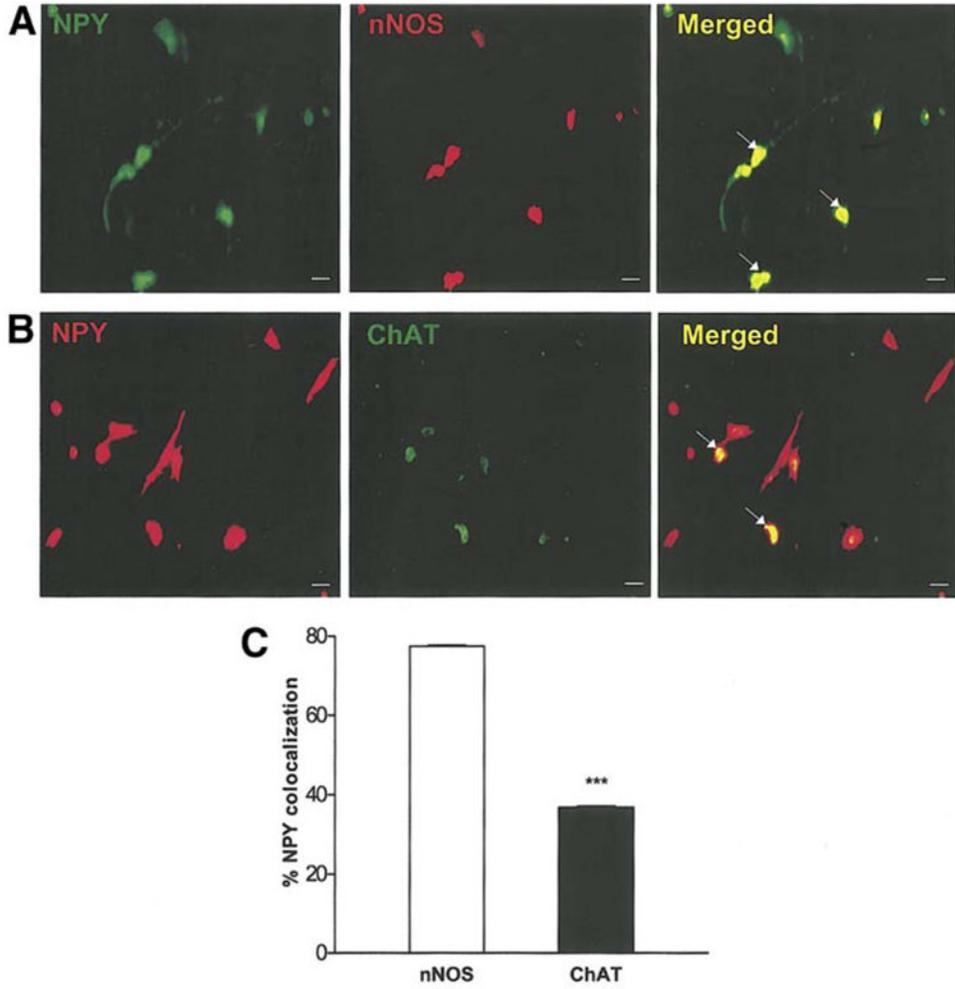
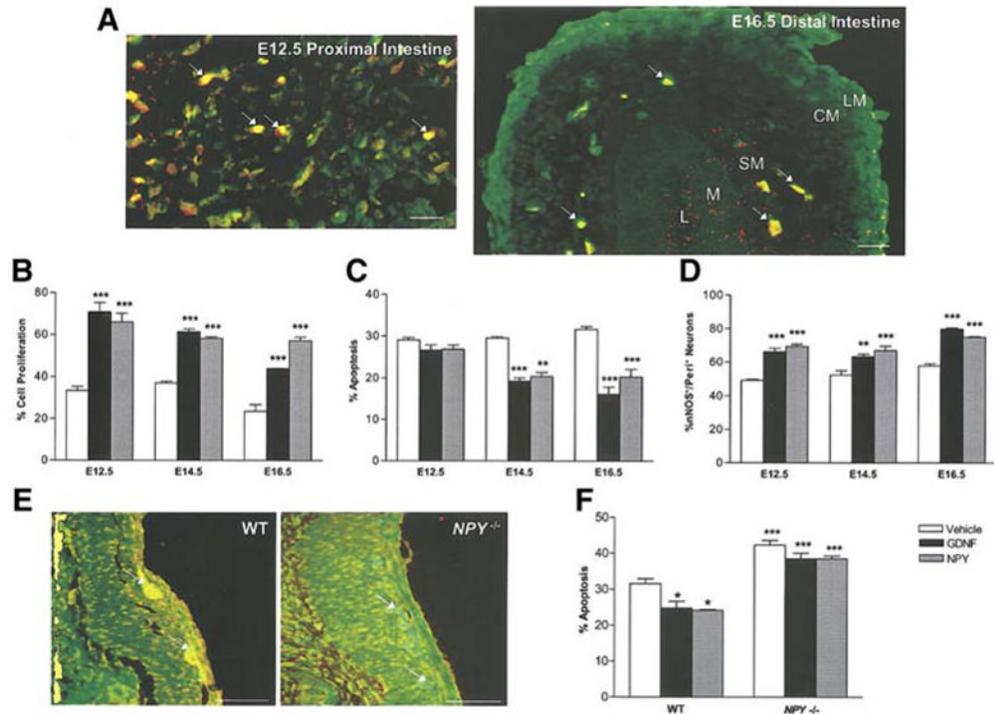


