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Agonism of free fatty acid receptors 1 and 4 generate peptide YY-mediated inhibitory responses in mouse colon

Short title: FFA1 and FFA4 activities in mouse colon

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RM, IRT and HMC have nothing to disclose. DMS and MS are currently employed by and hold shares in AstraZeneca Plc.

Background and purpose:

Free fatty acid receptor 1 (FFA1) and FFA4 are located on enteroendocrine L cells with the highest gastrointestinal (GI) expression in descending colon. Their activation causes the release of glucagon-like peptide 1 and peptide YY (PYY) from L cells. Additionally, FFA1 agonism releases insulin from pancreatic β-cells. As these receptors are modulators of nutrient-stimulated glucose regulation, this study's aim was to compare the pharmacology of commercially available agonists (TUG424, TUG891, GW9508) with proven selective agonists (JTT, TAK-875, AZ423, Metabolex-36), in the mouse.

Experimental approach:

Mouse (>10 weeks old; C57BL/6J) mucosa was mounted in Ussing chambers, voltage-clamped and the resultant short-circuit current (I_{sc}) recorded continuously. The pretreatments included FFA1 antagonists or $Y_1 \pm Y_2$ antagonists. Glucose-sensitivity was investigated by mannitol replacement apically and colonic and upper GI transit was assessed *in vitro* and *in vivo*.

Key Results:

FFA1 and FFA4 agonism required glucose and reduced I_{sc} in a PYY-Y_1-dependent manner. The novel compounds were more potent than GW9508. The FFA1 antagonists (GW1100 and ANT825) blocked FFA1 activity only and revealed FFA1 tonic activity. The FFA4 agonist, Metabolex-36 slowed colonic transit *in vitro* but increased intestinal transit *in vivo*.

Conclusion and Implications:

The selective FFA1 and FFA4 agonists were more potent than GW9508, a dual FFA1 and FFA4 agonist. We found that a PYY-Y_1 paracrine epithelial mechanism mediated their responses which were glucose-sensitive, potentially limiting hypoglycaemia. ANT825 revealed tonic activity and the possibility of endogenous FFA1 ligands causing PYY release. Finally, FFA4 agonism induced regional differences in transit.

Keywords: Free fatty acid receptors 1 and 4, peptide YY, glucagon-like peptide 1, mucosal ion transport, colonic transit

Abbreviations

ANOVA, analysis of variance;
ap, Apical;
AZ, AstraZeneca;
BIBO, BIBO3304;
BIIE, BIIE0246;
bl, Basolateral;
CCK, Cholecystokinin;
CNS, Central Nervous System;
DMSO, dimethyl sulphoxide;
FFA, free fatty acid;
FFA1, free fatty acid receptor 1;
FFA4, free fatty acid receptor 4;
GI, gastrointestinal;
GIP, Gastric inhibitory peptide;
GLP-1, glucagon-like peptide 1;
GLP-2, glucagon-like peptide 2;
GPCR, G protein-coupled receptor;
GSIS, Glucose stimulated insulin secretion;
HPMC, Hydroxylpropyl methylcellulose;
i.p., Intraperitoneal;
I_{sc}, Short-circuit-current;
KH, Krebs Henseleit;
KO, Knockout;
MC4 receptor, Melanocortin-4 receptor;
Met-36, Metabolex-36;
MMC, Myoelectric migrating complex;
OXM, oxyntomodulin;
Phlor, Phloridzin;
PLC, Phospholipase C;
PVP, polyvinylpyrrolidinone;
PYY, peptide YY;
PYY (3-36), peptide YY(3-36);

SDS, sodium dodecyl sulfate;
SGLT1, Sodium-glucose co-transporter 1;
SST, Somatostatin;
T2DM, type 2 diabetes mellitus;
UGIT, Upper gastrointestinal transit;
VIP; vasoactive intestinal polypeptide;
WT, wild type

Introduction

Free fatty acid receptors 1 (FFA1, previously known as GPR40) and 4 (FFA4, previously known as GPR120) are modulators of nutrient-stimulated glucose regulation and therefore are therapeutic targets for the treatment of type 2 diabetes mellitus (T2DM). FFA1 selective agonists have been shown to cause glucose-sensitive insulin secretion (Tsujihata et al. 2011). FFA1 and FFA4 were deorphanised in 2003 and 2005 respectively, as receptors for medium to long chain unsaturated fatty acids (Hirasawa et al. 2005, Itoh et al. 2003). As endogenous free-fatty acids (FFAs) often activate more than one receptor type (Kostenis 2004) and are metabolised to act as intracellular signalling molecules (Warnotte et al. 1994), they have limited utility as selective tools to establish the pharmacology and functional significance of specific FFA receptors. The recent discovery of selective, small molecule agonists for FFA1 and FFA4 therefore provided a pharmacological advantage over non-selective endogenous FFAs and this study set out to utilise some of these new tool compounds to investigate FFA1 and FFA4 signalling specifically.

Apart from FFA1 and FFA4 there are alternative FFA receptors in the gastrointestinal (GI) tract including, FFA2 (formerly GPR43), FFA3 (GPR41) and the acylethanolamide receptor, GPR119, all of which are selectively expressed in L cells together with FFA1 and FFA4 (Overton et al. 2006, Hirasawa et al. 2005, Edfalk et al. 2008, Karaki et al. 2006). Both FFA1 (Briscoe et al. 2003) and FFA4 (Hirasawa et al. 2008) have the capacity to couple to $G\alpha_{q/11}$, activate phospholipase C (PLC) and elevate Ca^{2+} levels, leading to the release of L cell peptide hormones. Colonic L cells contain peptide YY (PYY), the incretin glucagon-like peptide 1 (GLP-1) plus GLP-2 and oxyntomodulin (OXM) (Habib et al. 2013, Egerod et al. 2012, Cho et al. 2014). Additionally, small intestine L cells express neurotensin and cholecystokinin (CCK) (Egerod et al. 2012, Habib et al. 2013). In this study, we utilised the descending colon because this colonic region exhibits a greater degree of PYY-Y_1 signalling compared with the ascending colon (Cox et

al. 2001) and L cell density is greater in the distal colon in mouse (Arantes and Nogueira 1997) and human (Adrian et al. 1985). GLP-1 slows gastric emptying and enhances satiety (Little et al. 2006) while PYY is a well-known mediator of the ileal and colonic brake (Lin et al. 1996) and a major satiety-inducing agent together with its product, $PYY_{(3-36)}$ (Batterham et al. 2002). PYY is also a significant inhibitor of intestinal epithelial ion transport and we have previously utilised this activity to monitor the acute endogenous peptide functionality following L cell-specific stimulation with GPR119 agonists (Cox et al. 2010, Patel et al. 2014) or other L cell mediated mechanisms (Joshi et al. 2013, Panaro et al. 2014). In mouse and human distal colon, local mucosal PYY antisecretory actions are predominantly mediated via epithelial Y_1 receptors with a smaller component of submucosal neuron Y_2 activity (Tough et al. 2006, Tough et al. 2011), that together underpin endogenous PYY paracrine responses. FFA1 and FFA4 are also potential nutrient-sensing receptors in L cells and therefore are possible targets for the treatment of T2DM and obesity (Engelstoft et al. 2008).

The FFA1 agonist TAK-875 (Negoro et al. 2010), which in phase II trials lowered blood glucose without apparently increasing GLP-1 levels, was shown to signal via $G\alpha_{q/11}$ alone, in a manner similar to the proposed endogenous agonist, α-linolenic acid. In type II diabetic rats, TAK-875 improved glucose tolerance and enhanced glucose-dependent insulin secretion (Tsujihata et al. 2011). Despite the liver toxicity concerns of TAK-875, it significantly improved glycaemic control in diabetic Japanese patients in a phase III study (Kaku et al. 2015). JTT, a novel selective FFA1-targeted agonist (from patent number, WO 2009/054479), TAK-875 and the commercially available FFA1 agonist, TUG424 (Christiansen et al. 2008) were selected to assess the pharmacology of FFA1 in this study.

FFA4 is also found abundantly in gastric-inhibitory peptide (GIP)-containing K cells of the small intestine, gastric somatostatin (SST)-containing D cells and gastric ghrelin-containing A cells (Ito et al. 2009, Parker et al. 2009, Engelstoft et al. 2013, Egerod et al. 2015). The functional roles of FFA4 include enhanced insulin secretion, GLP-1 secretion (Halder et al. 2013), reduced gastric emptying (Little et al. 2006) and anti-inflammatory effects (Oh et al. 2010). FFA4 knockout (KO) mice exhibited a significantly reduced L cell GLP-1 secretion (Xiong et al. 2013) and K cell GIP secretion (Iwasaki et al. 2015) indicating that signalling via FFA4 in wild type (WT) tissues can cause incretin peptide release. The novel selective agonists selected for this study, AZ423 (Compound 34, McCoull et al. 2017) and Metabolex-36 (Ma et al. 2010), show 100-fold selectivity for murine FFA4 compared with FFA1 (Stone et al. 2014). Furthermore, AZ423 and

Metabolex-36 have exhibited a reduction in blood glucose excursion in an oral glucose tolerance test (Halder et al. 2013, McCoull et al. 2017). Additionally, the selected commercially available FFA4 agonist, TUG891 is selective for murine and human FFA4 (Hudson et al. 2013). Whether intestinal FFA4 agonism resembles FFA1 activities and is also glucose-sensitive in enteroendocrine L cells has yet to be determined.

As few functional studies have been performed in native preparations containing L cells, one aim of this study was to determine the potencies and efficacies of the commercially available agonists (TUG424 and TUG891) compared with the non-selective FFA1 and FFA4 agonist GW9508, and importantly the novel selective agonists JTT, TAK-875, AZ423 and Metabolex-36 in mouse colon. We also assessed the selectivity of the FFA1-preferring antagonist GW1100 (Briscoe et al. 2006) comparing it with ANT825, a selective FFA1 antagonist (referred to as compound 39 in Waring, 2015) and determined their relative abilities to reveal tonic FFA1 activity. We also determined the involvement of endogenous PYY in these responses in colonic mucosae by blocking peptide activity pharmacologically. Finally, the effects of FFA1 and FFA4 agonism upon colonic motility *in vitro* and *in vivo* were established.

Methods

Mucosal preparation and I_{sc} measurement

Mice (C57BL/6J, 12-20 weeks old, 20-30g, female and male) were procured from Charles River Laboratories (Margate, UK) had free access to standard chow (Rat and Mouse No 3 breeding diet, Special Diets Services, Braintree, UK) and water *ad libitum*. We used mice because the Y_1, Y_2 and Y_4 receptor localisation in the GI tract is the same in this species as in human colon (Cox and Tough. 2001, Tough et al. 2011). Mice were housed in open top conventional cages with Lignocel poplar bedding material along with the appropriate environmental enrichment. A maximum of 5 mice were housed in a single cage. Housing rooms were maintained at 20-24 °C, humidity 55% ± 10% and 12/12 h light and dark cycle. All animal care and experimental procedures complied with the Animals (Scientific procedures) Act 1986 and were approved by UK Home Office (licence number: PPL70/7887). Mice were killed by cervical dislocation and the whole colon was dissected (noting proximal and distal ends) and placed in fresh Krebs-Henseleit (KH), with the following composition (in mM); NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-glucose 11.1. Mucosae from the descending colon were prepared by removing the

overlying smooth muscle layers and associated myenteric plexi by micro-dissection. Mucosal lengths were cut in to 4-6 adjacent pieces, each of which was mounted between two halves of an Ussing chamber (WPI UK, Hitchin, Herts, UK) (exposed area, 0.14 cm^2), voltage-clamped at 0 mV (DVC1000 and amplifiers; WPI UK, Hitchin, Herts, UK) and equilibrated in oxygenated KH (95% O_2/5% CO_2, at 37˚C) for 15 min. Drug additions were made to either the basolateral (bl) or apical (ap) reservoirs and changes in short-circuit current (I_{sc}) were measured continuously.

