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RESEARCH PAPER

Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y_1 and Y_2 receptors

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BACKGROUND AND PURPOSE

Peptide YY (PYY) and neuropeptide Y (NPY) activate Y receptors, targets under consideration as treatments for diarrhoea and other intestinal disorders. We investigated the gastrointestinal consequences of selective PYY or NPY ablation on mucosal ion transport, smooth muscle activity and transit using wild-type, single and double peptide knockout mice, comparing mucosal responses with those from human colon.

EXPERIMENTAL APPROACH

Mucosae were pretreated with a Y_1 (BIBO3304) or Y_2 (BIIE0246) receptor antagonist and changes in short-circuit current recorded. Colonic transit and colonic migrating motor complexes (CMMCs) were assessed *in vitro* and upper gastrointestinal and colonic transit measured *in vivo*.

KEY RESULTS

Y receptor antagonists revealed tonic Y_1 and Y_2 receptor-mediated antisecretory effects in human and wild-type mouse colon mucosae. In both, Y_1 tone was epithelial while Y_2 tone was neuronal. Y_1 tone was reduced 90% in $PYY^{-/-}$ mucosa but unchanged in $NPY^{-/-}$ tissue. Y_2 tone was partially reduced in $NPY^{-/-}$ or $PYY^{-/-}$ mucosae and abolished in tetrodotoxin-pretreated $PYY^{-/-}$ tissue. Y_1 and Y_2 tone were absent in $NPYPYY^{-/-}$ tissue. Colonic transit was inhibited by Y_1 blockade and increased by Y_2 antagonism indicating tonic Y_1 excitation and Y_2 inhibition respectively. Upper GI transit was increased in $PYY^{-/-}$ mice only. Y_2 blockade reduced CMMC frequency in isolated mouse colon.

CONCLUSIONS AND IMPLICATIONS

Endogenous PYY and NPY induced significant mucosal antisecretory tone mediated by Y_1 and Y_2 receptors, via similar mechanisms in human and mouse colon mucosa. Both peptides contributed to tonic Y_2-receptor-mediated inhibition of colonic transit *in vitro* but only PYY attenuated upper GI transit.

Abbreviations

BIBO3304 (*R*)-*N*-[[4-(aminocarbonylaminomethyl)-phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate); BIBP3435, [(*S*)-N^2-diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide; BIIE0246, (*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide; CMMCs, colonic migrating motor complexes; DMSO, dimethyl sulphoxide; DPP IV, dipeptidyl peptidase IV; FPO, faecal pellet output; GI, gastrointestinal; GLP, glucagon-like

peptide; I_{sc}, short-circuit current; NPY, neuropeptide Y; NPY(3–36), neuropeptide Y(3–36); PP, pancreatic polypeptide; PYY, peptide YY; PYY(3–36), peptide YY(3–36); RT-PCR, reverse transcriptase polymerase chain reaction; TTX, tetrodotoxin; WT, wild-type

Introduction

Neuropeptide Y (NPY) is contained within many enteric neurons, but little is known about its physiological role within the enteric nervous system. By contrast, a recent study has shown that endogenous peptide YY (PYY) exerts a significant physiological paracrine effect in the colon (Cox *et al.*, 2010). Both peptides activate Y_1 and Y_2 receptors, which are widespread in the colon, but their relative contributions to gastrointestinal (GI) mucosal and smooth muscle activities are unknown. It is important to understand these mechanisms because Y receptors are potential targets for the treatment of malabsorptive diarrhoea. Infused PYY reduces ileal electrolyte secretion in man and has been described as a defender against diarrhoea (Playford *et al.*, 1990). PYY is located in enteroendocrine L cells of the distal ileum and colon (Böttcher *et al.*, 1984) whereas NPY is contained in several subtypes of enteric neurons and may act as an inhibitory enteric neurotransmitter (Fantaguzzi *et al.*, 2009). PYY is co-expressed (Böttcher *et al.*, 1984; Ku *et al.*, 2003) and co-released postprandially with the proglucagon products, glicentin and glucagon-like peptide (GLP)-1 and GLP-2 and it slows intestinal transit, acting as an 'ileal brake' (Lin *et al.*, 1996; Maljaars *et al.*, 2008) a mechanism that contributes to its satiety-inducing effects. Peripherally administered PYY and PYY(3–36) activate vagal afferents (as does GLP-1) thus providing a link between GI and central feeding mechanisms (Abbott *et al.*, 2005).

Peptide YY, NPY, PYY(3–36), NPY(3–36) and pancreatic polypeptide (PP) act on different Y receptors. In human and mouse colon, three Y receptors (Y_1, Y_2 and Y_4, nomenclature follows Alexander *et al.*, 2009) mediate mucosal antisecretory responses (Cox and Tough, 2002; Hyland *et al.*, 2003). Y receptors preferentially couple to the G_i-signalling pathway and this results in sustained inhibition of epithelial chloride ion secretion (Cox *et al.*, 1988; termed an antisecretory response) that is amplified by prior secretagogue treatment (Cox and Cuthbert, 1988; Cox *et al.*, 2001). It is notable that the distributions of Y_1, Y_2 and Y_4 receptors are the same in human and mouse colon. Immunohistochemical and functional studies have shown that Y_1 and Y_4 receptors are trafficked selectively to basolateral epithelial membranes where they mediate the responses to PYY and NPY (Y_1) or to PP (Y_4) respectively (Mannon *et al.*, 1999; Tough *et al.*, 2006; Cox, 2007). Y_2 receptors in contrast are neuronal, and activated by NPY, NPY(3–36), PYY and PYY(3–36), but not PP (Cox and Tough, 2002; Hyland *et al.*, 2003; Cox, 2007; Wang *et al.*, 2010). Because of the close similarities in human and mouse colon Y receptor localization, the latter species was chosen for comparison with human tissue.

Previous studies showed that exogenous PYY and NPY exert a similar combination of epithelial Y_1 receptor-mediated and submucosal neuron Y_2 receptor-mediated antisecretory responses in human and mouse colon mucosae (see Cox, 2007) while PP acts directly on epithelial Y_4 receptors (Tough *et al.*, 2006). In preliminary experiments application of a competitive, selective Y_1 receptor antagonist (BIBO3304; Wieland *et al.*, 1998) or a Y_2 receptor antagonist (BIIE0246; Doods *et al.*, 1999) revealed the presence of endogenous Y_1- or Y_2 receptor-mediated antisecretory tone in colonic mucosa from human and mouse colon (Cox and Tough, 2002; Hyland *et al.*, 2003). These observations were the first indication that endogenous PYY and/or NPY are released tonically and are functionally effective in the gut. Therefore, we set out to determine which peptide was primarily responsible for each Y receptor-mediated tone using mice lacking PYY, NPY or both peptides. We showed recently that epithelial Y_1 receptors were the primary target for endogenous PYY released following activation of L cell Gpr119 receptors and that this mucosal response was dependent on PYY, not NPY (Cox *et al.*, 2010). Y_1 receptors do not apparently mediate PYY or NPY effects on colonic smooth muscle (Pheng *et al.*, 1999; Ferrier *et al.*, 2000). Rather, a combination of Y_2 and Y_4 receptors mediate contractile effects stimulated by exogenous NPY, PYY and PP (Pheng *et al.*, 1999; Hyland *et al.*, 2003). A further aim of the present study was therefore to establish selective changes in motility and transit functions in $PYY^{-/-}$ or $NPY^{-/-}$ mice and to associate each of these with Y_1 or Y_2 receptors.

With these intended aims and the availability of selective Y_1 and Y_2 antagonists and peptide knockout mice, we set out to establish loss-of-functions specific to either NPY or PYY and to compare individual functional deficits in peptide null mice with antagonist-treated preparations of human colon mucosa.

Methods

Targeted deletion of PYY, NPY and both peptides

All animal care and experimental procedures complied with the Animals (Scientific procedures) Act 1986 and were approved by the local Ethical Committees. $PYY^{-/-}$ and $NPY^{-/-}$ mice, in which the entire coding sequence including the initiation start codon was removed, were generated by homologous recombination in embryonic stem cells as described previously (Boey *et al.*, 2006; Karl *et al.*, 2008). $NPY^{-/-}$ and $PYY^{-/-}$ mice were crossed to generate double heterozygotes and subsequent double knockout $NPYPYY^{-/-}$ mice, as confirmed by Southern blot analysis (Doyle *et al.*, 2008). All mice were on the same C57BL/6–129/SvJ background, had free access to standard chow (except before assay of *in vivo* upper GI transit) and water *ad libitum*. There was no difference between wild-type (WT) and knockout mice derived from heterozygous breeding, compared with WT mice bred separately out of these lines (Edelsbrunner

et al., 2009). Mice were age-matched (19.1 ± 0.9 weeks, $n = 44$), killed by CO_2 asphyxiation and GI tissues excised for *in vitro* experimentation.

