SUPPLEMENTAL FIGURES, FIGURE LEGENDS, AND EXPERIMENTAL PROCEDURES

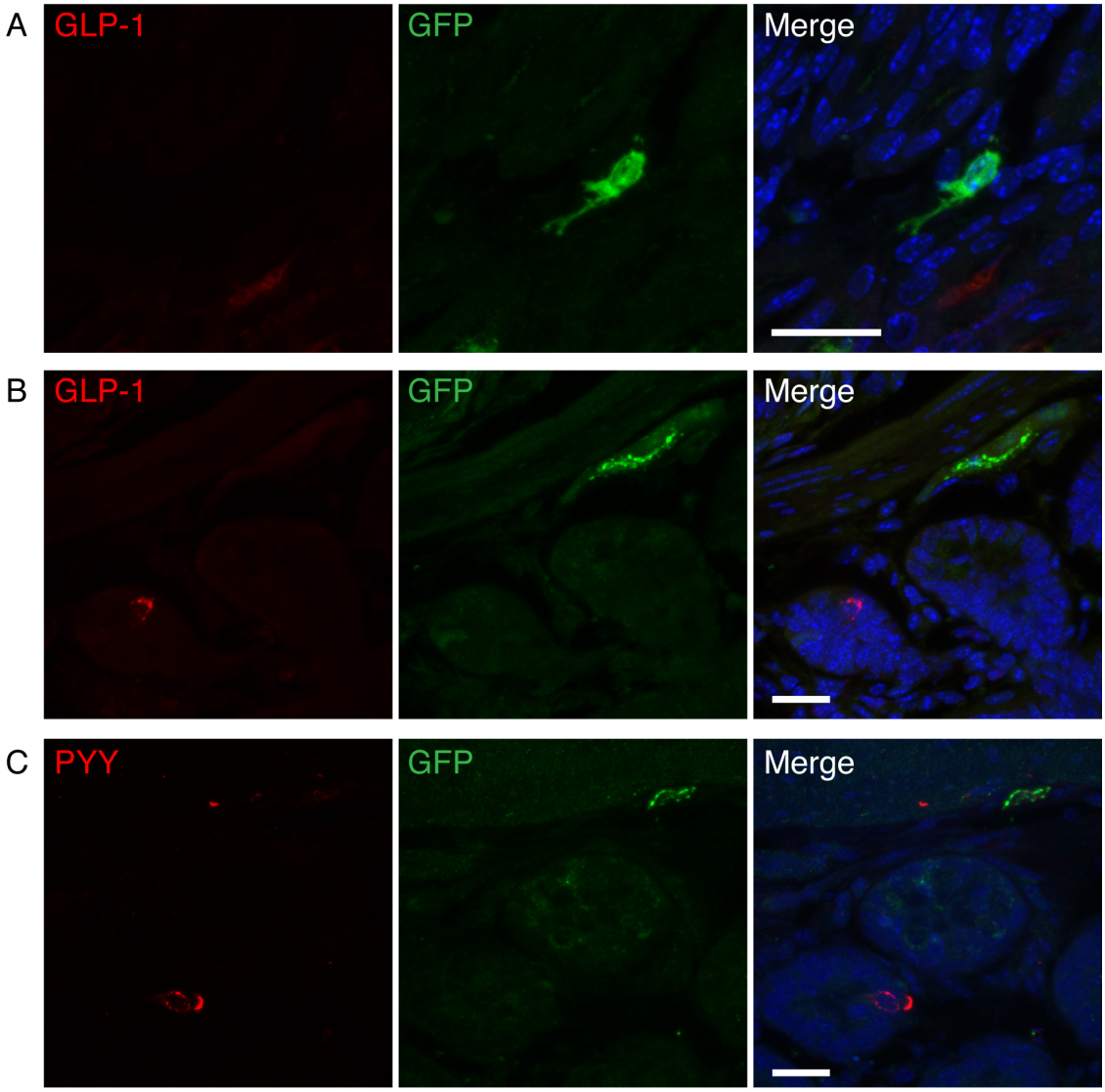

Figure S1, related to Figure 2. Presence of non-L cell MC4R expression in different regions of the mouse GI-tract. MC4R-Sapphire mice were used to visualize MC4R expressing cells in tissue segments from from **(A)** stomach, **(B)** jejunum and **(C)** ileum. MC4R-positive cells are marked with an antibody against GFP; L cells are marked with an antibody against either GFP or PYY; cell nuclei are marked with DAPI. The images are 2-dimensional projections of 3-dimensional multi-plane Z-stack images. Scale bars are 20 μm.