Sidedness, potency and efficacy of the FFA1 and FFA4 compounds

Mucosae were pretreated with vasoactive intestinal polypeptide (VIP, 30 nM, bl) to provide a degree of vectorial epithelial ion secretion upon which subsequent antisecretory signalling is more readily observed (as optimised previously; Tough et al. 2006; Cox et al. 2010). Once VIP responses had reached a maximum, a FFA1 or FFA4 agonist (TUG424 (300 nM), TUG891 (300 nM), GW9508 (10 μM)) was added to either reservoir as indicated, to determine the approximate sidedness of the FFA1 and FFA4 responses. Single additions of apical FFA1 or FFA4 agonists were used to construct concentration-response curves and calculate single EC_{50} values with GraphPad Prism v6.0. Changes in I_{sc} to certain FFA1 agonists (TAK-875, JTT and GW9508) and FFA4 agonists (Metabolex-36, AZ423) were biphasic; the first component (1˚) being a transient increase in I_{sc} (within 5 min) followed by a longer-lasting reduction in I_{sc} (the second component, 2˚) that was extrapolated from the waning VIP signal and reached its maximum within 10-15 min of FFA drug addition. When the biphasic changes in I_{sc} were observed, the 1˚ and 2˚ components were analysed separately. To investigate whether there was a neuronal component to FFA1 (TAK-875, 200 nM) and FFA4 (Metabolex-36, 100 nM) signalling, mucosae were pretreated with the neurotoxin, tetrodotoxin (TTX, 100 nM, bl) or vehicle (H_2O) for 15 min, followed by VIP and subsequently the FFA1 or FFA4 agonist. The responses to TAK-875 and Metabolex-36 were monitored for 20 min and finally PYY (10 nM, bl) was added as an internal control. Changes in I_{sc} to each drug or peptide were pooled and converted to $\mu A.cm^{-2}$.

Establishing the selectivity of FFA1 and FFA4 agonism using antagonists

In order to establish an IC_{50} of the FFA1 antagonist, ANT825, basal I_{sc} levels were allowed to stabilise before the addition of varying single concentrations (1 nM - 10 μM) of the FFA1 antagonist. After 10-15 min, VIP was added, followed by the selected FFA1 agonist, JTT (300

nM) and consequent reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. PYY (10 nM) was added finally as an internal control. The antagonist concentration (10 µM, ANT825) that abolished JTT responses was used in subsequent selectivity studies.

Blockade of the commercially available FFA1 agonists (TUG424, GW9508 and TAK-875) was confirmed using the antagonists, GW1100 (10 µM) (Briscoe et al. 2006) and ANT825 (10 µM). Here mucosae were pretreated apically for 10 min with the appropriate vehicle (0.1% DMSO) or the chosen FFA1 antagonist prior to VIP addition. TUG424 (100 nM), GW9508 (1 µM) were the FFA1 agonists tested initially, followed by TAK-875 (200 nM) and JTT (300 nM) while TUG891 (100 nM), Metabolex-36 (100 nM) and AZ423 (100 nM) were used to preferentially activate FFA4 receptors unless otherwise quoted. Finally, PYY (10 nM) was added as an internal control. ANT825 was chosen as the preferred FFA1 antagonist in further studies.

Y_1 and Y_2 receptor antagonist (BIBO3304 and BIIE0246) studies

Tissues were pretreated with previously optimised concentrations of the Y_1 antagonist, BIBO3304 (BIBO, 300 nM, bl) or the Y_2 antagonist, BIIE0246 (BIIE, 1 µM, bl) or both, versus their respective DMSO (0.003-0.1 %) controls. After 10-15 min VIP was added and once this response had stabilised, a single apical concentration of the FFA1 or FFA4 agonists was added and the consequent reduction in I_{sc} was converted to $\mu A.cm^{-2}$. PYY and the α_2-adrenoceptor agonist, UK14,304 (1 µM) were used as internal controls.

Mucosal glucose-sensitivity studies

Excised whole colon was placed in fresh glucose-free KH with the following composition (in mM); NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-mannitol 11.1. Mucosae were dissected as described above, placed in Ussing chambers and were bathed in KH containing glucose on both sides or replacing glucose with mannitol (at 11.1 mM) on the ap side only. When investigating FFA1 and FFA4 agonism in the presence of lower or higher glucose; the glucose concentration was altered (either 5 mM or 25 mM in KH) on ap and bl sides simultaneously. Mucosal basal I_{sc} levels were allowed to stabilise before the addition of VIP and once stabilised, a single apical addition of the FFA1 or FFA4 agonists was added and consequent reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. Phloridzin (50 µM, ap), the sodium-

glucose co-transporter 1 (SGLT1) inhibitor was used to block apically located SGLT1 and this electrogenic response should be lost when mannitol replaced apical glucose.

Faecal pellet propulsion in vitro

The colon (from the caeco-colonic junction to the rectum) was excised, photographed (t = 0 min) and bathed in KH at 37˚C with either vehicle (0.1% DMSO) or agonist (300 nM TUG424, TUG891, TAK-875 or Metabolex-36) as indicated. After 20 min (t = 20 min) each colon length was re-photographed and the distance travelled by the remaining pellets was measured from the rectum, as described previously (Tough et al. 2011). The effects on transit of the agonists were compared in the absence and presence of the FFA1 antagonist, GW1100 (10 μM). In these experiments, the colon was bathed in vehicle (0.1% DMSO) or antagonist, photographed at 20 min, and then bathed in KH containing the agonist of choice for 20 min. The colon was re-photographed at t = 40 min. The pellet movement was measured as mean distance travelled relative to the total colonic length and was used to calculate the % colonic transit.

Colonic bead excretion in vivo

Mice were acclimatised to handling 3 days prior to experimentation and were fasted 16 h prior to testing, although water was provided *ad libitum*. Plasma glucose before the fast was 10.5 ± 0.9 mmol/l (mean ± 1 SEM, *n*=9) and 4.8 ± 0.3 mmol/l after fasting (*n*=9). Distal colonic propulsion was measured according to the methods described by Forbes et al (2012). One hour after administration of vehicle, or drug (FFA1 agonist, TAK-875; FFA4 agonist, Metabolex-36, FFA1 antagonist, ANT825) or the positive control (loperamide hydrochloride (HCl) by oral gavage or intraperitoneal (i.p.) injection, mice were placed under 4-5% isoflurane anaesthesia (Isoflurane-VET, Merial Animal Health Ltd, Harlow, UK) and a 2 mm bead was inserted 2 cm intrarectally into the distal colon using blunt tubing (Portex, 1.7 × 0.4 mm). The mice were subsequently placed into a grid bottom cage, monitored and the time to bead expulsion was measured. Once the bead was excreted, the mouse was killed by cervical dislocation. All drugs were suspended in their respective vehicles and sonicated for 30 min.

Upper GI transit in vivo

Mice were acclimatized to handling 3 days prior to experimentation and were fasted for 16 h prior to testing. TAK875, Metabolex-36, ANT825, their respective vehicles or loperamide HCl was administrated via i.p. injection, 60 min prior to testing. In the experiments that used the SST_2 antagonist, CYN 154806, mice were pretreated with the antagonist (3 mg/kg) or saline for 15 min, prior to Metabolex-36 (50 mg/kg, i.p.). A charcoal meal (10% plant charcoal in 5% gum acacia (Tough et al. 2011)) was given by intragastric gavage, and 30 min later the animal was killed by cervical dislocation and the small intestine was isolated from the pyloric to ileocecal junctions. Upper GI transit (UGIT), encompassing gastric emptying and small intestinal motility was determined as previously described (Forbes et al. 2012).

Statistical analysis

Pooled responses (as $\mu A.cm^{-2}$) are expressed as mean ± SEM from the numbers of observations as shown, using GraphPad Prism version 6.0 (GraphPad Prism Inc., La Jolla, CA, USA). Single comparisons between groups of data were analysed by unpaired, two-tailed Student's *t*-tests or one-way ANOVA for multiple comparisons with *post hoc* Dunnett's test. *Post hoc* tests were run only when F achieved $P < 0.05$ and there was no significant variance inhomogeneity. $P < 0.05$ was considered statistically significant. Mice (males and females) were randomised in this study. Additionally, drug treatments added to each mucosal preparation were randomised using the Latin square design technique. Furthermore, vehicle and drug treatments were alternated in the *in vivo* studies for each cohort. Explicit blinding was not performed however; each repeat was compared with one another and the drug-pretreated repeats. Any discrepancies between n number cohorts were due to the loss of a single mucosal preparation from a single animal *in vitro,* or a value was removed when it was more than two standard deviations from the mean *in vitro* and *in vivo*. The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015).

Materials

FFA1 (JTT and TAK-875) and FFA4 agonists (AZ423 and Metabolex-36) and the FFA1 antagonist (ANT825) were obtained from AstraZeneca, Gothenburg, Sweden. Stock solutions of drugs were dissolved in neat DMSO (at 10^{-2} M or 10^{-3} M). Initial 1:10 dilutions were in neat DMSO and subsequent serial dilutions were in distilled water (excluding the FFA1 agonist, TAK-

875 and the FFA4 agonist, AZ423, where all serial dilutions were performed in distilled water). BIBO3304, (*N*-[(1*R*)-1-[[[[4-[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate), BIIE0246, (*N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide), GW9508 (4-[[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid), TUG424, (3-(4-(o-Tolylethynyl)phenyl)propanoic acid), TUG891 **(**4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid) and CYN 154806 (Ac-(4-NO_2-Phe)-*cyc*(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)-D-Tyr-NH_2) were purchased from Tocris Bioscience (Bristol, UK). GW1100 (4-[5-[(2-ethoxy-5-pyrimidinyl)methyl]-2-[[(4-fluorophenyl)methyl]thio]-4-oxo-1(4H)-pyrimidinyl]-benzoic acid,ethyl ester) and loperamide HCl was from Cambridge Bioscience (Cambridge, UK). VIP and PYY were purchased from Cambridge Bioscience (Cambridge, UK) and TTX from Abcam, (Cambridge, UK). VIP, PYY and TTX were dissolved in distilled water. All peptide stocks were stored at -20˚ C until required and underwent a single freeze-thaw cycle. Phloridzin (1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl]-1-propanone) was purchased from Sigma-Aldrich (Dorset, UK) and it too was dissolved in distilled water. The following *in vivo* vehicles were all suspended in warm saline, methylcellulose and polyvinylpyrrolidinone (PVP) from Sigma-Aldrich (Dorset, UK), sodium dodecyl sulfate (SDS) from VWR International (Leicestershire, UK), hydroxylpropyl methylcellulose (HPMC) from Alfa Aesar (Lancashire, UK) and Tween-80 from Boston BioProducts (Ashland, USA).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Results

The sidedness, potency and efficacy of FFA1 and FFA4 agonists in descending colon mucosa

First, basal resistance and I_{sc} levels after stabilisation of the colonic mucosae were within ranges published previously (Figure 1A) (Tough et al. 2011). The activity of the non-selective FFA1 and FFA4 agonist, GW9508 (Figure 1B), as well as the TUG compounds added apically or basolaterally induced a biphasic change in I_{sc}. When the reductions in I_{sc} were compared, there were no significant differences between the response sizes to each agonist added to either side (Figure 1C). The more selective FFA1 agonists, TAK-875 and JTT and FFA4 agonists, Metabolex-36 and AZ423 were added apically and induced a biphasic I_{sc} response. The rapid first I_{sc} component was attributed to the vehicle, 0.1% DMSO (3.0 ± 1.1 µA.cm^{-2}, *n*=12) when compared with the same component for JTT (1.7 ± 0.9 µA.cm^{-2}, *n*=5) and Metabolex-36 (3.2 ± 1.7 µA.cm^{-2}, *n*=4) or 0.01% DMSO (3.0 ± 1.1 µA.cm^{-2}, *n*=6) when compared with TAK-875 (2.5 ± 1.3 µA.cm^{-2}, *n*=6) and AZ423 (4.6 ± 1.4 µA.cm^{-2}, *n*=6). Only the later reductions in I_{sc} to varying concentrations of each FFA1 and FFA4 agonists are shown in Figure 1 D - F. To determine whether the FFA1 and FFA4 responses were neuronal, we pretreated the mucosae with TTX (100 nM, bl). The responses to TAK-875 (-11.2 ± 2.0 µA.cm^{-2}, *n*=5) and Metabolex-36 (-8.3 ± 2.3 µA.cm^{-2}, *n*=5) were unaffected by TTX compared to their DMSO controls (-7.5 ± 1.8 µA.cm^{-2} and -8.1 ± 2.2 µA.cm^{-2} respectively, *n*=5), indicating FFA1 and FFA4 responses are most likely epithelial in origin and their responses are not mediated by TTX-sensitive neurons.