Measurement of changes in vectorial ion transport [short-circuit current (I_{sc})] across mucosal preparations

Colonic tissue was obtained from patients undergoing elective bowel resection surgery, with informed consent (6 male and 2 female, 72.8 ± 4.1 year), as described previously (Cox and Tough, 2002) and as approved by the Guy's and St Thomas' Hospitals Research Ethics Committee. Human mucosa was dissected and mounted in Ussing chambers within 2 h of excision. Intestinal mucosae from WT or knockout mice of either gender were dissected from overlying smooth muscle layers and voltage-clamped at 0 mV in Ussing chambers within 45 min post excision, as described in detail previously (Cox and Tough, 2002; Hyland *et al.*, 2003). Vectorial ion transport was measured continuously as I_{sc} ($\mu A \cdot cm^{-2}$) and all additions were basolateral unless otherwise stated. Once stable basal I_{sc} levels were achieved these preparations, which are a combination of mucosa and underlying submucosa with intact submucosal innervation, were pretreated with vehicle, the dipeptidyl peptidase IV (DPP IV) inhibitor (1 μM; compound 3, des-fluoro-sitagliptin; Lankas *et al.*, 2005) or neuronal activity was abolished with tetrodotoxin (TTX; 100 nM). In a separate set of experiments the effects of the DPP IV inhibitor *per se* were investigated in the absence or presence of 10 nM vasoactive intestinal polypeptide a secretagogue used to optimize observation of subsequent antisecretory responses (Cox *et al.*, 2001). Treatment periods were 20–30 min before addition of, the Y_1 receptor antagonist BIBO3304 (300 nM, throughout), BIBP3435 (1 μM; the inactive enantiomer of the Y_1 receptor antagonist BIBP3226) or the Y_2 receptor antagonist BIIE0246 (1 μM). In some tissues, a combination of the DPP IV inhibitor (1 μM) and BIBO3304 (300 nM) was added together 20–30 min before the addition of BIIE0246.

Measurement of colonic muscle contractility and faecal pellet propulsion in vitro

Murine ascending colon longitudinal muscle tension was measured as described previously (Hyland *et al.*, 2003). Colonic faecal pellet propulsion was monitored from the caeco-colonic junction to the rectum. Pellet positions were recorded photographically ($t = 0$ min) and the colon then placed in aerated Krebs–Henseleit solution (composition in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 11.1 (pH 7.4) maintained at 37°C, with vehicle or drug (1 μM BIIE0246, 300 nM BIBO3304 or 1 μM compound 3). After 20 min the colon was re-photographed, the distances of the remaining pellets from the rectum were measured and colonic transit calculated as the mean distance travelled relative to total length (% colonic transit).

In a separate series of experiments, the entire colon (from C57BL/6 mice) was allowed to empty in physiological saline (in mM: NaCl 118, KCl 4.6, NaH_2PO_4 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 11.1) at 37°C and then cannulated at either end and mounted in a continuously superfused horizontal bath. BIIE0246 (1 μM) was infused into the lumen, or was added to the superfusate from which it might be expected to act on myenteric neurons. Video recordings were made of contractile activity in control solutions, in the presence of BIIE0246 and after washout of the Y_2 receptor antagonist. The videos were converted to spatiotemporal maps of colonic diameter using in-house software and these were used to characterize colonic migrating motor complexes (CMMCs) as described in detail by Roberts *et al.* (2007).

Measurement of faecal pellet output (FPO) in vivo

Mice of each genotype were placed individually in a novel environment (a rat cage with grid bottom) for 15 min, with free access to food and water at the same time of day (between 10.00–11.00 h) and the number of faecal pellets they excreted over this 15 min period was counted. This was taken to be their FPO. In a separate experiment, WT or $NPY^{-/-}$ mice were acclimatized to the grid bottom cage for 3 days. They were then injected with vehicle [100 μL of 10% dimethyl sulphoxide (DMSO) in saline], BIBO3304 (100 μL of 0.4 mM) or BIIE0246 (60 $\mu g \cdot 100\ \mu L^{-1}$), i.p. and were allowed to equilibrate (15 min). After equilibration, FPO was measured over the next 15 min.

Measurement of upper GI transit in vivo

Male mice were fasted for 16 h (water *ad libitum*) and experiments were conducted between 10.00–13.00 h. Mice were conditioned to handling and vehicle (100 μL of 10% DMSO) or drug (60 $\mu g \cdot 100\ \mu L^{-1}$ BIIE0246 or 100 μL of 0.4 mM BIBO3304) was given i.p. 15 min before intragastric delivery of a charcoal meal (10% plant charcoal in 5% gum acacia; Sagar *et al.*, 2005). After 30 min mice were killed (by cervical dislocation), the small intestine was isolated from pyloric to ileo-caecal junctions, and the upper GI transit rate calculated as the distance travelled by the charcoal front, relative to the total length of small intestine (% upper GI transit).

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Human and mouse Y_1 and Y_2 receptor mRNA levels were determined using the RT-PCR method. Colonic tissue from six patients was dissected into discrete layers (mucosa, submucosa and muscle) and a superficial mucosal scraping was also produced (using a glass slide). Mouse ascending and descending colon (from three animals) muscle and mucosal layers were separated by dissection. Tissues were homogenized and the RNA isolated using RNeasy kits. To remove contaminating genomic DNA, human RNA samples were treated with DNase during RNA isolation, whereas equal quantities of mouse RNA ($n = 3$) were pooled, DNase treated and then purified with RNeasy kits a second time. 2 μg of RNA was reverse-transcribed in the presence or absence of M-MLV reverse transcriptase to determine any genomic DNA contamination. Semi-quantitative measurements of cDNA levels for Y_1 and Y_2 receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β actin, were performed by PCR using Thermo-Start PCR Master Mix and the primers listed (Supporting Information Table S1). PCR products were resolved on 2% agarose gels stained with ethidium bromide. Digital images of bands were captured using GeneSnap

(SynGene, Cambridge, UK) and quantified (Adobe Photoshop and Microsoft Excel, Adobe System Inc., San Jose, CA, USA).

Data and statistical analyses

Changes in I_{sc} or g tension were expressed as the mean ± SEM from a minimum of three experiments and multiple comparisons of these data groups were performed using one-way ANOVA with Dunnett's post-test. For *in vitro* colon pellet movement and *in vivo* upper GI transit and FPO measurements, single comparisons between data groups were performed using Student's unpaired *t*-test, whereas multiple comparisons used one-way ANOVA with Bonferoni's post-test, to compare data within a genotype. Two-way ANOVA with Bonferoni's post-test was used to compare each genotype and WT response to vehicle and antagonist treatments and each other. P values ≤ 0.05 were statistically significant.

Materials

BIBO3304, BIBP3435 and BIIE0246 were gifts from Boehringer-Ingelheim Pharma KG (Biberach an der Riss, Germany) and stock solutions were dissolved in 10% DMSO (at 1 mM) and stored at –20°C. Peptides were from Bachem Laboratories Inc. (St Helens, UK) and aliquots were stored at –20°C, undergoing a single freeze–thaw cycle. The DPP IV inhibitor, compound 3 was a gift from Dr R. Roy (Merck Inc., Rahway, NJ, USA; Lankas *et al.*, 2005). TTX was from Ascent Scientific (Bristol, UK). RNeasy kits and DNase were purchased from Qiagen, M-MLV reverse transcriptase (Fermentas, Cambridge, UK) and Thermo-Start PCR Master Mix (Abgene, Epsom, UK). RT-PCR primers and all other compounds were of analytical grade (Sigma-Aldrich, Poole, UK).

Results

The basal electrophysiological parameters of human and mouse colon mucosae (Table 1) were not significantly different from those described previously and the sensitivities to exogenous peptides were also similar in mucosae from the different genotypes (Cox *et al.*, 2010).