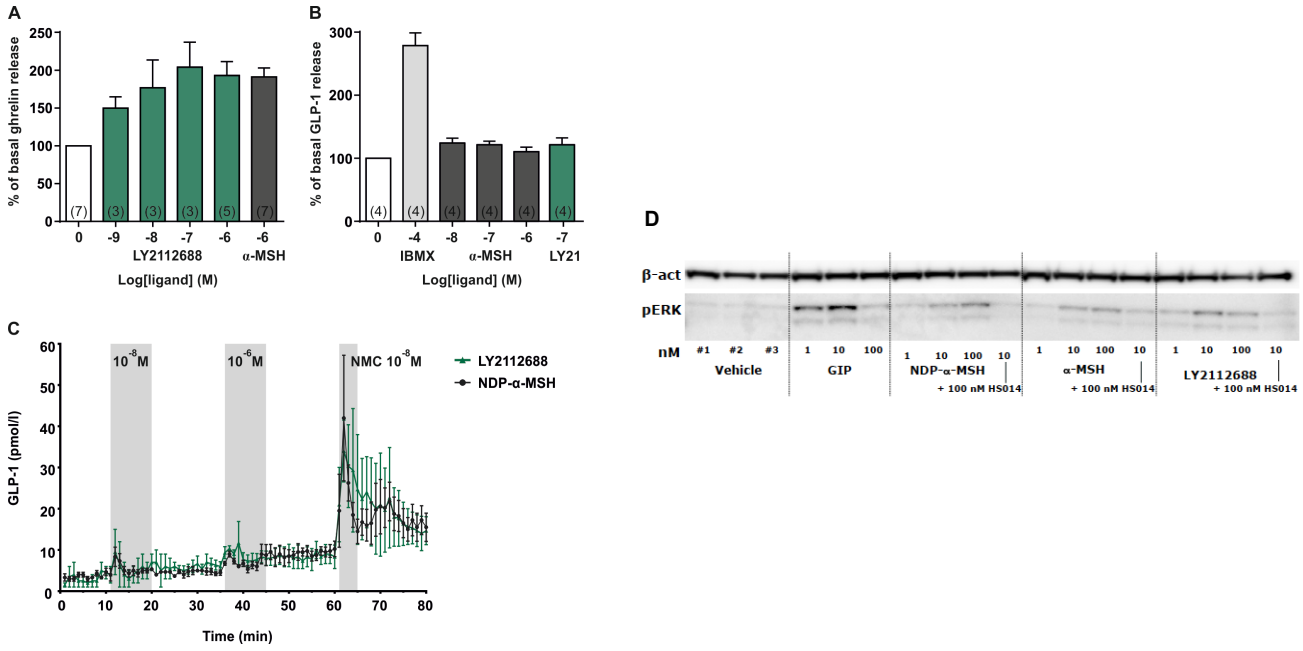

Figure S2, related to Figure 3. Detection of functional activities of MC4R in L cells and a L cell-derived cell line. (A) Acyl-ghrelin release from gastric mucosal cells treated with increasing concentrations of LY2112688 or 1 μM α-MSH. (B) Total GLP-1 release from colonic crypt cultures treated with 100 μM 3-isobutyl-1-methylxanthine (IBMX), increasing concentrations of α-MSH or 100 nM LY2112688 (LY21). The data has been normalized to the basal secretion from vehicle-treated cells and shown as means ± SEM. Number of repeated experiments is written in brackets in each bar. (C) The distal small intestine was perfused via the artery, and infusions of 10 nM NDP-α-MSH followed by 1 μM NDP-α-MSH (dark grey, n=3), or 10 nM LY2112688 followed by 1 μM LY2112688 (green, n=2) were conducted for ten minutes. The concentration of GLP-1 was measured in the venous effluent. Neuromedin C (NMC) was used as a positive control. (D) Western blot analysis of phosphorylation of ERK in GLUTag cells treated with GIP, NDP-α-MSH, α-MSH or the MC4 specific agonist LY2112688 (concentrations in nM) for five minutes. The MC4R specific antagonist HS014 was added prior to NDP-α-MSH, α-MSH or LY2112688 (each at 10 nM) in the experiments shown in the last column for each ligand.