The reductions in I_{sc} to each agonist were concentration-dependent, with TUG424 and TUG891 being similarly potent, approximate EC_{50} values were 57.1 nM (24.7 – 131.8 nM) and 62.5 nM (24.1 – 162.3 nM) respectively (Figure 1D). GW9508 was less potent with an EC_{50} value of 354.8 nM (191.6 – 656.8 nM) and appeared to be slightly more efficacious; however, this was not significantly different from the TUG agonists (Figure 1D) or the selective FFA1 or FFA4 agonists. JTT was more potent (EC_{50} of 20.7 nM (12.7 – 34.0 nM)) than TAK-875 (EC_{50} of 67.6 nM (30.6 – 149.4 nM)) and they exhibit similar efficacy (Figure 1E). Furthermore, the FFA4 agonist Metabolex-36 (EC_{50} of 15.4 nM (7.9 – 30.4 nM)) and AZ423 (EC_{50} of 17.3 nM (3.6 – 83.8 nM)) were similarly potent and efficacious (Figure 1F). In comparison to the selective FFA1 and FFA4 agonists, GW9508 appeared to be a dual agonist.

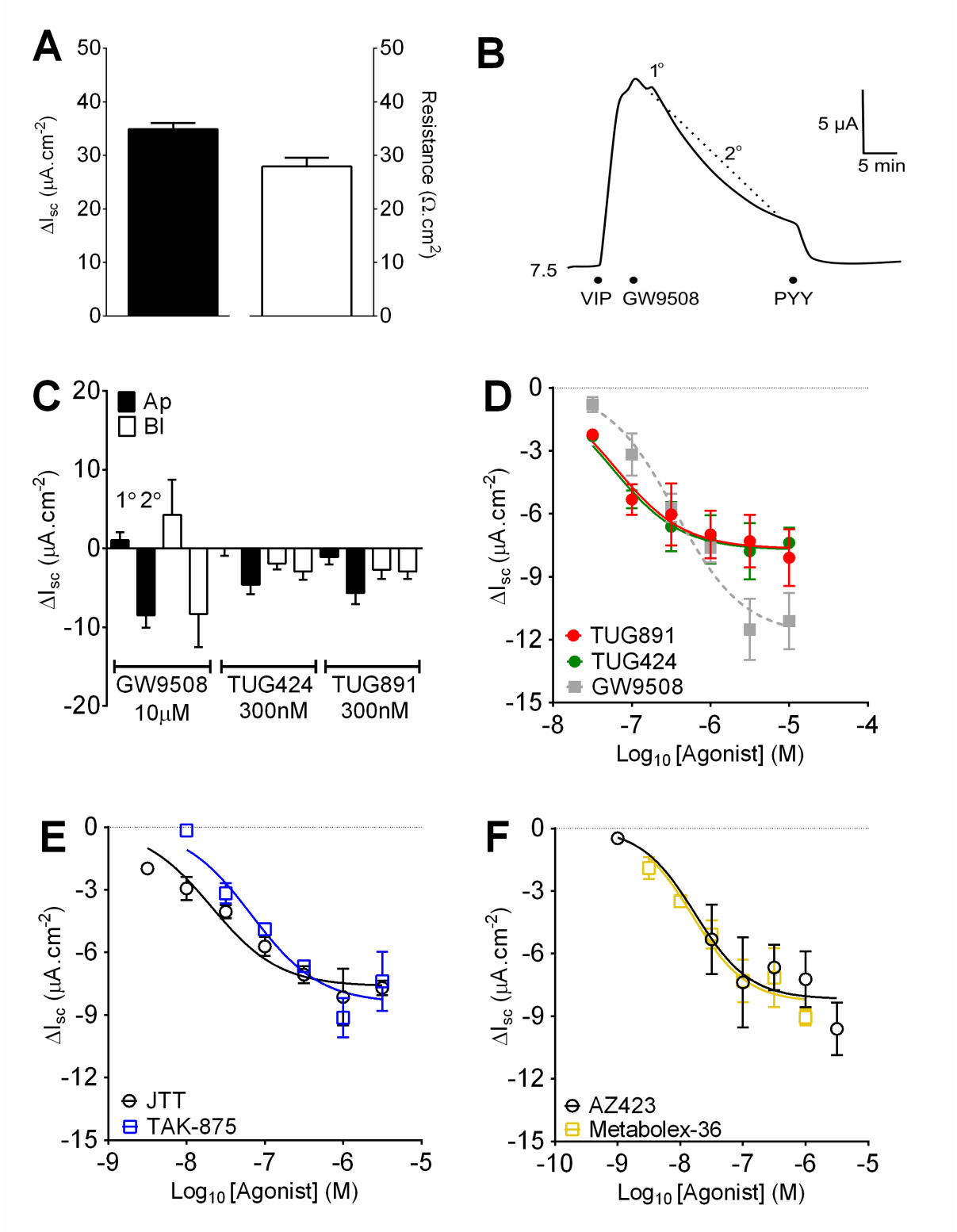
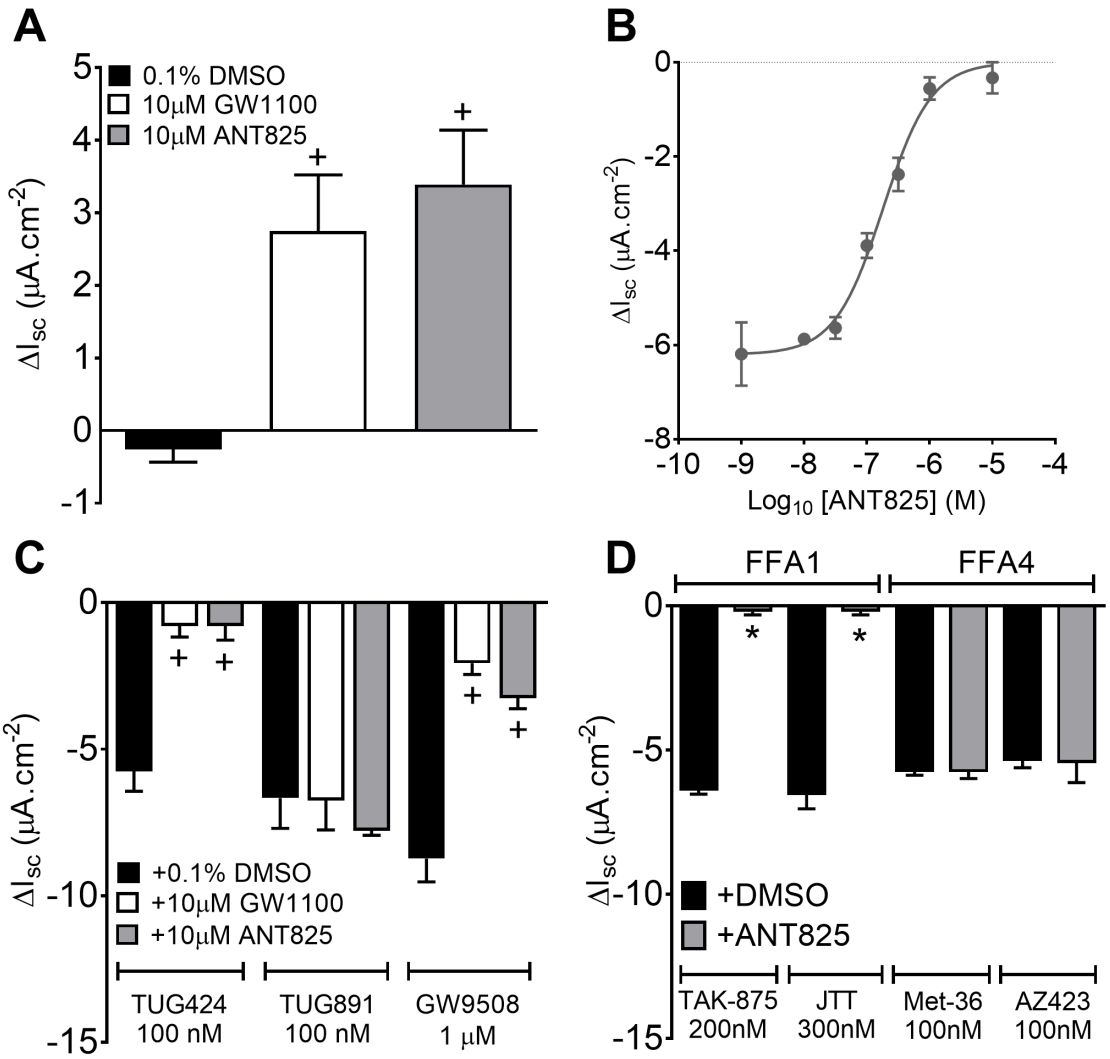


Figure 1. FFA1 and FFA4 responses in descending colon mucosa. In A: basal I_{sc} and resistance values after stabilisation of mucosal preparations (*n*=20). In B: representative trace showing the biphasic nature of apical GW9508 (10 μM) response in descending colon mucosa after VIP (30 nM) and followed by PYY (10 nM). In C: pooled data showing the primary (1˚) and

secondary (2˚) responses to apical (ap) or basolateral (bl) additions of GW9508 (*n*=5), TUG424 (*n*=5) or TUG891 (*n*=5). The 1˚ responses were recorded within 0-5 min of agonist addition (and were due to vehicle), whereas 2˚ reductions in I_{sc} occurred within 10-15 min. Concentration-response curves for the 2˚ effects of TUG424 (*n*=5 for all concentrations, except 300 nM (*n*=6)) and TUG891 (*n*=5) compared with GW9508 (*n*=5) shown in grey in D; for JTT (*n*=5) and TAK-875 (10 nM (*n*=5), 30 nM (*n*=5), 100 nM (*n*=6), 300 nM (*n*=5), 1 µM (*n*=7) and 3 µM (*n*=8)) in E; and AZ423 (*n*=5) and Metabolex-36 (*n*=5) in F. Bars and points are the means ± 1SEM.