Y_1 and Y_2 receptor antagonists reveal antisecretory tone in human and mouse colon mucosae

Y_1 or Y_2 receptor blockade was achieved using BIBO3304 (a Y_1 receptor antagonist; Wieland *et al.*, 1998) or BIIE0246 (a Y_2 receptor antagonist; Doods *et al.*, 1999). BIBO3304 raised I_{sc} levels in human and mouse colon mucosae (Figure 1A and B). Because BIBO3304 is a competitive Y_1 receptor antagonist its ability to increase I_{sc} *per se* was probably due to blockade of tonically released endogenous NPY or PYY, either of which could inhibit epithelial ion secretion via Y_1 receptors in the absence of a blocker. BIBP3435, the inactive stereoisomer of the Y_1 receptor antagonist BIBP3226, a predecessor of BIBO3304 that also increases I_{sc} in human colon (Cox and Tough, 2002), had no effect on human or mouse colon mucosae (Figure 1A and B). The Y_2 receptor antagonist BIIE0246 also raised I_{sc} levels after a short lag period, reaching a plateau at ~15 min in human and mouse tissue (Figure 1C and D). DMSO (0.01%) vehicle controls were without effect in human (0.3 ± 0.6 μA·cm^{-2}, n = 5) or WT mouse colon (0.7 ± 0.4 μA·cm^{-2}, n = 6) mucosae. Next we set out to determine whether these tonic activities were neuronal (i.e. preferentially NPY-mediated) or not, in which case a contribution to this tone would be provided by PYY, as no other Y receptor ligands exhibit significant Y_1 receptor affinity. TTX reduced basal I_{sc} slightly (Table 1) and had no effect on Y_1 receptor-mediated tone in either tissue (Figure 1A and B). However, the neurotoxin virtually abolished the Y_2 receptor-mediated tonic effects in human and mouse mucosae (Figure 1C and D, respectively).

Both receptor antagonists were tested in WT mouse jejunum mucosa where L cell numbers are fewer compared with the distal colon. BIBO3304 increased I_{sc} by 1.3 ± 1.3 μA·cm^{-2} (n = 3) compared with 6.4 ± 0.8 μA·cm^{-2} (n = 17) in colon (P < 0.02), while BIIE0246 (1 μM) raised basal I_{sc} by

Table 1

Basal resistance and changes in baseline I_{sc} levels following addition of TTX or DPP IV inhibitor (Compound 3) to basolateral surface of colon mucosa from human and mouse tissue

	Resistance (Ω·cm^2)	Basal I_{sc} (μA·cm^{-2})	100 nM TTX (μA·cm^{-2})	1 μM Compound 3 (μA·cm^{-2})
Human colon	82.2 ± 4.0 (37)	60.2 ± 5.6 (37)	–17.9 ± 3.4 (7)	2.8 ± 1.0 (8)
Mouse jejunum				
WT	24.4 ± 1.1 (6)	82.4 ± 13.0 (6)	ND	ND
Mouse colon				
WT	34.2 ± 1.8 (23)	55.7 ± 5.7 (70)	–3.9 ± 1.3 (17)	–1.3 ± 0.3 (6)
NPY$^{-/-}$	30.1 ± 3.1 (18)	80.8 ± 13.1 (18)	–9.4 ± 1.8 (7)	0.4 ± 0.2 (8)*
PYY$^{-/-}$	40.0 ± 2.9 (20)	66.5 ± 9.8 (20)	–8.6 ± 2.8 (6)	–1.3 ± 0.5 (8)
NPYPYY$^{-/-}$	24.4 ± 3.3 (8)	49.6 ± 15.6 (8)	–13.3 ± 3.5 (7)**	ND

Each value is the mean ± SEM from the number of observations shown in parenthesis.
*P ≤ 0.05; **P ≤ 0.01 compared with mouse wild-type (WT) controls. ND, not determined.

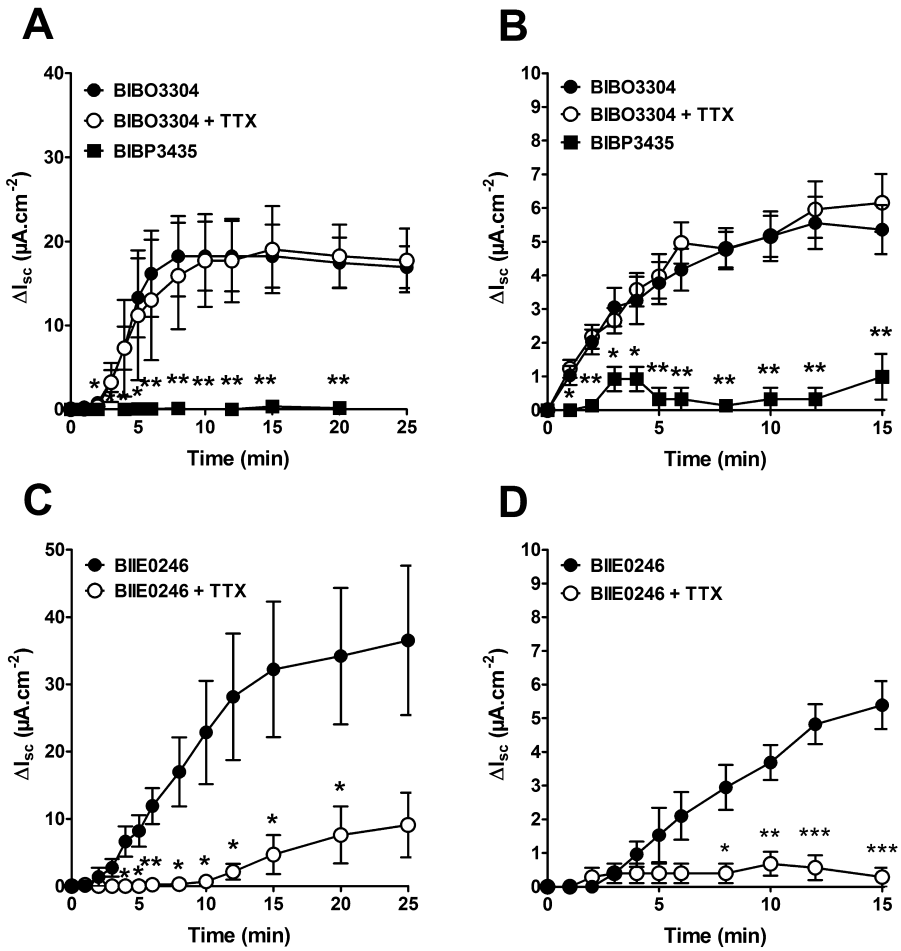

Figure 1

The effect of Y_1 and Y_2 receptor antagonists (BIBO3304; in A, B or BIIE0246 C, D added at $t = 0$ min) on basal levels of I_{sc} in human or mouse colon mucosae. Y_1 receptor blockade by BIBO3304 (300 nM) added to human mucosa (A) or wild-type (WT) mouse mucosa (B) increased I_{sc} and neither effect was altered by pretreatment with tetrodotoxin (TTX) (100 nM). There was no change in I_{sc} after addition of the inactive isomer of a Y_1 receptor antagonist, BIBP3435 (1 μM). Y_2 receptor antagonism with BIIE0246 (1 μM) was reduced significantly by pretreatment with TTX in human (C) and in WT mouse mucosa (D). Values are the mean ± SEM from 3–10 observations and statistical differences between control and experimental groups are shown (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

9.9 ± 3.4 μA·cm^{-2} (n = 3) compared with colonic (7.1 ± 1.3 μA·cm^{-2}, n = 15, $P > 0.05$) responses.

DPP IV inhibition selectively amplifies Y_2 antisecretory tone

Because DPP IV is the enzyme responsible for metabolizing NPY to NPY(3–36) [and to a lesser degree PYY to PYY(3–36) (Mentlein *et al.*, 1993)] thereby losing their affinities for the Y_1 receptor but maintaining activities at Y_2 receptors, and because DPP IV inhibitors are therapeutically proven as anti-diabetic therapies (Demuth *et al.*, 2005) we next set out to investigate whether DPP IV inhibition would modulate the tone revealed by either Y antagonist. First, the DPP IV inhibitor, compound 3 (Lankas *et al.*, 2005) added alone reduced basal I_{sc} levels in WT and PYY$^{-/-}$ colon (Table 1) and following vasoactive intestinal polypeptide this effect was increased significantly (WT: –3.3 ± 0.4 μA·cm^{-2}, n = 6, $P \leq 0.01$; PYY$^{-/-}$: –3.7 ± 0.9 μA·cm^{-2}, n = 8, $P \leq 0.05$); however, compound 3 was without effect *per se* in NPY$^{-/-}$ mucosa (Table 1). Y_1 receptor-mediated tone was insensitive to enzyme inhibition (Figure 2A and B) but Y_2 receptor-mediated tone was increased significantly in human and WT mouse colon following DPP IV blockade (Figure 2C and D). In WT mucosa pretreated with compound 3 and the Y_1 receptor antagonist, subsequent BIIE0246 effects were reduced compared with the responses to BIIE0246 alone (Figure 2D) indicating that Y_1 receptor mechanisms contribute to a proportion of Y_2 receptor-mediated antisecretory tone when DPP IV activity is inhibited.