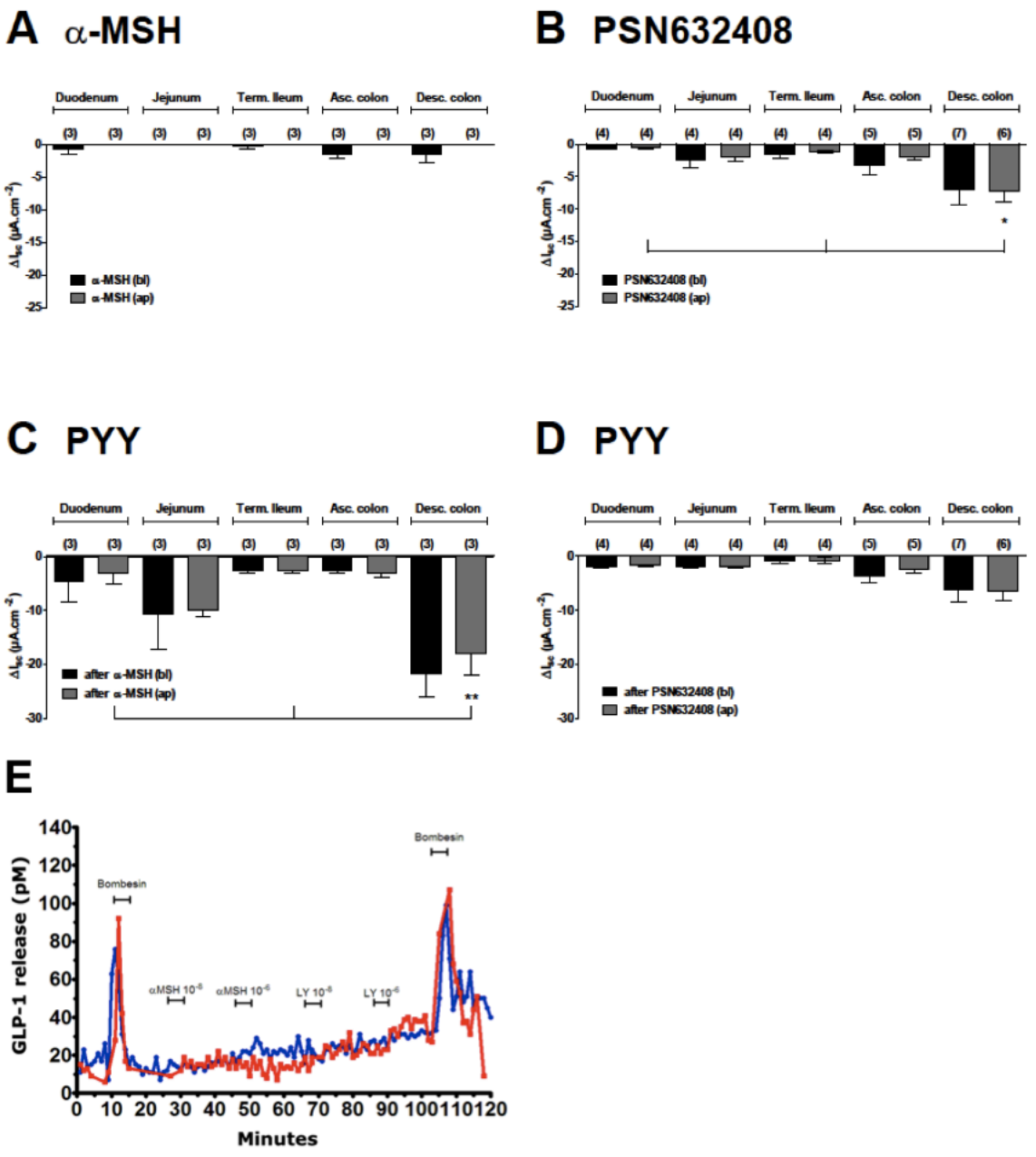

Figure S3, related to Figure 4. Mucosal responses to α-MSH, PSN632408 and PYY, and GLP-1 release following melanocortin ligand infusion are not detectable in the rat intestine. (A-D) Regional sensitivity to basolateral (bl) or apical (ap) α-MSH (1 μM, in **A**) or GPR119 agonist, PSN632408 (10 μM, in **B**) in duodenum, jejunum, terminal (Term.) ileum, ascending (Asc.) and descending (Desc.) colon. Subsequent reductions in I_{sc} to PYY (10 nM bl) after either α-MSH (in **C**) or PSN632408 (in **D**). Values are the mean - 1SEM with *n* numbers in parenthesis. **(E)** GLP-1 release from perfused rat small intestine. The small intestine was perfused via the artery, and infusions of 10 nM bombesin, 10 nM α-MSH, 1 μM α -MSH, 10 nM LY2112688 or 1 μM LY2112688 were conducted for five minutes.