GW1100 and ANT825 each inhibit agonist-induced FFA1 responses in the descending colon

The FFA1 antagonists, GW1100 and ANT825 alone revealed a similar degree of FFA1 tonic activity under basal conditions in the distal colon (Figure 2A). ANT825 inhibited JTT responses competitively (Figure 2B), with an IC_{50} of 219 nM. Optimal blocking concentrations of each FFA1 antagonist, GW1100 or ANT825 revealed the selectivity of TUG424 and GW9508 for the FFA1 receptor. TUG891 responses were not affected by either FFA1 antagonist. Additionally, only part of the GW9508 response was reduced in the presence of ANT825 or GW1100 (Figure 2C). The FFA1 selective agonists (TAK-875 and JTT) were significantly inhibited by ANT825 whereas the FFA4 selective agonists, Metabolex-36 and AZ423 responses were unaffected (Figure 2D).



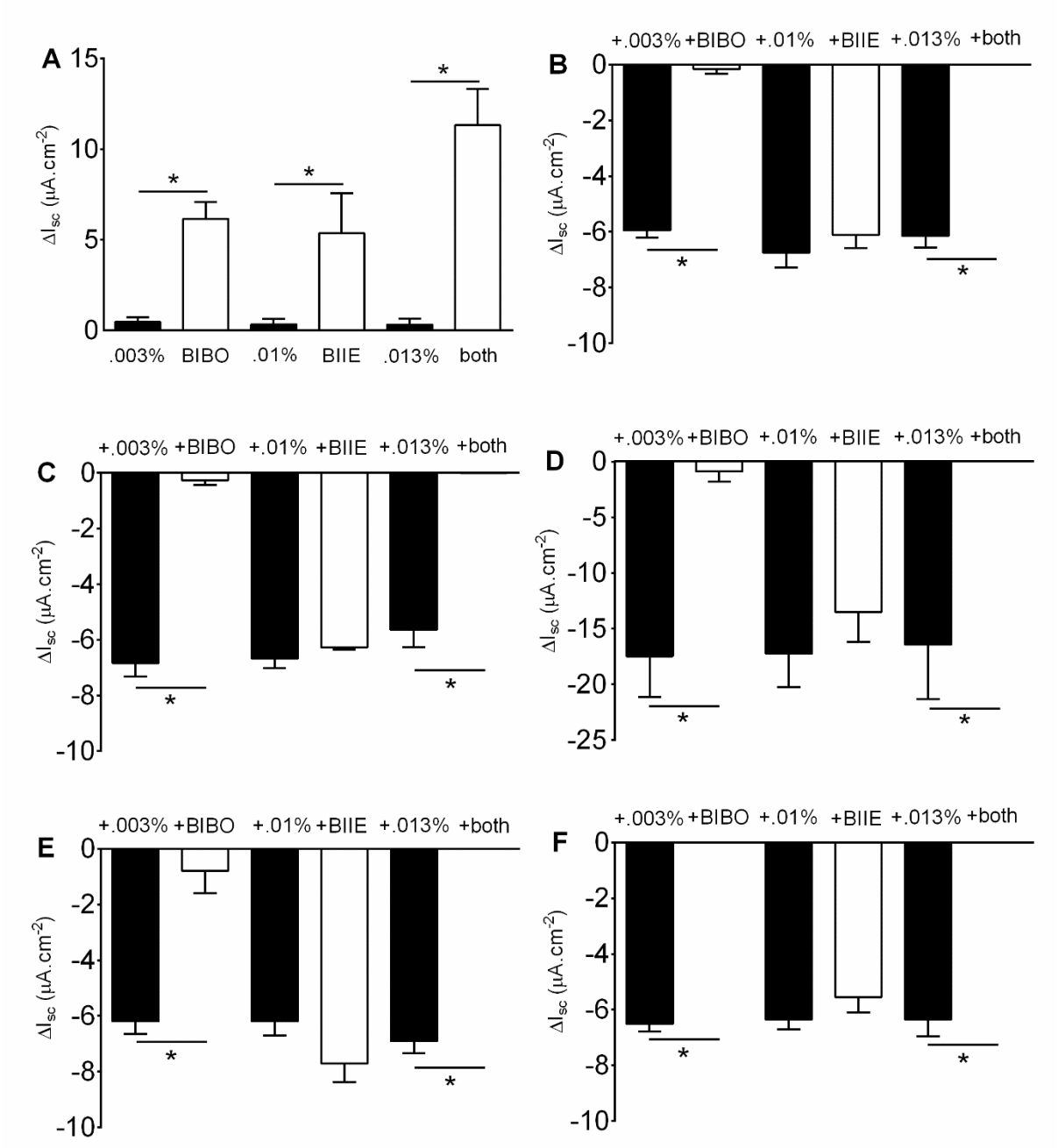
391
392

393 **Figure 2. Tonic FFA1 activity and inhibition of FFA1 responses by GW1100 or ANT825.** In
394 A: changes in I_{sc} to vehicle, DMSO or the FFA1 antagonists, GW1100 or ANT825 alone. In B:
395 competitive inhibition of the FFA1 agonist, JTT responses by pretreatment with the FFA1
396 antagonist, ANT825. Each point is the mean ±1SEM (*n*=5 for all concentrations of JTT except 10
397 μM (*n*=6)). In C: pooled data shows the selective inhibition of apical TUG424 and GW9508
398 responses, but not TUG891 responses following apical GW1100 or ANT825 treatment compared
399 with respective vehicle controls (+DMSO controls for TUG424 (*n*=6), for TUG891 (*n*=5) and for
400 GW9508 (*n*=5)). In D: selective inhibition of the FFA1 agonists TAK-875 and JTT responses but
401 not the FFA4 agonist, AZ423 and Metabolex-36 (Met-36) responses following apical ANT825
402 treatment (10 μM, *n*=5). Statistical differences from respective vehicle controls are shown as

follows; * $P < 0.05$ (Student's *t* test) and $^{+}$ $P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

FFA1 and FFA4 colonic responses are BIBO3304 but not BIIE0246 sensitive

Endogenous PYY mediation of the FFA1 (JTT and TAK-875) and FFA4 (AZ423 and Metabolex-36) agonist responses were determined by blocking with the Y_1 antagonist, BIBO3304, the Y_2 antagonist, BIIE0246 or both antagonists together. Each antagonist revealed endogenous PYY-Y_1 and Y_2 tonic activity under basal conditions (Figure 3A) similar to that observed in the mouse colon previously (Tough et al. 2011, Hyland et al. 2003). FFA1 responses to JTT (Figure 3B) and TAK-875 (Figure 3C) were abolished by the Y_1 antagonist, indicating PYY-Y_1 signalling predominantly mediates FFA1 responses. The FFA1 responses in the presence of the Y_2 antagonist were slightly reduced, suggestive of a minor role for Y_2 receptors as seen previously (Tough et al. 2011). Notably, there was no influence of the I_{sc} level at the time of adding the FFA1 agonist on subsequent FFA1 efficacy. Taken together these observations confirm selective Y_1-mediation of FFA1 responses. Subsequent exogenous PYY responses were also abolished by the Y_1, but not by the Y_2 antagonist alone, while the combination abolished PYY activity (Figure 3D). The responses to FFA4 agonists, AZ423 (Figure 3E) and Metabolex-36 (Figure 3F) were also abolished by the Y_1 but not Y_2 antagonist and thus both FFA1 and FFA4 responses in the mouse colon are Y_1 receptor mediated.



426

427 **Figure 3. Y_1- but not Y_2-receptor sensitivity of FFA1 and FFA4 agonism in the descending**
428 **colon.** The effects of the Y_1 (BIBO3304, BIBO, 300 nM) ± the Y_2 (BIIE0246, BIIE, 1 μM)
429 antagonist and corresponding DMSO controls (+.003 %, +.01 %, +.013 %) on baseline I_{sc} levels
430 are shown in A. The effect of the FFA1 agonists, JTT (*n*=5 for all data groups except +.003% and
431 +.013% (*n*=6)) and TAK-875 (*n*=5 for all data groups except +BIBO (*n*=6)) in the absence or
432 presence of Y antagonists are shown in B and C respectively. Exogenous PYY (10 nM) responses
433 (*n*=5 for all data groups except +.003% and +.013% (*n*=6)) after JTT treatment, in the presence of
434 DMSO or Y_1 ± Y_2 antagonists are shown in D. The FFA4 agonist's responses, AZ423 (*n*=5) and

Metabolex-36 (n=5 for all data groups except +.01% (n=6)) are shown in E and F respectively. Bars represent the mean ± 1 SEM. Statistical differences between vehicle controls (0.003 %, 0.01 % and 0.013 % DMSO) and respective antagonists BIBO, BIIE or both are shown as follows; * $P < 0.05$ (Student's t test).

FFA1 and FFA4 responses are glucose-sensitive in the descending colon mucosa

In order to establish the glucose-sensitivity of FFA signalling in mucosal preparations, the responses to the FFA1 and FFA4 agonists were compared in the presence or absence of apical glucose. Mannitol replacement of apical glucose abolished the FFA1 responses (Figure 4A & B) and inhibited FFA4 activity (Figure 4C & D) compared to vehicle. Internal controls using phloridzin (Phlor, added ap) decreased the I_{sc}, but as expected only in the presence of glucose (because SGLT1 requires glucose to function and is targeted apically). Thus, FFA1 and FFA4 receptors can be activated in a glucose-sensitive manner. In contrast, PYY responses were not glucose-sensitive (Figure 4A-D). Furthermore, TAK-875 and Metabolex-36 responses were not affected when the glucose concentration (ap + bl) was varied to represent a hypoglycaemic (5.0 mM) or hyperglycaemic (25.0 mM) environment (Figure 4E).