PYY deletion abolishes Y_1 tone and partially inhibits Y_2 antisecretory tone

In order to establish which peptide primarily mediated each Y receptor tone, we assessed the relative loss of tone from NPY$^{-/-}$, PYY$^{-/-}$ or NPYPYY$^{-/-}$ colonic tissue. We included TTX in this assessment in order to determine whether either tone

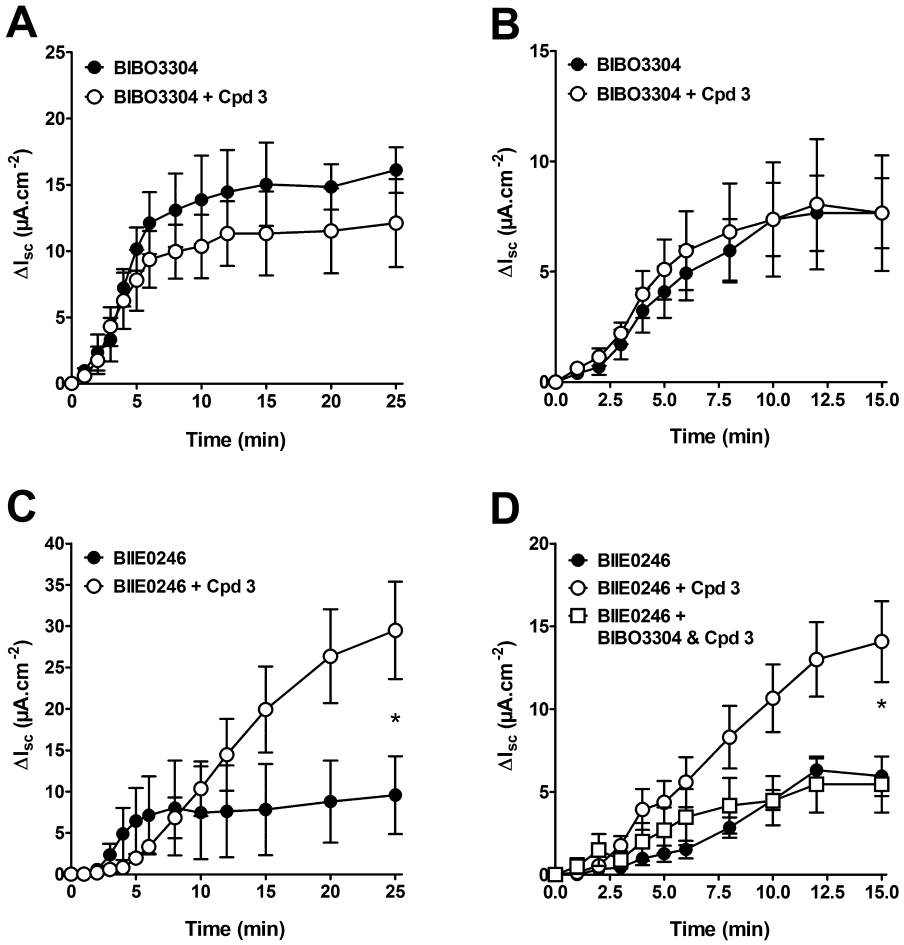

Figure 2

DPP IV inhibition does not affect Y_1 receptor-mediated antisecretory tone but it amplifies Y_2 tone. BIBO3304 (added at t = 0 min, 300 nM) increases in I_{sc} were insensitive to pretreatment with the DPP IV inhibitor, compound 3 (1 μM, +Cpd 3) in human (A) and mouse (B) colon mucosa. Significant amplification of Y_2 receptor-mediated tone was observed 25 min after BIIE0246 addition (1 μM throughout) to human mucosa (C) and 15 min after the Y_2 receptor antagonist was added to wild-type (WT) mouse mucosa (D). Additionally in D, pretreatment of mouse colon with BIBO3304 (300 nM) before BIIE0246 and Cpd 3, reduced I_{sc} levels to those after BIIE0246 alone. Values are the mean ± SEM from 4–8 observations and statistical differences between BIIE0246 alone and BIIE0246 + Cpd 3, are shown (*$P \leq 0.05$).

was neuronal (and therefore NPY-mediated) in origin. First, the effects of Y_1 receptor antagonism in $NPY^{-/-}$ colon were not statistically different from those in WT colon (Figure 3A). A proportion of Y_2 receptor-mediated tone was however significantly reduced in $NPY^{-/-}$ (between 3–5 min, Figure 3B). TTX-pretreatment of $NPY^{-/-}$ mucosa did not significantly alter Y_1 or Y_2 receptor-mediated tone (Figure 3A and B). In contrast, $PYY^{-/-}$ tissue exhibited significantly lower levels of Y_1 and Y_2 tone compared with WT colon (Figure 3C and D). The residual levels of Y_1 and Y_2 tone in $PYY^{-/-}$ mucosa were abolished by TTX (Figure 3C and D). $NPYPYY^{-/-}$ tissue was insensitive to both receptor antagonists (Figure 3A and B) indicating the absence of Y_1 and Y_2 receptor-mediated antisecretory tone from this null tissue. Because PYY deletion reduced Y_1 tone by ~90%, we concluded that PYY rather than NPY was responsible for Y_1 receptor-mediated tone. In contrast, Y_2 tone was inhibited similarly (50–60%) by ablation of either peptide. Neuronal mechanisms contributed more to Y_2 than to Y_1 receptor-mediated tone, as indicated by the significantly greater TTX sensitivity of Y_2 tone (Figure 3B and D) compared with Y_1 receptor-mediated antisecretory tone (Figure 3A and C).

Expression of Y_1 and Y_2 receptors in different layers of human and mouse colon

Y_1 and Y_2 receptor mRNA was observed in mucosa scraped from the luminal surface (Figure 4A and C) as well as intact mucosa (as used in Ussing chambers) of human colon. Mucosa from mouse ascending and descending colon expressed similar mRNA levels of each receptor (Figure 4B and D). Y_1 and Y_2 receptor mRNA expression was also observed in the submucosal layer of human colon specimens (Figure 4A and C) and in muscle layers from both species, particularly in mouse descending colon (Figure 4B and D).