The concentration of GLP-1 was measured in the venous effluent; colors indicate two independent experiments. In A-D, values are the mean - 1SEM with *n* numbers in parenthesis. In B, * $P < 0.05$ compares apical PSN632408 responses with those from more proximal intestinal areas as shown, while in C, ** $P < 0.01$ for comparisons as shown and in both one-way ANOVA with Bonferroni's post-test was performed.

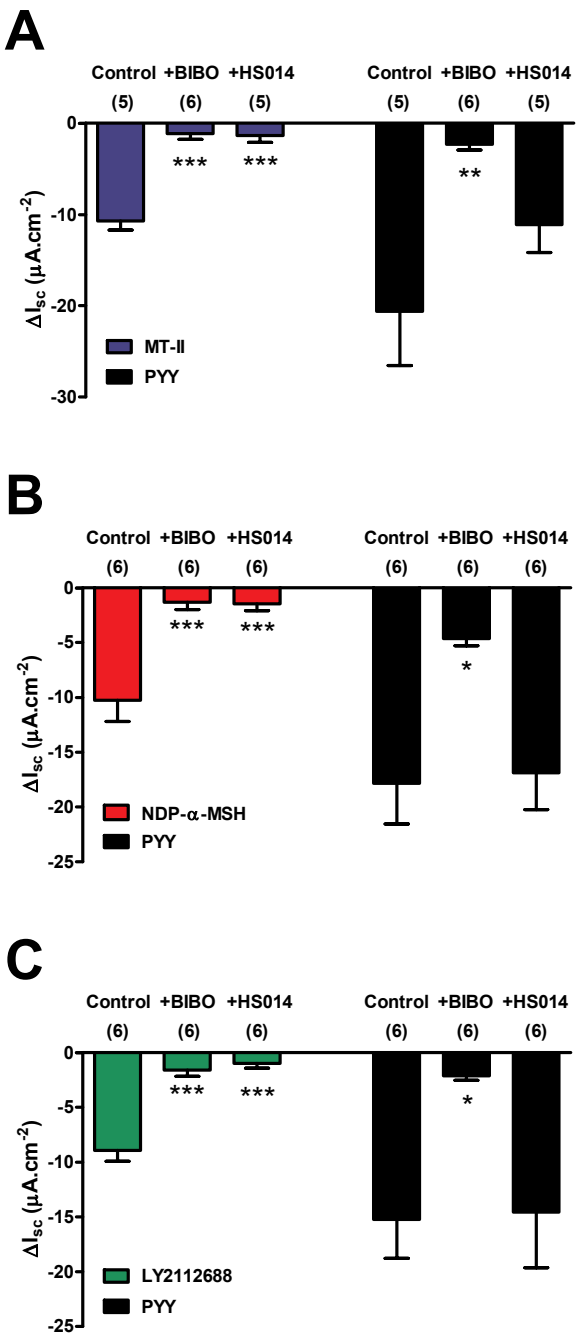

Figure S4, related to Figure 4. MC4R agonism in C57BL/6J **mouse colon mucosa are Y1 receptor-mediated**. MC4R responses activated by basolateral MT-II (3 nM in A) or NDP-α-MSH (3 nM in B) or LY2112688 (30 nM in C) with subsequent PYY responses (10 nM, bl). Pooled agonist responses (mean -1SEM) are shown after vehicle (Controls) or Y1 receptor blockade (+BIBO (BIBO3304); bl, 300 nM), or MC4R antagonism (+HS014; bl, 30 nM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compare data points with control α-MSH responses using 1-way ANOVA with Dunnett's post-test.