Figure 7. NPY co-localizes with nNOS. Primary enteric neurons were treated with GDNF for 24 h, fixed and assessed for nNOS (red) and peripherin (green) (**a**), or ChAT (green) and peripherin (red) (**b**). (**c**) Percent NPY colocalization with nNOS or ChAT is shown. Results are mean \pm S.E., $n=3$, *** = $p<0.001$. Scale bar: 20 μ m.

**Figure 8.**

Expression and effects of NPY at different developmental stages. **(a)** NPY is expressed in the proximal intestine of E12.5 rat embryos and extends into the distal intestine in E16.5 rat embryos. NPY is stained with cy3 (red) and peripherin with FITC (green). Arrows show the NPY⁺ ganglia in E12.5 and E16.5 gut. In the E16.5 intestine the intestinal layers are labeled L (lumen), M (mucosa), SM (submucosal), CM (circular muscle layer), LM (longitudinal muscle layer). Scale bar for **(a)**: 20 μ m. **(b)** The effect of GDNF and NPY on apoptosis and proliferation was assessed in rat embryos at different developmental stages. NPY affects proliferation more in the early stages and apoptosis in the later embryonic stages. **(d)** nNOS neurons were assessed by immunostaining at different developmental stages. Both GDNF and NPY caused an increase in the number of nNOS⁺ neurons starting from developmental stage E12.5. **(e)** Immunohistochemistry for NPY was performed to demonstrate the absence of NPY expression in *NPY*^{-/-} mice. The ganglia are stained with peripherin (green) and NPY (red). The overlap of red and green is seen in NPY⁺ neurons. **(f)** GDNF did not suppress the apoptosis in enteric neurons cultured from *NPY*^{-/-} mice. The effect of GDNF and NPY on apoptosis in enteric neurons obtained from *NPY*^{-/-} mice was assessed by the peripherin/TUNEL method. Results are mean + S.E., *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. Scale bar for **(e)**: 100 μ m. P value is with respect to the neurons cultured in the presence of vehicle in each respective condition.

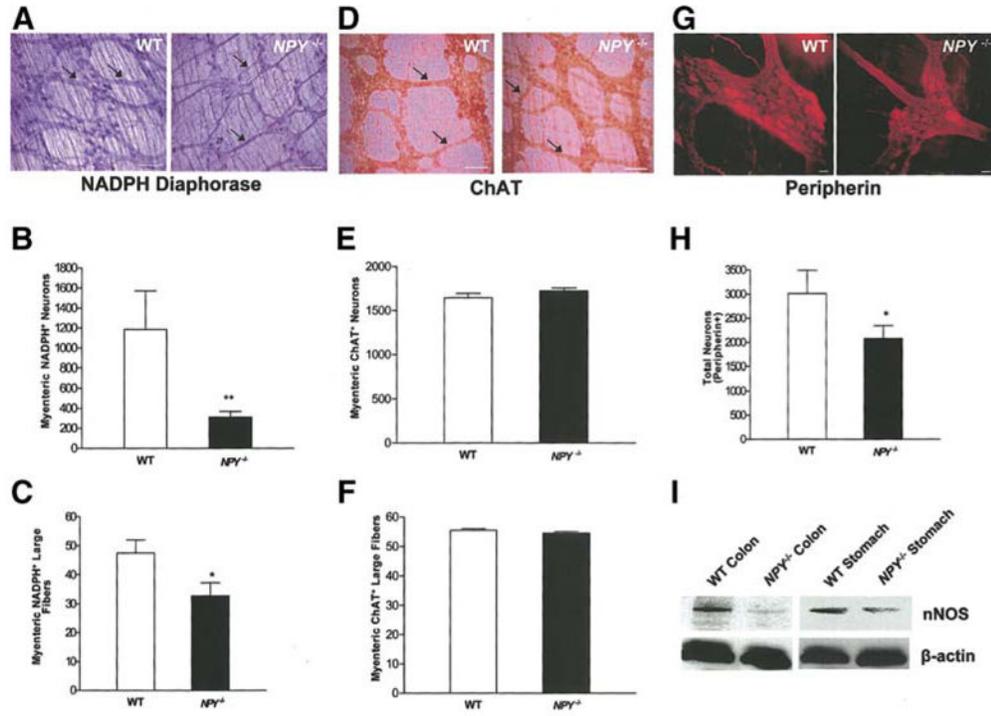


Figure 9. *NPY*^{-/-} mice have reduced colonic nNOS⁺ containing neurons compared to WT mice. (a, b, c) Longitudinal muscle strips along with myenteric plexus from mouse colon were fixed and stained for nNOS⁺ neurons using the NADPH-diaphorase staining. The nNOS⁺ neurons and large fibers (arrows) of the myenteric plexus were significantly decreased in the colon of the *NPY*^{-/-} mice compared to WT. (d, e, f) ChAT⁺ neurons and large fibers (arrows) of the myenteric plexus were similar in the *NPY*^{-/-} and WT colon. Total number of neurons was assessed by peripherin staining (g, h). (i) nNOS expression in WT and *NPY*^{-/-} mouse stomach and colon was assessed by Western blot. Scale bar: 100 μm (a, d) and 20 μm (g). Results are mean + S.E., n=6 in each group, ** = p<0.01, * = p<0.05.