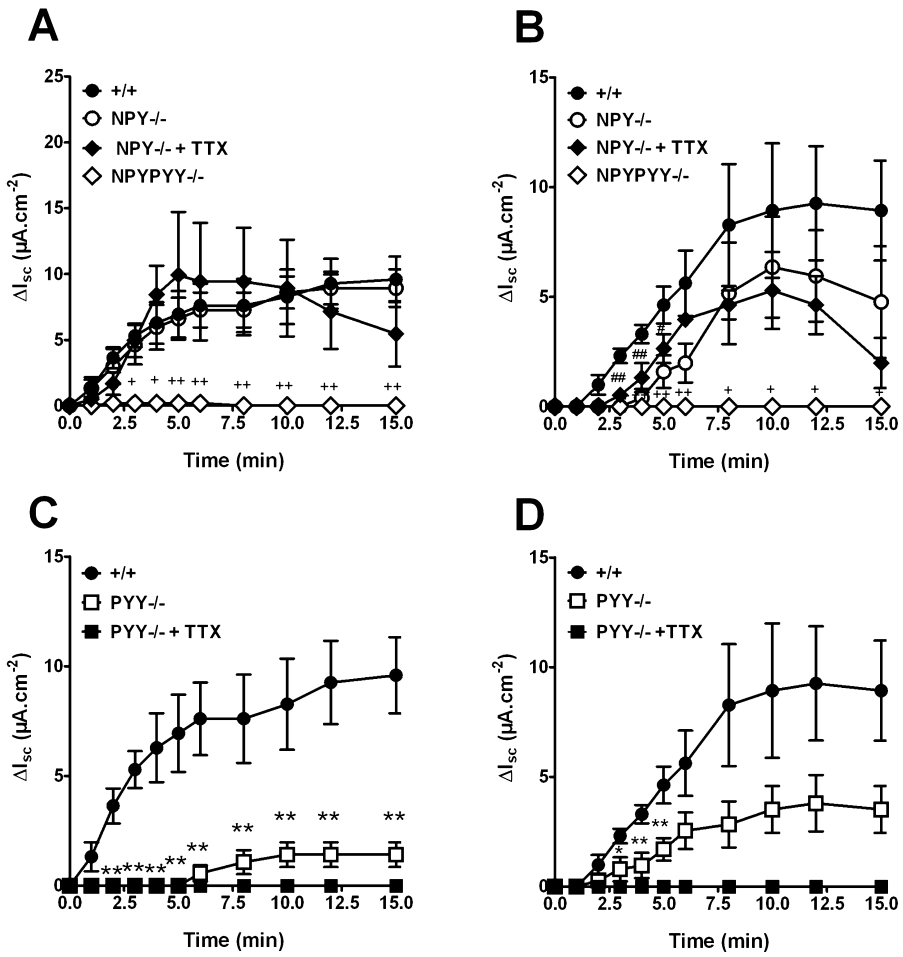

Figure 3

The effect of ablating NPY, PYY or both peptides on Y_1 (A, C) or Y_2 receptor-mediated antisecretory tone (B, D) in mouse colon mucosa compared with wild-type (WT) mucosa (+/+). In A: Y_1 receptor blockade (300 nM BIBO3304 added at $t = 0$ min) in WT and $NPY^{-/-}$ mucosa was similar, but was absent from $NPYPYY^{-/-}$ tissue. In B: Y_2 receptor blockade (BIIE0246, 1 μM) was partially lost in $NPY^{-/-}$ tissue (significantly so from 3–5 min, $\#P \leq 0.05$, $\#\#P \leq 0.01$) and was absent from $NPYPYY^{-/-}$ colon mucosa (significantly, $+P \leq 0.05$, $++P \leq 0.01$). In C: Y_1 antisecretory tone was lost from $PYY^{-/-}$ mucosa and residual Y_1 tone was abolished by tetrodotoxin (TTX) (100 nM) pretreatment. In D: Y_2 receptor-mediated tone was partially reduced in $PYY^{-/-}$ colon mucosa and abolished in this knockout tissue after addition of TTX. Points are the mean ± SEM from 3–7 observations with statistical differences between WT mucosa and peptide null mucosa as shown ($*P \leq 0.05$, $**P \leq 0.01$).

Colon smooth muscle contractility, transit and CMMCs

Given the Y_1 and Y_2 mRNA levels in muscle regions of WT mouse colon, and the presence of tonic mucosal Y_1 and Y_2 receptor-mediated activity, it was logical to establish whether tonic muscle activity was present and whether faecal pellet propulsion was dependent on the presence of either peptide. PYY (30 nM) or NPY (100 nM) added to WT ascending colon resulted in transient longitudinal muscle contractions (Figure 5A for PYY) that were unaltered in $NPY^{-/-}$, $PYY^{-/-}$ and $NPYPYY^{-/-}$ tissue (data not shown). Neither BIBO3304 (data not shown) nor BIIE0246 (Figure 5B) altered basal tension or spontaneous activity in WT colon, but the Y_2 receptor antagonist abolished subsequent PYY responses (Figure 5B and C) and both antagonists together produced a similar inhibition (Figure 5C, +Both). These data corroborate previous studies showing PYY(3–36)-mediated contraction of $Y_2^{+/+}$, but not $Y_2^{-/-}$ colonic muscle (Hyland *et al.*, 2003). Furthermore, inhibition of DPP IV resulted in an elevation of NPY-induced contractions; however, this was not statistically significant and PYY responses were unaltered (Figure 5D).

We next determined whether colonic transit *in vitro* differed between the genotypes or whether the Y antagonist sensitivity was altered in null, compared with WT tissues. First, colonic transit was elevated, although not significantly in $NPY^{-/-}$, $PYY^{-/-}$ and $NPYPYY^{-/-}$ compared with WT transit (Figure 6A). The Y_1 receptor antagonist, BIBO3304 reduced WT colonic transit and this inhibition was consistent across the genotypes (statistically so when comparing transit in $NPY^{-/-}$ control and Y_1 receptor antagonist-treated $NPY^{-/-}$ tissue; Figure 6A). In WT colon in contrast, the Y_2 receptor antagonist BIIE0246 increased transit significantly compared with BIBO3304's inhibition. In each of the peptide null

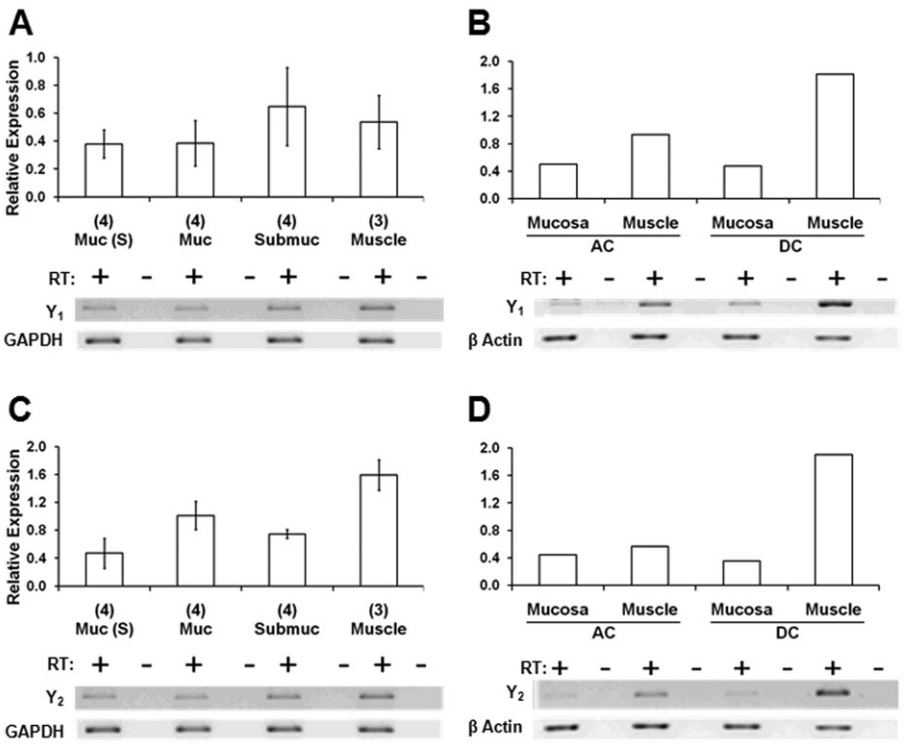

Figure 4

Y_1 receptor (A, B) and Y_2 receptor (C, D) expression in human (A, C) and mouse (B, D) colonic tissue. Semi-quantitative RT-PCR was performed to determine Y_1 or Y_2 receptor mRNA levels. GAPDH (379 bp) or β-actin (321 bp) housekeeping gene expression were used as loading controls. Band intensities were quantified by densitometry and the ratios of Y receptor/loading control are depicted. Values in A and C are means ± SEM (*n* as indicated). In B and D, values are pooled mRNA ($n = 1$) from 3 mouse colon samples. Muc, mucosa; (S), scraped; Submuc, submucosa; AC, ascending colon; DC, descending colon.

groups, however, Y_2 receptor blockade was consistently inhibitory and significantly so in $NPY^{-/-}$ colon (Figure 6A). *In vivo* assessment of stress-induced defaecation showed $NPY^{-/-}$ mice excreted significantly more faecal pellets than the other genotypes (Figure 6B). FPO in $NPY^{-/-}$ mice after acclimatization was 40% greater than in WT; however, this was not statistically significant, nor were the effects of blocking Y_1 or Y_2 receptors on FPO in WT or $NPY^{-/-}$ mice (Figure 6C).

In view of the significant effect of the Y_2 antagonist on colonic transit *in vitro*, we examined the effects of BIIE0246 on CMMCs, which are thought to represent a spontaneous analogue of the peristaltic contractions that propel faecal pellets (Dickson *et al.*, 2007; 2010). Luminal infusion of BIIE0246 into isolated WT colon had no effect on CMMCs (data not shown) but adding the same concentration (1 μM) of the Y_2 receptor antagonist to the serosal solution significantly and reversibly depressed the frequency of CMMCs (Figure 7A and B).