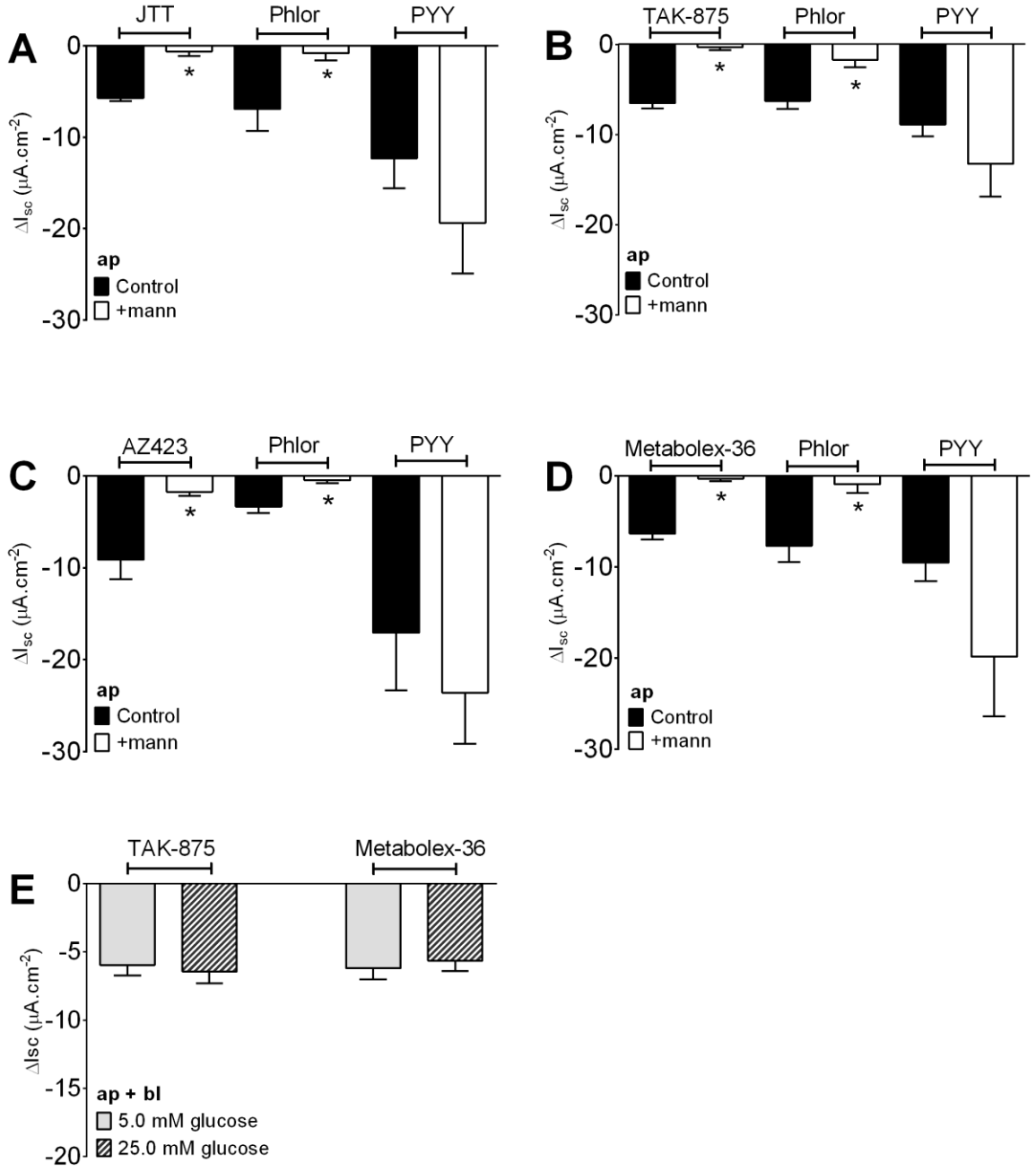


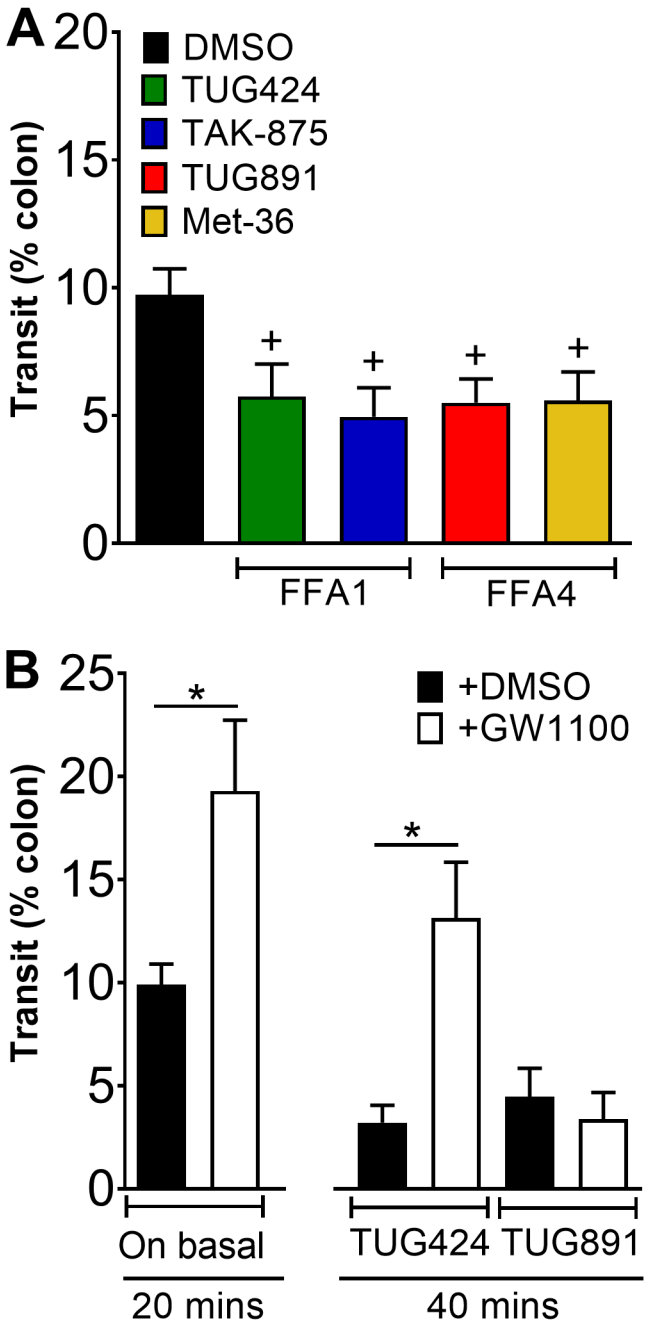
Figure 4. Glucose-sensitivity of FFA1 and FFA4 agonism in the descending colon. Glucose-sensitivity of apical FFA1 agonists, JTT (300 nM, *n*=5) in A, TAK-875 (200 nM, *n*=5) in B and apical FFA4 agonists, AZ423 (100 nM, *n*=5) in C and Metabolex-36 (100 nM, *n*=5) in D, in the presence (black bars) and absence (white bars) of 11.1 mM glucose. Control mucosae were bathed in glucose both sides whereas, mannitol (+ mann, 11.1 mM) replaced glucose apically only. Phloridzin (Phlor, 50μM, apically only) and PYY (10 nM) responses are also shown. In E: TAK-875 (200 nM, *n*=5) and Metabolex-36 (100 nM, *n*=5) antisecretory responses in the presence of apical and basolateral (ap + bl) 5.0 mM glucose or 25.0 mM glucose. Bars represent the mean -

1SEM. Statistical differences between agonist or phloridzin responses in the presence or absence of glucose, are shown as follows; * $P < 0.05$ (Student's *t* test).

Caeco-colonic transit is inhibited by FFA1 and FFA4 agonists

Since FFA1 and FFA4 mucosal responses in the colon were mediated by PYY and this endogenous peptide is known to slow colonic transit (Tough et al. 2011), we next assessed whether the FFA1 and FFA4 agonists reduced transit at single optimal concentrations (300 nM; Figure 5A). In isolated colons, TUG424, TUG891, TAK-875 and Metabolex-36 significantly decreased transit in comparison to vehicle-treated (DMSO, 0.1%) controls.

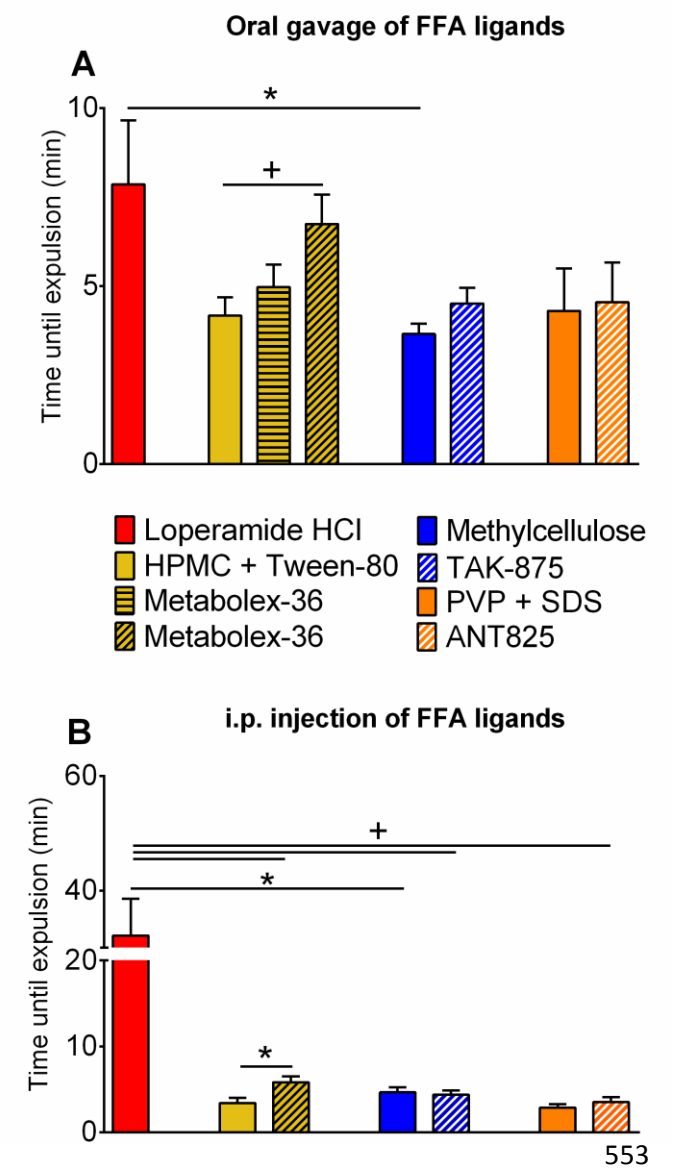
Since GW1100 (10 µM) inhibited FFA1 responses in colonic mucosa, we set out to determine whether GW1100 alone could increase basal faecal transit and reverse the effect of the FFA1 agonist on colonic transit, *in vitro*. Figure 5B shows that GW1100 alone significantly increased basal colonic transit, again indicating endogenous FFA1 inhibitory tone. After 20 min pretreatment with the FFA1 antagonist, GW1100 the effect of TUG424 and TUG891 was assessed in the presence and absence of GW1100. In the presence of GW1100, the inhibitory effect on colonic transit of TUG424 was reversed, but GW1100 had no effect on TUG891 (FFA4) activity, showing FFA1 selectivity (Figure 5B).



506 **Figure 5**. **Colonic transit is slowed by FFA1 and FFA4 agonists in isolated colons *in vitro***.
507 Colonic transit is slowed by 300 nM of TUG424 (*n*=6), TAK-875 (*n*=6), TUG891 (*n*=6) and
508 Metabolex-36 (Met-36, *n*=6) in A at t =20 min. In B: the effects of FFA1 antagonist GW1100 (10
509 μM) are compared on basal faecal transit at t=20 min and on subsequent inhibition of transit
510 following 20 min treatment with TUG424 or TUG891 at t=40 min (n = 5). Each bar represents
511 the mean + 1SEM. Statistically significant differences from controls were; * $P < 0.05$ (Student's *t*
512 test) and $^{+}$ $P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

Colonic bead propulsion is slowed by Metabolex-36 not TAK875 or ANT825 in vivo

As the FFA1 and FFA4 agonists, (TAK-875 and Metabolex-36 respectively) slowed transit in isolated colons *in vitro;* we assessed the ability of these agonists and loperamide HCl to slow colonic transit *in vivo*, comparing oral administration versus i.p. injection. Additionally, we investigated whether ANT825 had the potential to reveal FFA1 tone *in vivo*. Oral administration of the positive control, loperamide HCl significantly slowed transit compared to its vehicle control. Two doses of Metabolex-36 were assessed orally; the lower dose had no effect on transit whereas the higher dose significantly slowed transit. Furthermore, oral gavage of TAK-875 and ANT825 had no effect on colonic transit (Figure 6A). Both loperamide HCl and Metabolex-36 significantly slowed colonic transit when given via i.p. injection whereas TAK-875 and ANT825 had no effect (Figure 6B). As i.p. injection of loperamide HCl slowed transit more efficiently in comparison to oral gavage, we used this route of administration for each drug in the subsequent *in vivo* studies.