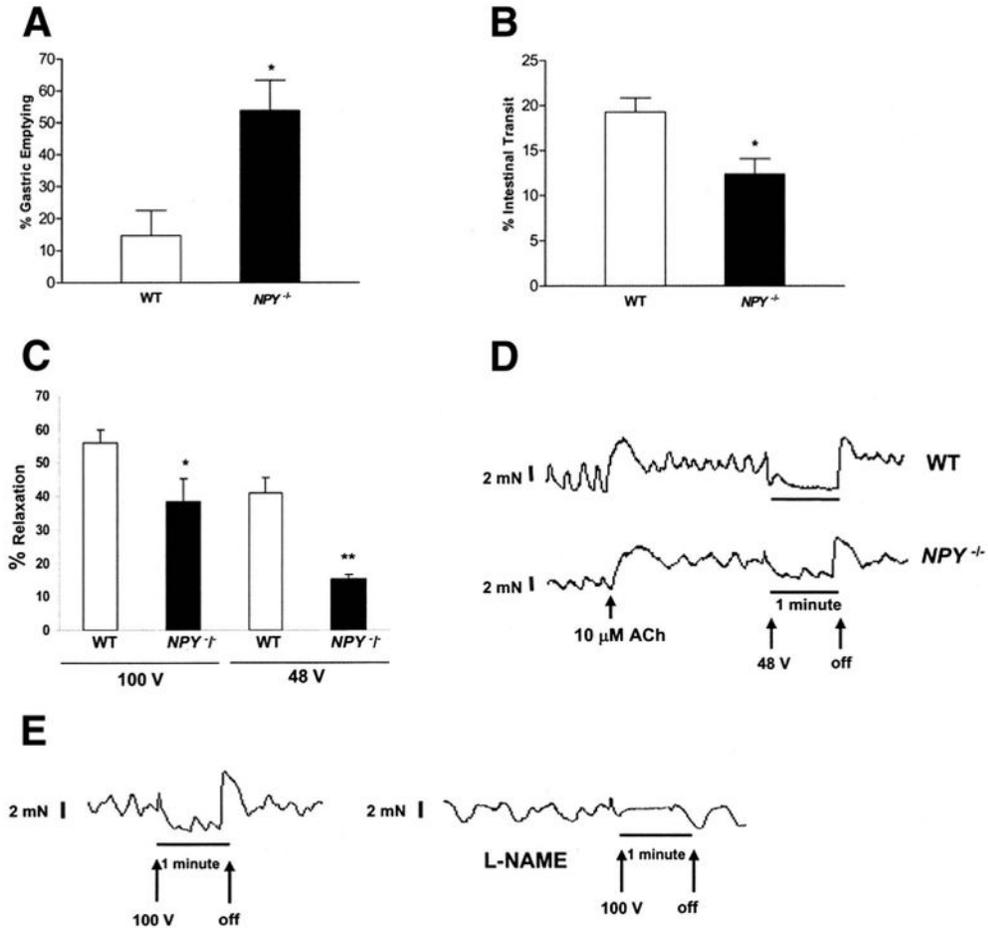


Figure 10. Altered motility in *NPY*^{-/-} mice (a) Gastric emptying and intestinal transit was assessed in WT and *NPY*^{-/-} mice. Gastric emptying was significantly higher in *NPY*^{-/-} compared to WT mice. Results are mean + S.E., n=4, * = p<0.05. (b) Intestinal transit was significantly lower in *NPY*^{-/-} compared to WT mice. Results are mean + S.E., n=4, * = p<0.05. (c) Assessment of colonic relaxation with electrical field stimulation in isolated colonic muscle strips containing the myenteric plexus. Transmural electrical field stimulation (100 V/48 V, 20 Hz, 5 msec, 60 s) evoked relaxation of the proximal colon was significantly impaired in *NPY*^{-/-} compared to WT mice. Results are mean + S.E., n=4, * = p<0.05, ** = p<0.01. (d) Representative tracings of electrical field stimulation. The X-axis represents time and the Y-axis represents force in mN. The arrows represent when the electrical stimulation was turned on and off. (e). Effect of L-NAME on electrical field- induced stimulation. The isolated muscle strips from WT mice were pretreated with L-NAME or buffer alone, for 30 min and EFS was performed at 100V, 20 Hz, 5 msec, 60 s. The tracing shows the lack of relaxation in the presence of L-NAME.