Upper GI transit in WT and peptide knockouts

Because PYY is a mediator of the 'ileal brake' it was important to establish how ablation of the peptide affected upper GI transit *in vivo*. $PYY^{-/-}$ mice exhibited a significantly increased transit (20%) compared with WT mice (Figure 8). This was not the case in $NPYPYY^{-/-}$ mice although $NPY^{-/-}$ mice exhibited transit intermediate between WT and $PYY^{-/-}$ rates. Upper GI transit was slightly increased, but not significantly so, after i.p. administration of the Y_1, or the Y_2 receptor antagonist to WT mice (Figure 8) indicating that endogenous PYY and NPY slow transit. These transit rates were not statistically different from those measured with either receptor antagonist in $NPY^{-/-}$ mice, although the peripherally acting BIIE0246 reduced $NPY^{-/-}$ transit to WT levels. The upper GI transit of $PYY^{-/-}$ mice was, however, inhibited to a similar degree by each Y receptor antagonist (two-way ANOVA highlighted a statistical difference between antagonism in WT and $PYY^{-/-}$ groups) indicating that endogenous NPY may contribute to the raised upper GI transit in $PYY^{-/-}$ mice.

Discussion

Competitive antagonism of Y_1 or Y_2 receptors in human and mouse colon mucosae results in prolonged I_{sc} elevation, revealing tonic antisecretory effects that involve endogenous PYY and NPY. The Y_1 receptor-mediated tone revealed by BIBO3304 was similar to that observed with BIBP3226, as shown previously (Cox and Tough, 2002), whereas

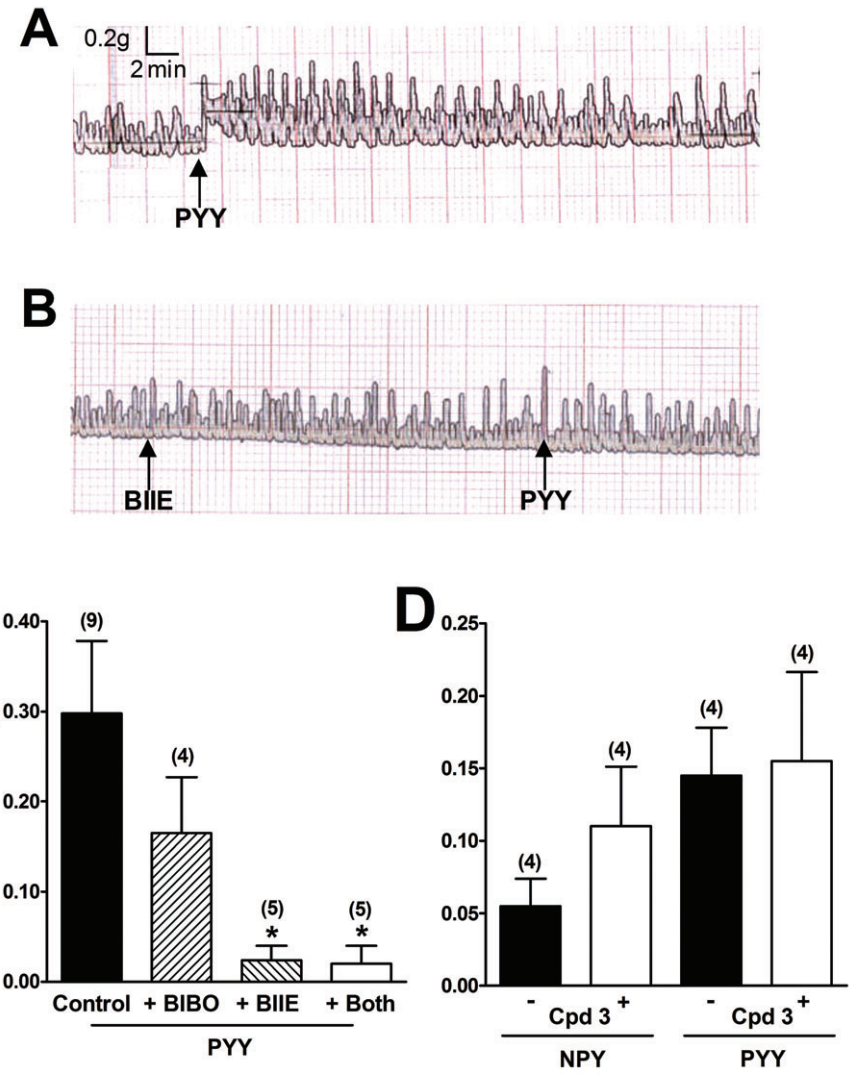

Figure 5

Contractile effects of PYY (30 nM) in wild type ascending colon longitudinal muscle (in A) were abolished by pretreatment with BIIE0246 (1 μM, B). Pooled data (in C) show significantly decreased PYY responses following BIIE0246 alone or in combination with BIBO3304 (300 nM, +Both) but not after BIBO3304 alone. In C, significant differences, $*P \leq 0.05$ are shown compared with control levels of isometric tension. In D, pretreatment of tissues with the DPP IV inhibitor, compound 3 (1 μM, Cpd 3) did not significantly increase contractile responses to either NPY (100 nM) or PYY (30 nM) ($n = 4$). Bars are the mean + SEM from n numbers shown in parenthesis.

BIBP3435, the inactive enantiomer of the latter antagonist, was without effect in the present study. Submucosal innervation was not required for Y_1 receptor-mediated tone, but is a prerequisite for Y_2 receptor-mediated tone. PYY mediates Y_1 tone as it is absent from $PYY^{-/-}$ mucosa. $NPY^{-/-}$ and WT tissue exhibit the same levels of Y_1 receptor-mediated tone and only when both peptides are ablated are Y_1 and Y_2 tonic activities lost. We therefore conclude that Y_1 antisecretory tone is predominantly epithelial in origin and PYY-mediated, while Y_2 receptor-mediated tone is neuronal and predominantly NPY-mediated although L cell derived PYY does contribute to Y_2 tone. NPY mediation of Y_2 tone is probably due to inhibition of a tonically active neural circuit in the mucosa with the source of NPY being one or more neurons in that circuit. As no input from the myenteric plexus or the extrinsic nerve supply is possible in mucosa/submucosa preparations, this tonic activity is likely to arise from recurrent excitatory circuits within the submucous plexus (Chambers *et al.*, 2005) with NPY released from these same submucosal neurons acting as an inhibitory brake. The PYY component of Y_2 receptor-mediated tone may be via an action at secretomotor nerve terminals in the mucosa, thus depressing their output (Hirai *et al.*, 1997). NPY and PYY can hyperpolarize colonic submucosal neurons (Hirai *et al.*, 1997) as well as myenteric neurons (Browning and Lees, 2000) most probably via Y_2 receptors, although Y_1 receptors have been identified on mucosal enteric nerves in human colon (Mannon *et al.*, 1999). In combination with Y_1 receptor-linked PYY released from L cells, Y_1 and Y_2 receptor-mediated tonic activities result in significant antisecretory effects (Cox,