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554 **Figure 6. The effect of oral gavage versus i.p. injection of FFA ligands and loperamide HCl**
555 **on colonic bead expulsion *in vivo*.** The effect on colonic bead expulsion (expressed as time until
556 bead expelled (min)) after oral gavage or i.p. injection of loperamide HCl (10 mg/kg, red bar,
557 *n*=11) compared with its vehicle control, 0.5% methylcellulose (blue bar, *n*=11); the FFA4
558 agonist, Metabolex-36 at two doses: 6 mg/kg (yellow bar with black horizontal stripes, *n*=6) and
559 50 mg/kg (yellow bar with black diagonal stripes, *n*=10) compared with their vehicle control,
560 0.5% hydroxylpropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar, *n*=10); the FFA1
561 agonist, TAK-875 (27 mg/kg, blue bar with white diagonal stripes, *n*=11) compared with its
562 vehicle control (0.5% methylcellulose, blue bar) and finally the FFA1 antagonist, ANT825 (29
563 mg/kg, orange bar with white diagonal stripes, *n*=7) compared with its vehicle (0.25%
564 polyvinylpyrrolidone (PVP) + 0.05% sodium dodecyl sulfate (SDS), orange bar, *n*=7). Each bar

represents the mean + 1SEM. Statistically differences from controls were; * $P < 0.05$ (Student's *t*-test). Differences between the oral doses of Metabolex-36 from its control and additionally between loperamide HCl (i.p.) and the FFA drugs were; $^{+}$ $P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

Upper GI transit is increased by Metabolex-36 in vivo

Here we investigated whether the FFA agonists (i.p.) and loperamide HCl could slow intestinal transit and additionally whether ANT825 could increase intestinal transit (revealing FFA1 tone), after mice were given a charcoal meal (as described in the methods). The positive control, loperamide HCl significantly slowed GI transit, as expected compared to its vehicle control. Unexpectedly, Metabolex-36 significantly increased intestinal transit whereas TAK-875 and ANT825 had no effect (Figure 7).

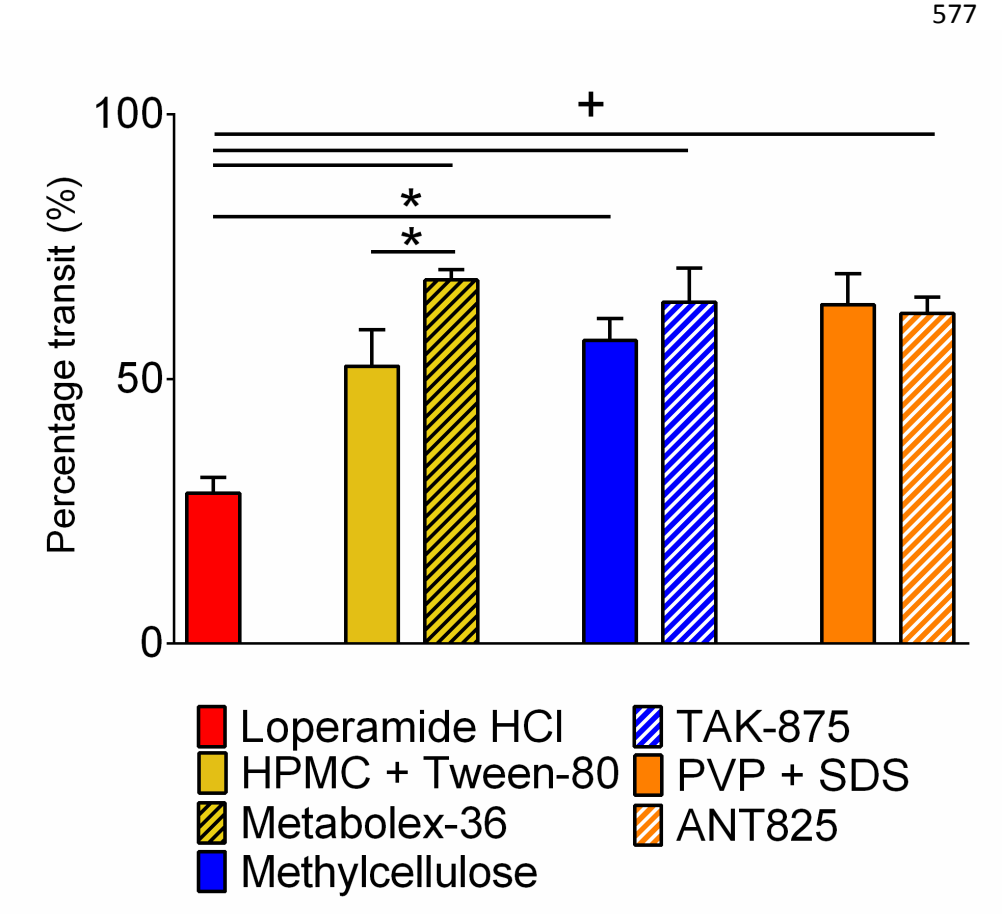
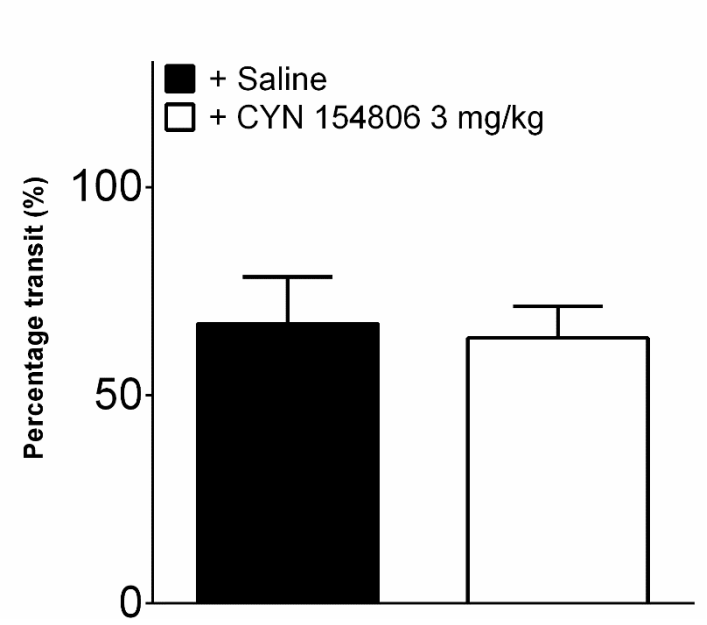


Figure 7. The effect of an i.p. injection of loperamide HCl and FFA ligands on UGIT *in vivo*. The intestinal transit of a charcoal meal (expressed as a percentage of the small intestine length) after i.p. injection of the positive control, loperamide HCl (10 mg/kg, red bar, *n*=6) compared with its vehicle control, 0.5% methylcellulose (blue bar, *n*=6); the FFA4 agonist, Metabolex-36 (50 mg/kg, yellow bar with black diagonal stripes, *n*=6) compared with its vehicle control, 0.5% hydroxylpropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar, *n*=6); the FFA1 agonist, TAK875 (27 mg/kg, blue bar with white diagonal stripes, *n*=5) compared with its vehicle control (0.5% methylcellulose, blue bar) and the FFA1 antagonist, ANT825 (29 mg/kg, orange bar with white diagonal stripes, *n*=5) compared with its vehicle (0.25% polyvinylpyrrolidone (PVP) + 0.05% sodium dodecyl sulfate (SDS), orange bar, *n*=5). Each bar represents the mean + 1SEM. Statistically differences from controls were; * $P < 0.05$ (Student *t*-test) whereas differences between loperamide HCl and FFA drugs were; $^{+}$ $P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

SST_2 inhibition has no effect on Metabolex-36 induced UGIT effect in vivo

As Metabolex-36 increased UGIT *in vivo*, we investigated whether this was due to an inhibitory FFA4 effect (via a $G\alpha_i$-mediated mechanism) on somatostatin-containing D cells in the GI tract. This inhibition would result in disinhibition and ultimately a pro-motile effect, as somatostatin is a known inhibitor of GI motility. Mice were pretreated with the SST_2 antagonist, CYN 154806 or saline before the effect of Metabolex-36 was tested. CYN 154806 + Metabolex-36 had no effect on the UGIT of the charcoal meal, in comparison to mice pretreated with saline (Figure 8).



626 **Figure 8. CYN 154806 had no effect on the Metabolex-36 induced increase in UGIT *in vivo*.**
627 The effect of Metabolex-36 (50 mg/kg, i.p. injection) on UGIT in the absence (saline, *n*=5) or
628 presence (*n*=5) of the SST_2 antagonist, CYN 154806. Bars represent mean + 1SEM.

629

630 **Discussion**

631

632 *The improved selectivity of FFA1 and FFA4 agonists versus commercially available agonists*

633 FFA1 and FFA4 agonism was independent of the surface of administration and the time-
634 dependence of I_{sc} changes was similar, suggesting that these receptors are located on both the
635 apical and basolateral surfaces. However, it should be noted that all the drugs used were lipid
636 soluble requiring DMSO as a vehicle therefore, drugs added on the apical surface could activate
637 FFA receptors on the basolateral side and vice versa. Interestingly, Christensen et al. (2015)
638 showed preferential but not exclusive vascular (basolateral) FFA1-induced GLP-1 secretion using
639 endogenous and synthetic agonists, in rat small intestine. We utilised apical administration and
640 the reductions in I_{sc} we observed occurred within the same time frame as other apically located L
641 cell $G\alpha_q$-coupled receptors, some activated by water-soluble compounds (Joshi et al. 2013, Forbes
642 et al. 2015, Alamshah et al. 2016) thus indicating apical receptors.

643 GW9508 is reported to act via FFA1 and FFA4 with 100x higher affinity for FFA1 than FFA4
644 (Briscoe et al. 2006). GW9508 is a dual agonist at the FFA1 and FFA4 and this may explain its

slightly greater efficacy than more selective agonists we used. We found that the selective FFA1 and FFA4 agonists exhibited a similar potency which was more potent than the potency of GW9508. Both FFA4 agonists, Metabolex-36 and AZ423 have >100-fold higher selectivity for FFA4 in comparison to the FFA1. This phenomenon of higher potency but lower efficacy has been observed previously with a GPR119 agonist, PSN-GPR119 (Patel et al. 2014) and may indicate acute receptor desensitisation.

FFA1 agonists are preferentially $G\alpha_{q/11}$-linked and recent evidence suggests that '$G\alpha_{q/11}$-only' FFA1 agonists (e.g. TAK-875) stimulate the release of incretin hormones, GLP-1 and GIP, with reduced efficacy compared to FFA1 agonists that signal via $G\alpha_{q/11}$ and $G\alpha_s$ pathways; to cause a more robust release of GLP-1 and GIP (Hauge et al. 2015). Our FFA1 agonists appear to be $G\alpha_{q/11}$-coupled preferentially as mucosal responses were transient in comparison with $G\alpha_s$-coupled L cell signalling e.g. GPR119 or MC4 (Cox et al. 2010, Panaro et al. 2014).

FFA1 antagonism reveals FFA1 mucosal and anti-motility tone and agonist-specificity.

The FFA1 antagonists, ANT825 and GW1100 revealed, for the first time, a degree of endogenous FFA1 tonic activity in colonic mucosa. The degree of tonic activity was similar to that observed with the MC4 receptor antagonist, HS014 (3.6 $\mu A.cm^{-2}$) (Panaro et al. 2014). Since endogenous FFA1 and MC4 agonism stimulates PYY release from L cells, blockade of these receptors inhibits endogenous PYY release. Crivellato et al (2002) demonstrated that enteroendocrine cells in mouse colon undergo piecemeal degranulation, hormone release that may be related to the tonic activity we observe in mucosa. Endogenous lipids, natural ligands of the FFA1 (Itoh et al. 2003) may be responsible for the observed tonic activity via FFA1.

The potency of the FFA1 competitive antagonist, ANT825 was not dissimilar from its potency (pEC_{50} of 6.8) obtained in a human embryonic kidney 293 cell line expressing human FFA1, measuring inositol monophosphate (Waring 2015). We found GW1100 (pIC_{50} of 5.99; (Briscoe et al. 2006)) abolished TUG424 responses but not TUG891 responses, showing FFA1 selectivity as seen previously (Briscoe et al. 2006). Like GW1100, ANT825 abolished FFA1 responses but both FFA1 antagonists' also partially inhibited GW9508 responses, indicating that at this concentration (1 µM); GW9508 exerts a dual agonism via FFA1 and most likely FFA4. Briscoe et al (2006) revealed that GW1100 had no effect on the ability of GW9508 to activate FFA4. We conclude that GW9508 is a dual FFA1 and FFA4 agonist.

FFA1 (TUG424, TAK-875) and FFA4 (TUG891, Metabolex-36) agonists attenuated colonic motility *in vitro* to a similar degree as seen previously with MC4 receptor activation and GPR119 agonism (Panaro et al. 2014). They decreased colonic motility presumably via the release of PYY, which mediates the colonic brake. Pretreatment with the FFA1 antagonist, GW1100 revealed basal colonic mucosal tone (as seen with Ussing chamber studies) indicative of endogenous FFA1 activity. In the presence of the FFA1 antagonist GW1100, the colonic brake induced by the FFA1 agonist (TUG424) was disinhibited and therefore colonic transit increased whereas transit induced by FFA4 agonist (TUG891) remained unaffected, showing selectivity of the FFA1 agonist.

As the FFA1 agonist, TAK875 and the FFA4 agonist, Metabolex-36 slowed transit *in vitro*; we investigated their ability to slow transit *in vivo*. Following the overnight fast, plasma glucose levels were reduced to 5 mM. However, we established that FFA1 and FFA4 signalling is unchanged at 5 mM compared with 11.1 mM glucose as seen previously for GPR119 agonism (Patel et al. 2014). Metabolex-36 slowed colonic transit significantly presumably via PYY, whereas TAK-875 failed to show significant inhibition of bead colonic transit when administered orally or via i.p. injection. This apparent lack of efficacy may be due to TAK-875's partial agonism or its administration as a suspension. In our *in vitro* transit studies, the isolated colon is severed from the central nervous system (CNS) and TAK-875 acts directly upon colonic FFA1 receptors. In the *in vivo* model, it's possible that the ability of TAK-875 to release PYY is not sufficient to alter colonic motility, with the added complication of modulatory CNS pathways. In addition, there are concerns in the literature that isoflurane interferes with GI transit. In 2005, Torjman and colleagues reported that 6 min exposure to isoflurane in rats slows UGIT by 50% (Torjman et al. 2005). In our study, mice received a 2 min exposure of isoflurane 1 h after the drug, vehicle or positive control were administered, followed by bead insertion and excretion. Therefore, we conclude that this brief exposure of isoflurane in our protocol (at the time of maximal drug exposure) should have minimal effect on colonic motility. Furthermore, we note that there are no differences in colonic transit rates between different vehicle controls (oral gavage vs i.p.) in this study, compared with those in a previous investigation (Forbes et al. 2012).

Unexpectedly, Metabolex-36 was pro-motile in the upper intestine. FFA4 is expressed on I cells, K cells and L cells causing release of CCK, GIP and PYY (and GLP-1) respectively, via $G\alpha_q$-signalling (Iwasaki et al. 2015, Hirasawa et al. 2005, Tanaka et al. 2008). These peptides inhibit gastric emptying and small intestinal transit and additionally, PYY is responsible for both ileal and colonic brakes (Spiller et al. 1984, Spiller et al. 1988). However, recently FFA4 has been

identified in gastric ghrelin and somatostatin cells, signalling via $G\alpha_i$ proteins to inhibit peptide release (Engelstoft et al. 2013, Egerod et al. 2015). Somatostatin is known to inhibit motility and increase the interval between migrating myoelectric complexes (MMC), therefore slowing transit. We hypothesised that Metabolex-36 increased intestinal motility by inhibiting somatostatin release and tested this possibility with the SST_2 antagonist, CYN 154806 at an effective dose used previously in rat small intestine, *in vivo* (Booth et al. 2001). However, CYN 154806 was ineffective (at 3 mg/kg) and we conclude that either the antagonist dose used was too low in the mouse, or Metabolex-36 produces a pro-motile effect via an alternative FFA4 mechanism.

PYY-Y_1 but not PYY-Y_2 mediation of FFA1 and FFA4 signalling in mouse colonic mucosa

FFA1 and FFA4 agonist responses in the mouse colon were Y_1 receptor mediated. Additionally, these responses were unaffected by TTX indicating a predominantly epithelial origin for FFA1 and FFA4 activity, not involving TTX-sensitive submucosal neurons. The degree of antisecretory tone mediated by the Y_1 and Y_2 receptors was similar to that shown previously in mouse colon (Hyland et al. 2003, Tough et al. 2011). The FFA1 and FFA4 signalling in colonic mucosa are mediated by endogenous PYY acting on local epithelial Y_1 receptors. The epithelial Y_1 mediation and lack of any Y_2 (neuronal) involvement is consistent with our previous findings for other L cell-enriched receptors e.g. MC4 (Panaro et al. 2014), GPR119 (Patel et al. 2014), FFA2 (Forbes et al. 2015) and the calcium-sensing receptor (Joshi et al. 2013). There appears to be no GLP-1 mediation of FFA1 and FFA4 responses in this study. GLP-1 has a half-life of 1.5 min which limits the ability to detect its response, if any. Additionally, we have discovered that the GLP-1 response can vary across the colon, with largest GLP-1 responses observed in the ascending colon and smallest in the descending colon (Tough et al. 2017). Here we have used descending colon and in combination with the short half-life of GLP-1, this may explain why we do not see a GLP-1 mediated response.

FFA1 and FFA4 responses in mouse colon mucosa are glucose-sensitive

Our studies demonstrate for the first time that activation of FFA1 and FFA4 responses in native tissues are glucose-sensitive as seen in previous investigations of L cell-specific activation by GPR119 (Cox et al. 2010), MC4 receptor (Panaro et al. 2014), the activation of the calcium-sensing receptor by L-glutamine (Joshi et al. 2013) and FFA2 agonism (Forbes et al. 2015).

Theoretically these agonists should not therefore cause hypoglycaemia *in vivo*, a disadvantage of certain anti-diabetic drugs currently on the market. FFA1 and FFA4 receptors are currently clinically favourable targets as they are not only co-expressed in GI L cells but differentially expressed in the pancreas. FFA1 is expressed in pancreatic β-cells (Itoh et al. 2003) and FFA4 has recently been discovered in somatostatin-containing delta cells (Stone et al. 2014). PYY and GLP-1 released from the L cells should enhance satiety (an advantage for patients with associated obesity) and elevate insulin release respectively via activation of GLP-1 receptors on pancreatic β cells. Furthermore, an FFA1 agonist could simultaneously directly elevate insulin release after a meal. This combination of indirect and direct FFA1 effects should improve glucose tolerance without hypoglycaemia.

Final conclusions

We have shown that selective FFA1 and FFA4 agonism is PYY-Y_1 receptor mediated and glucose-sensitive in mouse descending colon mucosa. These compounds would limit potential hypoglycaemia, a side effect of certain commercially available anti-diabetic drugs. ANT825 and GW1100 revealed tonic FFA1 activity and the possibility of FFA1 endogenous ligands causing PYY release. Furthermore, we have shown for the first time that agonism of FFA1 and FFA4 inhibits colonic transit *in vitro* and FFA4 agonism induces regional differences in transit *in vivo.* In colonic L cells agonism of FFA4 is presumably $G\alpha_q$-mediated and leads to antisecretory mucosal responses that slows colonic transit, but leads to upper GI pro-motile activity *in vivo* in the mouse, which remains unresolved. FFA1 and FFA4 co-agonism may be beneficial therapeutically and would more closely mimic the combinatorial activity of long chain fatty acids in the diet.

Author contributions

RM and IRT performed and analysed the experiments and contributed with HMC to the design and interpretation of the data, and writing of the manuscript. DMS and MS provided AstraZeneca in-house data and contributed to the design and interpretation of the work. MS and HMC had the project idea and got funding. RM, IRT, DMS, MS and HMC reviewed and approved the final manuscript version and agreed to be accountable for all aspects of the work.

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References

Adrian, T. E., Ferri, G. L., Bacarese-Hamilton, A. J., Fuessl, H. S., Polak, J. M. and Bloom, S. R. (1985) 'Human distribution and release of a putative new gut hormone, peptide YY', *Gastroenterology,* 89(5), 1070-7.

Alamshah, A., McGavigan, A. K., Spreckley, E., Kinsey-Jones, J. S., Amin, A., Tough, I. R. *et al*. (2016) 'L-Arginine promotes gut hormone release and reduces food intake in rodents', *Diabetes Obes Metab,* 18(5), 508-18.

Alexander SPH, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Southan C, Davies JA and CGTP Collaborators (2015) The Concise Guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. Br J Pharmacol. 172(24): 5744-5869.

Arantes, R. M. and Nogueira, A. M. (1997) 'Distribution of enteroglucagon- and peptide YY-immunoreactive cells in the intestinal mucosa of germ-free and conventional mice', *Cell Tissue Res,* 290(1), 61-9.

Batterham, R. L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L. *et al.* (2002) 'Gut hormone PYY(3-36) physiologically inhibits food intake', *Nature,* 418(6898), 650-4.

Booth, C. E., Kirkup, A. J., Hicks, G. A., Humphrey, P. P. and Grundy, D. (2001) 'Somatostatin sst(2) receptor-mediated inhibition of mesenteric afferent nerves of the jejunum in the anesthetized rat', *Gastroenterology,* 121(2), 358-69.

Briscoe, C. P., Peat, A. J., McKeown, S. C., Corbett, D. F., Goetz, A. S., Littleton, T. R. *et al.* (2006) 'Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules', *Br J Pharmacol,* 148(5), 619-28.

Briscoe, C. P., Tadayyon, M., Andrews, J. L., Benson, W. G., Chambers, J. K., Eilert, M. M. *et al.* (2003) 'The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids', *J Biol Chem,* 278(13), 11303-11.

809 Cho, H. J., Robinson, E. S., Rivera, L. R., McMillan, P. J., Testro, A., Nikfarjam, M. *et al.* (2014)
810 'Glucagon-like peptide 1 and peptide YY are in separate storage organelles in enteroendocrine
811 cells', *Cell Tissue Res,* 357(1), 63-9.

812
813 Christensen, L.W., Kuhre, R.E., Janus, C., Svendsen, B. and Holst, J.J. (2015) 'Vascular, but not luminal,
814 activation of FFA1 (GPR40) stimulates GLP-1 secretion from isolated perfused rat small intestine',
815 *Physiol Rep,* 3 (9): e12551
816
817 Christiansen, E., Urban, C., Merten, N., Liebscher, K., Karlsen, K. K., Hamacher, A. *et al.* (2008)
818 'Discovery of potent and selective agonists for the free fatty acid receptor 1 (FFA(1)/GPR40), a
819 potential target for the treatment of type II diabetes', *J Med Chem,* 51(22), 7061-4.

820
821 Cox, H. M., Pollock, E. L., Tough, I. R. and Herzog, H. (2001) 'Multiple Y receptors mediate pancreatic
822 polypeptide responses in mouse colon mucosa', *Peptides,* 22(3), 445-52.

823

824 Cox, H. M. and Tough, I.R. (2001) 'Neuropeptide Y, Y1, Y2 and Y4 receptors mediate Y agonist responses
825 in isolated human colon mucosa', *Br J Pharmacol,* 135 (6), 1505 -1512.

826
827 Cox, H. M., Tough, I. R., Woolston, A. M., Zhang, L., Nguyen, A. D., Sainsbury, A. *et al.* (2010) 'Peptide
828 YY is critical for acylethanolamine receptor Gpr119-induced activation of gastrointestinal mucosal
829 responses', *Cell Metab,* 11(6), 532-42.