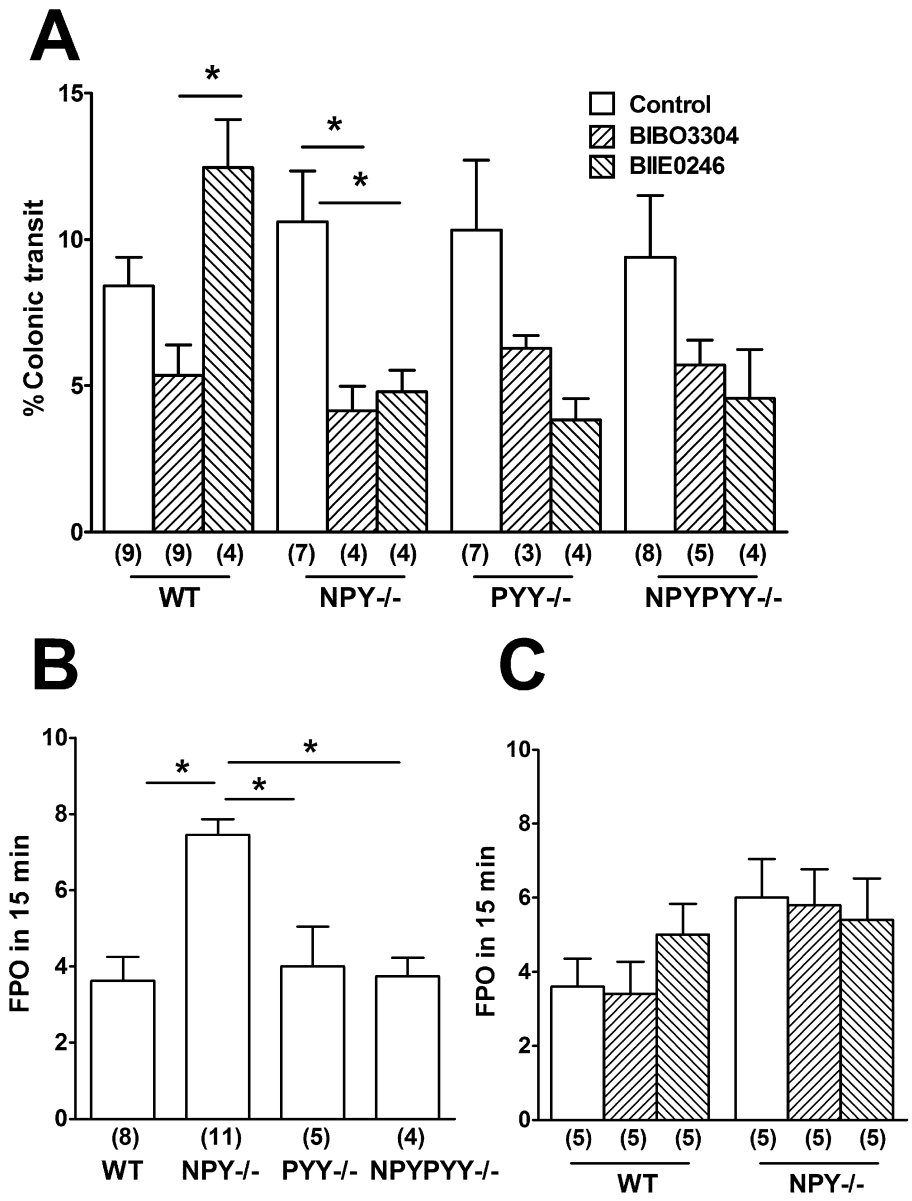

Figure 6

Colonic transit measurements *in vitro* (A) and *in vivo* (B, C) in wild-type (WT) and peptide knockout mice, in the absence or presence of Y_1 or Y_2 receptor antagonists Values are the mean + SEM from mice of different genotypes. In A, Y_1 receptor blockade (BIBO3304, 300 nM) inhibited colonic transit in all the genotypes but this was only significantly different from vehicle controls in $NPY^{-/-}$ colon ($^*P \leq 0.05$). Y_2 receptor antagonism with BIIE0246 (1 μM) significantly increased WT colonic transit compared with the effect of BIBO3304 (one-way ANOVA, $^*P \leq 0.05$). This difference in WT tissues between antagonist effects was absent from all peptide null mice, significantly so in $NPY^{-/-}$ ($P \leq 0.05$, two-way ANOVA). In B, the FPO observed in $NPY^{-/-}$ mice was significantly increased compared with all other genotypes after 15 min in a novel environment ($^*P \leq 0.05$, two-way ANOVA). In C, acclimatized, vehicle-treated, $NPY^{-/-}$ mice exhibited a trend for greater FPO, but this and the effects of Y_1 or Y_2 receptor antagonists were not significantly different from WT rates of defaecation (two-way ANOVA).

2007) and both receptor types have the potential to protect against diarrhoea.

We hypothesized that increasing the stability of endogenous NPY, and to a lesser degree PYY, by inhibiting DPP IV activity (Mentlein *et al.*, 1993) would alter the balance between Y_1 and Y_2 receptor-mediated tone in favour of the former. However, Y_1 tone was not affected by DPP IV inhibition and we conclude that endogenous Y_1 agonism is maximal in colonic mucosa, so increasing the stability of endogenous NPY (and PYY) cannot further increase Y_1 receptor-mediated activity. In contrast, DPP IV inhibition significantly increased Y_2 tone in human and mouse colon mucosa, indicating that this tonic activity may not be maximal when the enzyme is active. However, when both DPP IV and Y_1 receptors were blocked, inhibition of Y_2 receptors was less effective in increasing I_{sc} than in the presence of

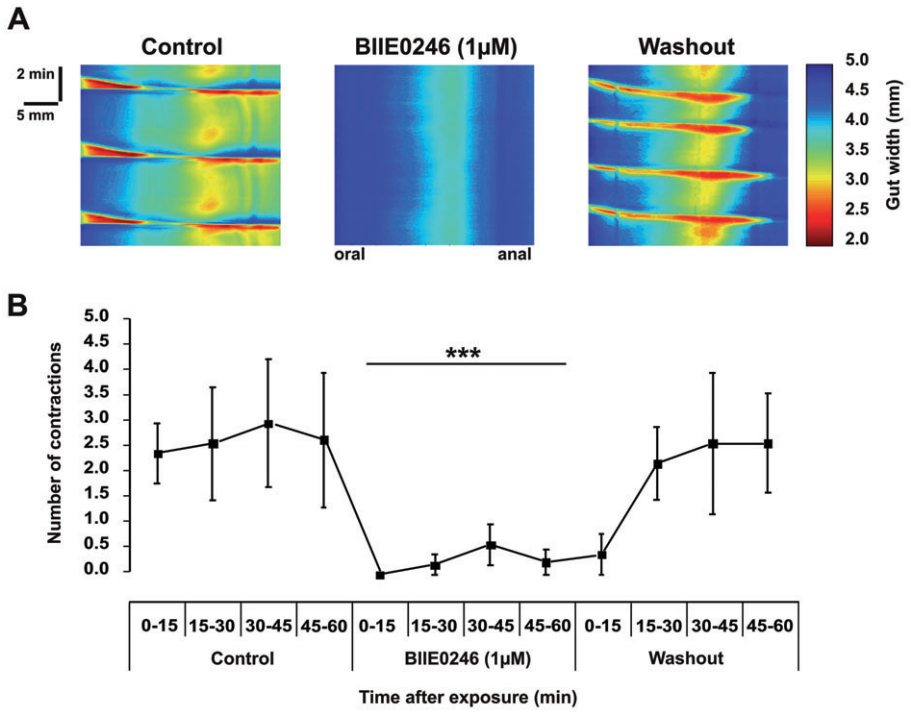

Figure 7

Spatiotemporal maps and contraction frequency in the mouse colon in response to the Y_2 receptor antagonist, BIIE0246. In A, spatiotemporal maps illustrating contractile activity (whole length colonic contractions) in physiological saline (control), BIIE0246 (1 μM) and following washout. In B, total whole-length contraction number observed during control conditions, with BIIE0246 (1 μM) and washout periods. Note that in these maps, colonic diameter is colour coded with red indicating maximally constricted and blue indicating maximally dilated, and time increases downward from the top of each map. ***$P \leq 0.001$, significant inhibition of numbers of contractions.

the DPP IV inhibitor alone. Thus, a proportion of the DPP IV-elevated Y_2 tone is Y_1 receptor-mediated in WT mouse mucosa. We suggest that together these mechanisms may contribute to the constipatory side effect of the DPP IV inhibitor, vildagliptin (Lauster *et al.*, 2007).

Peptide YY and NPY contract mouse longitudinal smooth muscle via Y_2 receptors as we observed significant inhibition of these responses following Y_2, but not Y_1 receptor antagonism. As activation of Y_2 receptors would be expected to inhibit neural activity, the most likely explanation for the contractions is inhibition of a tonic inhibitory drive to the longitudinal muscle. This might be due to either Y_2 receptors on inhibitory longitudinal muscle motor neurons, although such neurons appear to be sparse in the mouse colon (Sang and Young, 1996) or an action on the, as yet unidentified, neural circuit that maintains that drive. Y_2 receptors have been localized to many terminals within the myenteric plexus of mouse colon (Wang *et al.*, 2010) suggesting that the latter explanation might be more likely, but as yet the identities of the neurons expressing Y_2 receptors are unknown.