830
831 Crivellato, E., Ribatti, D., Mallardi, F. and Beltrami, C. A. (2002) 'Granule changes of human and murine
832 endocrine cells in the gastrointestinal epithelia are characteristic of piecemeal degranulation', *Anat*
833 *Rec,* 268(4), 353-9.

834
835 Curtis, M. J., Bond, R. A., Spina, D., Ahluwalia, A., Alexander, S. P., Giembycz, M. A. *et al.* (2015)
836 'Experimental design and analysis and their reporting: new guidance for publication in BJP', *Br J*
837 *Pharmacol,* 172(14), 3461-71.

838
839 Edfalk, S., Steneberg, P. and Edlund, H. (2008) 'Gpr40 is expressed in enteroendocrine cells and mediates
840 free fatty acid stimulation of incretin secretion', *Diabetes,* 57(9), 2280-7.

841
842 Egerod, K. L., Engelstoft, M. S., Grunddal, K. V., Nohr, M. K., Secher, A., Sakata, I. *et al.* (2012) 'A major
843 lineage of enteroendocrine cells coexpress CCK, secretin, GIP, GLP-1, PYY, and neurotensin but
844 not somatostatin', *Endocrinology,* 153(12), 5782-95.

845
846 Egerod, K. L., Engelstoft, M. S., Lund, M. L., Grunddal, K. V., Zhao, M., Barir-Jensen, D. *et al.* (2015)
847 'Transcriptional and Functional Characterization of the G Protein-Coupled Receptor Repertoire of
848 Gastric Somatostatin Cells', *Endocrinology,* 156(11), 3909-23.

849
850 Engelstoft, M. S., Egerod, K. L., Holst, B. and Schwartz, T. W. (2008) 'A gut feeling for obesity: 7TM
851 sensors on enteroendocrine cells', *Cell Metab,* 8(6), 447-9.

852

Engelstoft, M. S., Park, W. M., Sakata, I., Kristensen, L. V., Husted, A. S., Osborne-Lawrence, S. *et al.* (2013) 'Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells', *Mol Metab,* 2(4), 376-92.

Forbes, S., Herzog, H. and Cox, H. M. (2012) 'A role for neuropeptide Y in the gender-specific gastrointestinal, corticosterone and feeding responses to stress', *Br J Pharmacol,* 166(8), 2307-16.

Forbes, S., Stafford, S., Coope, G., Heffron, H., Real, K., Newman, R. *et al.* (2015) 'Selective FFA2 Agonism Appears to Act via Intestinal PYY to Reduce Transit and Food Intake but Does Not Improve Glucose Tolerance in Mouse Models', *Diabetes,* 64(11), 3763-71.

Habib, A. M., Richards, P., Rogers, G. J., Reimann, F. and Gribble, F. M. (2013) 'Co-localisation and secretion of glucagon-like peptide 1 and peptide YY from primary cultured human L cells', *Diabetologia,* 56(6), 1413-6.

Halder, S., Kumar, S. and Sharma, R. (2013) 'The therapeutic potential of GPR120: a patent review', *Expert Opin Ther Pat,* 23(12), 1581-90.

Hauge, M., Vestmar, M. A., Husted, A. S., Ekberg, J. P., Wright, M. J., Di Salvo, J. *et al.* (2015) 'GPR40 (FFAR1) - Combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo', *Mol Metab,* 4(1), 3-14.

Hirasawa, A., Hara, T., Katsuma, S., Adachi, T. and Tsujimoto, G. (2008) 'Free fatty acid receptors and drug discovery', *Biol Pharm Bull,* 31(10), 1847-51.

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M. *et al.* (2005) 'Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120', *Nat Med,* 11(1), 90-4.

Hudson, B. D., Shimpukade, B., Mackenzie, A. E., Butcher, A. J., Pediani, J. D., Christiansen, E. *et al.* (2013) 'The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism', *Mol Pharmacol,* 84(5), 710-25.

Hyland, N. P., Sjöberg, F., Tough, I. R., Herzog, H. and Cox, H. M. (2003) 'Functional consequences of neuropeptide Y Y 2 receptor knockout and Y2 antagonism in mouse and human colonic tissues', *Br J Pharmacol,* 139(4), 863-71.

Ito, J., Ito, M., Nambu, H., Fujikawa, T., Tanaka, K., Iwaasa, H. *et al.* (2009) 'Anatomical and histological profiling of orphan G-protein-coupled receptor expression in gastrointestinal tract of C57BL/6J mice', *Cell Tissue Res,* 338(2), 257-69.

Itoh, Y., Kawamata, Y., Harada, M., Kobayashi, M., Fujii, R., Fukusumi, S. *et al.* (2003) 'Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40', *Nature,* 422(6928), 173-6.

Iwasaki, K., Harada, N., Sasaki, K., Yamane, S., Iida, K., Suzuki, K. *et al.* (2015) 'Free fatty acid receptor GPR120 is highly expressed in enteroendocrine K cells of the upper small intestine and has a critical role in GIP secretion after fat ingestion', *Endocrinology,* 156(3), 837-46.

Joshi, S., Tough, I. R. and Cox, H. M. (2013) 'Endogenous PYY and GLP-1 mediate L-glutamine responses in intestinal mucosa', *Br J Pharmacol,* 170(5), 1092-101.

Kaku, K., Enya, K., Nakaya, R., Ohira, T. and Matsuno, R. (2015) 'Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial', *Diabetes Obes Metab,* 17(7), 675-81.

Karaki, S., Mitsui, R., Hayashi, H., Kato, I., Sugiya, H., Iwanaga, T. *et al.* (2006) 'Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine', *Cell Tissue Res,* 324(3), 353-60.

Kostenis, E. (2004) 'A glance at G-protein-coupled receptors for lipid mediators: a growing receptor family with remarkably diverse ligands', *Pharmacol Ther,* 102(3), 243-57.

Lin, H. C., Zhao, X. T., Wang, L. and Wong, H. (1996) 'Fat-induced ileal brake in the dog depends on peptide YY', *Gastroenterology,* 110(5), 1491-5.

Little, T. J., Pilichiewicz, A. N., Russo, A., Phillips, L., Jones, K. L., Nauck, M. A. *et al.* (2006) 'Effects of intravenous glucagon-like peptide-1 on gastric emptying and intragastric distribution in healthy subjects: relationships with postprandial glycemic and insulinemic responses', *J Clin Endocrinol Metab,* 91(5), 1916-23.

Ma, J., Novack, A., Nashashibi, I., Pham, P., Rabbat, C. J., Song, J. *et al.* (2010) 'Aryl GPR120 receptor agonists and uses thereof.', *International Patent WO 2010/048207*, Metabolex Inc.

McCoull, W., Bailey, A., Barton, P., Birch, A.M., Brown, J.H., Butler, H.S. *et al* (2017) 'Indazole-6-phenylcyclopropylcarboxylic Acids as Selective GPR120 Agonists with in Vivo Efficacy', *J. Med. Chem*, (60), 3187-3197.

Negoro, N., Sasaki, S., Mikami, S., Ito, M., Suzuki, M., Tsujihata, Y. *et al.* (2010) 'Discovery of TAK-875: A Potent, Selective, and Orally Bioavailable GPR40 Agonist', *ACS Med Chem Lett,* 1(6), 290-4.

Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W. *et al.* (2010) 'GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects', *Cell,* 142(5), 687-98.

Overton, H. A., Babbs, A. J., Doel, S. M., Fyfe, M. C., Gardner, L. S., Griffin, G. *et al.* (2006) 'Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents', *Cell Metab,* 3(3), 167-75.

Panaro, B. L., Tough, I. R., Engelstoft, M. S., Matthews, R. T., Digby, G. J., Møller, C. L. *et al.* (2014) 'The melanocortin-4 receptor is expressed in enteroendocrine L cells and regulates the release of peptide YY and glucagon-like peptide 1 in vivo', *Cell Metab*, 20(6), 1018-29.

Parker, H. E., Habib, A. M., Rogers, G. J., Gribble, F. M. and Reimann, F. (2009) 'Nutrient-dependent secretion of glucose-dependent insulinotropic polypeptide from primary murine K cells', *Diabetologia*, 52(2), 289-98.

Patel, S., Mace, O. J., Tough, I. R., White, J., Cock, T. A., Warpman Berglund, U. *et al*. (2014) 'Gastrointestinal hormonal responses on GPR119 activation in lean and diseased rodent models of type 2 diabetes' in *Int J Obes*, 38, 1365-73.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP et al. (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucl Acids Res 44: D1054-1068.

Spiller, R. C., Trotman, I. F., Adrian, T. E., Bloom, S. R., Misiewicz, J. J. and Silk, D. B. (1988) 'Further characterisation of the 'ileal brake' reflex in man - effect of ileal infusion of partial digests of fat, protein, and starch on jejunal motility and release of neurotensin, enteroglucagon, and peptide YY', *Gut*, 29(8), 1042-51.

Spiller, R. C., Trotman, I. F., Higgins, B. E., Ghatei, M. A., Grimble, G. K., Lee, Y. C. *et al*. (1984) 'The ileal brake - inhibition of jejunal motility after ileal fat perfusion in man', *Gut*, 25(4), 365-74.

Stone, V. M., Dhayal, S., Brocklehurst, K. J., Lenaghan, C., Sorhede Winzell, M., Hammar, M. *et al.* (2014) 'GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans', *Diabetologia*, 57(6), 1182-91.

Tanaka, T., Katsuma, S., Adachi, T., Koshimizu, T. A., Hirasawa, A. and Tsujimoto, G. (2008) 'Free fatty acids induce cholecystokinin secretion through GPR120', *Naunyn Schmiedebergs Arch Pharmacol*, 377(4-6), 523-7.

Torjman, M.C., Joseph, J.I., Munsick, C., Morishita, M. and Grunwald, Z. (2005) 'Effects of Isoflurane on gastrointestinal motility after brief exposure in rats', *International Journal of Pharmaceutics*, 294, 65-71.

Tough, I.R., Moodaley, R. and Cox, H.M. (2017) 'Mucosal glucagon-like peptide 1 (GLP-1) responses are mediated by calcitonin gene-related peptide (CGRP) in the mouse colon and both peptide responses are area-specific', *Neurogastroenterol Motil*, e13149

Tough, I. R., Forbes, S., Tolhurst, R., Ellis, M., Herzog, H., Bornstein, J. C. *et al.* (2011) 'Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y(1) and Y(2) receptors', *Br J Pharmacol*, 164(2b), 471-84.

Tough, I. R., Holliday, N. D. and Cox, H. M. (2006) 'Y(4) receptors mediate the inhibitory responses of pancreatic polypeptide in human and mouse colon mucosa', *J Pharmacol Exp Ther*, 319(1), 20-30.

Tsujihata, Y., Ito, R., Suzuki, M., Harada, A., Negoro, N., Yasuma, T. *et al*. (2011) 'TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats', *J Pharmacol Exp Ther*, 339(1), 228-37.

Waring, M. J., Baker D. J., Bennett, S.N.L., Dossetter, A.G., Fenwick, M., Garcia, R. *et al.* (2015) 'Discovery of a series of 2-(pyridinyl)pyrimidines as potent antagonists of GPR40', *Med Chem Commun*, 6, 1024-9.

Warnotte, C., Gilon, P., Nenquin, M. and Henquin, J. C. (1994) 'Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells', *Diabetes*, 43(5), 703-11.

Xiong, Y., Swaminath, G., Cao, Q., Yang, L., Guo, Q., Salomonis, H. *et al.* (2013) 'Activation of FFA1 mediates GLP-1 secretion in mice. Evidence for allosterism at FFA1', *Mol Cell Endocrinol*, 369(1-2), 119-29.