Similar levels of Y_1 and Y_2 receptors are expressed in muscle layers (with embedded myenteric nerves) as confirmed by our semi-quantitation of Y_1 and Y_2 receptor gene expression, but Y_1 receptor antagonism did not significantly reduce longitudinal contractile responses to PYY. Y_1 receptors have been identified on mucosal enteric nerves (Mannon *et al.*, 1999) and cell bodies of nitrergic myenteric neurons (Peaire *et al.*, 1997), most of which are circular muscle motor neurons (Sang and Young, 1996). We found that Y_1 receptor antagonism inhibited colonic transit in WT and peptide null mice and conclude that this inhibition results from blockade of tonic Y_1 receptor-mediated motility. Activation of Y_1 receptors on nitrergic motor nerves would most likely cause hyperpolarization and thus inhibit these neurons. The presence of Y_1 receptor antagonists would prevent this tonic inhibition, inducing relaxation and a slowing of transit. This mechanism is apparently unaltered in single and double peptide null colon, which raises the question of the identity of the endogenous ligand, and it is also notable that tonic Y_1 receptor-mediated antisecretory activity in $NPY^{-/-}$ colon mucosa is the same as WT activity. In rat colon, tonic Y_1 receptor-mediated inhibition of ascending substance P-mediated contractions has been described (Grider and Langdon, 2003). In our studies, loss of PYY or NPY *per se* had no significant effect on basal colonic transit, but a significant increase in pellet transit with the Y_2 receptor antagonist was observed *in vitro*, indicating that in the absence of extrinsic innervation there is a tonic Y_2 receptor-mediated inhibition of this complex motor pattern. The tonic Y_2 receptor-mediated inhibition of motility is absent from each peptide knockout, unlike the intramural Y_1 receptor-mediated effects, and we conclude that the tonic activation of Y_2 receptors depends on both NPY and PYY.

The Y_2 receptor-enhanced colonic motility observed in the current study is consistent with recent findings, which

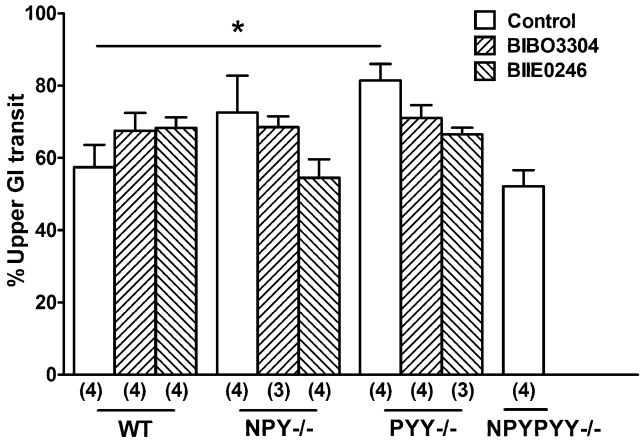

Figure 8

Upper GI transit *in vivo* in wild-type (WT), single and double peptide null mice following vehicle (control) or Y_1 or Y_2 receptor antagonists. Transit was increased significantly in $PYY^{-/-}$ compared with WT transit rates (Student's unpaired *t*-test; $*P \leq 0.05$). The effects of Y_1 (BIBO3304, 0.4 mM·100 μL^{-1}, i.p.) or Y_2 (BIIE0246, 60 $\mu g \cdot 100\ \mu L^{-1}$, i.p.) receptor antagonists on transit are shown for each genotype. There were no statistical differences between antagonist-treated transit rates and controls in WT, $NPY^{-/-}$ or $PYY^{-/-}$ mice. However, the inhibitory effects of both antagonists in $PYY^{-/-}$ were significantly different from the slight prokinetic effects of both antagonists in WT controls (two-way ANOVA, no asterisk shown). Each bar is the mean + SEM from number of assays in parenthesis.

show exogenous PYY and the Y_2 receptor agonist PYY(3–36) (but not a Y_1 agonist) slow both the increased defaecation induced by novel environmental stress and the enhanced colonic transit induced by tryptophan in conscious WT mice (Wang *et al.*, 2010). Moreover, PYY(3–36) reduces colonic hypersecretion and motility induced by prostaglandin E_2 (Moriya *et al.*, 2010). Neither study identified tonic Y_1 or Y_2 receptor activity, and we also observed that conscious $NPY^{-/-}$ mice defaecated significantly more pellets than the other genotypes when placed in a novel environment in this study, in line with their anxiogenic phenotype (Karl *et al.*, 2008).

In marked contrast to the effects of BIIE0246 on natural colonic pellet propulsion *in vitro*, we found that this antagonist virtually abolished spontaneous CMMCs in isolated murine colon. This was only seen when the antagonist was applied serosally, suggesting that the Y_2 receptors blocked were in the myenteric plexus. It also indicates that mucosally released PYY is not acting on Y_2 receptors on the mucosal terminals of intrinsic sensory neurons and this inability to activate Y_2 receptors on these or other components of the enteric neural circuitry, may contribute to the lack of effect of Y_2 receptor blockade on FPO in $NPY^{-/-}$ mice.

It has been suggested strongly that CMMCs are the basic mechanism responsible for faecal pellet propulsion in the mouse colon (Dickson *et al.*, 2007; 2010; Heredia *et al.*, 2009) but our data suggest that these two processes can be dissociated pharmacologically. While the mechanisms responsible for CMMC initiation are still under question, with conflicting reports about whether removal of mucosa prevents spontaneous initiation (Dickson *et al.*, 2010; Keating *et al.*, 2010), there is no disagreement that faecal pellets act to release 5-HT from the mucosa to enhance propulsion (Heredia *et al.*, 2009). Computer simulation indicates that propagation of migrating activity patterns like the CMMC depends on activity-dependent inhibition within these networks (Thomas *et al.*, 2004). Thus, if neurally released NPY is involved in this activity-dependent inhibition, then Y_2 receptor antagonism would block initiation and propagation of CMMCs from the proximal part of the colon. By contrast, because faecal pellets stimulate 5-HT release, they can activate local enteric circuitry responsible for propulsion and blocking neuronal Y_2 receptors would allow this circuitry to operate more powerfully, thereby enhancing propulsion.

Peptide YY ablation selectively and significantly increases upper GI transit. However, double peptide knockout results in a basal rate the same as that of WT mice as is the case for colonic transit. This may be due to lack of both peripheral (PYY's inhibition of gastric emptying and upper GI transit; Savage *et al.*, 1987; Lin *et al.*, 1996; Maljaars *et al.*, 2008) and central mechanisms (NPY's activation of the dorsal vagal complex to increase gastric emptying; Chen *et al.*, 1997, and hypothalamic stimulation of colonic motility; Mönnikes *et al.*, 2000) that negate each other. In upper GI and colonic transit assays *in vivo*, the effects of BIBO3304 and BIIE0246, given i.p., are predominantly peripheral as both antagonists exhibit minimal entry into the CNS (H.N. Doods, pers. comm.). Neither antagonist had a significant effect on WT or $NPY^{-/-}$ mouse upper GI transit, while the more rapid rate of $PYY^{-/-}$ mice was sensitive to both Y receptor antagonists. We conclude, first, that in $PYY^{-/-}$ mice NPY can contribute to the peripheral Y_1- and Y_2 receptor-mediated motility and, second, that in WT mice endogenous PYY plays a more significant (Y_2 receptor-mediated) inhibitory role than NPY.

In conclusion, endogenous NPY and PYY exert discrete but consistent inhibitory effects that are similar in human and mouse colon mucosae. In both, NPY mediates antisecretory tone predominantly via neuronal Y_2 receptors, although the exact identity of the submucosal neurons (and Henle's plexi in human colon) remains unclear. PYY co-activates epithelial Y_1- and neural Y_2 receptor-mediated antisecretory effects and the absence of PYY and NPY renders colonic mucosa incapable of displaying either antisecretory tone. Upper GI transit, on the other hand, appears to depend on PYY rather than NPY, while colonic transit depends on both peptides acting via Y_2 receptors to slow transit. Diarrhoea and constipation are a combination of mucosal and motor function and the absence of PYY or NPY results in a pro-secretory state, while PYY deletion results in increased upper GI transit. The link between endogenous PYY and its cognate Y_1 and Y_2 receptors may help explain why patients with PYY-expressing tumours can suffer from severe constipation (Matsuda *et al.*, 2002) while surgical removal of PYY-rich distal colon frequently results in protracted post-operative diarrhoea (Imamura, 2002) resembling the functional phenotype we describe for $PYY^{-/-}$ mice.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Human and mouse primers for RT-PCR semi-quantification of target genes

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