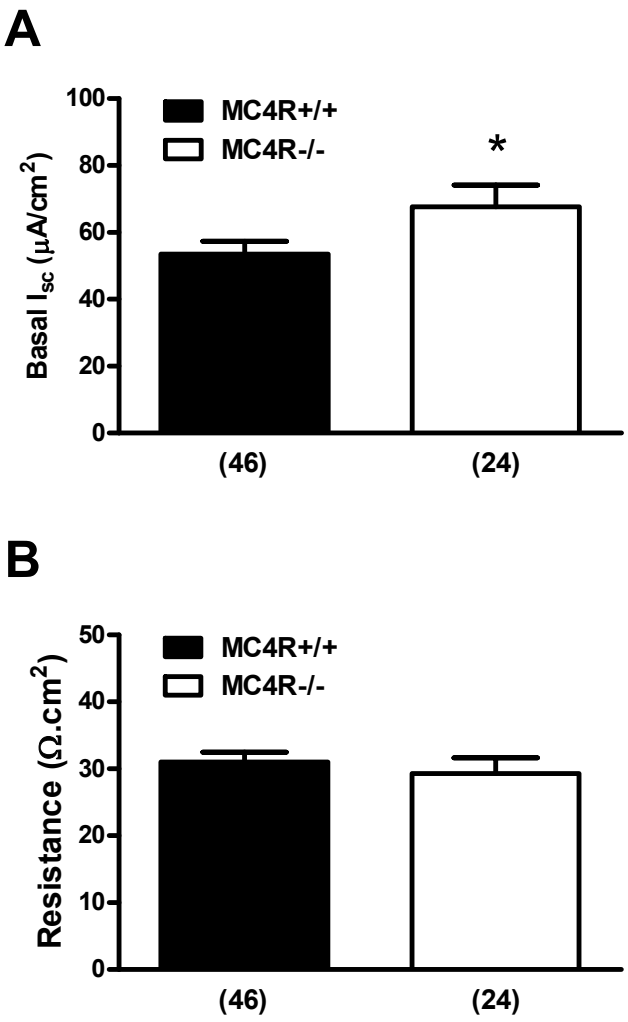

Figure S5, related to Figure 4. Basal I_{SC} levels are elevated in MC4R-/- colon mucosa. Basal I_{SC} (in A) are observed in colon preparations from MC4R-/- compared with MC4R+/+ mice. Basal resistances from the same preparations are unchanged. Bars are the mean + 1SEM with *n* numbers in parenthesis (* $P < 0.05$, Student's unpaired *t*-test).

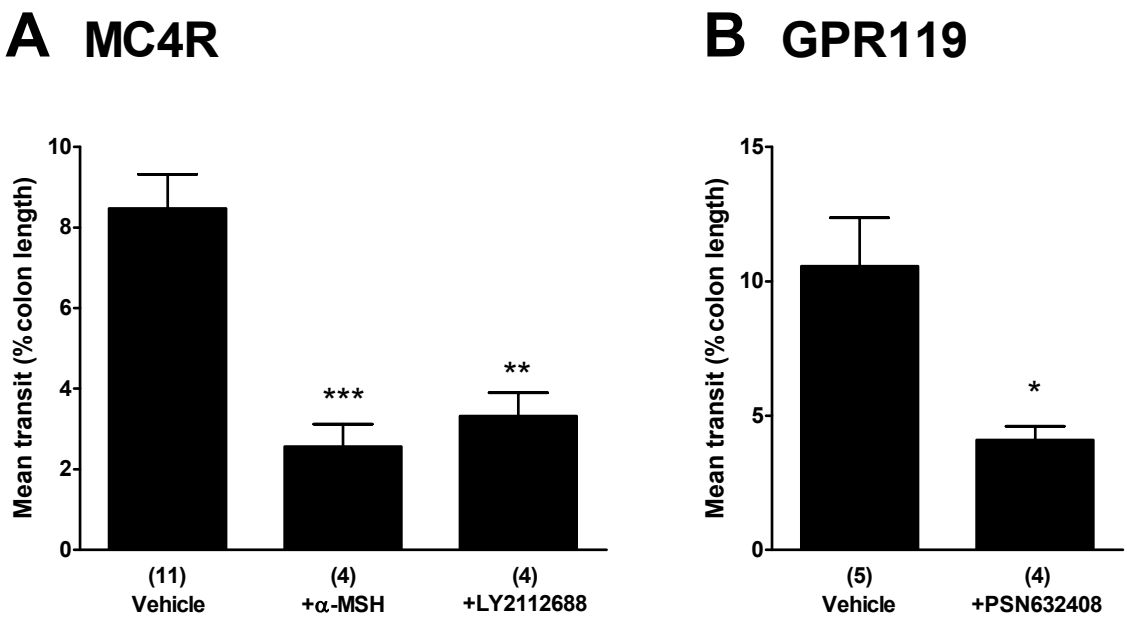

Figure S6, related to Figure 4. C57BL/6J mouse colon transit is attenuated by MC4R activation *in vitro*. Descending fecal pellet movement (as a % of colon length) in A: the presence of KH (Vehicle), α-MSH (1 μM) or LY2112688 (100 nM) and in B: Vehicle (95% ethanol) or GPR119 agonist, PSN632408 (10 μM). Values are the mean +1SEM with *n* numbers in parenthesis. * $P < 0.05$, ** $P < 0.01$, Student's unpaired *t*-test.

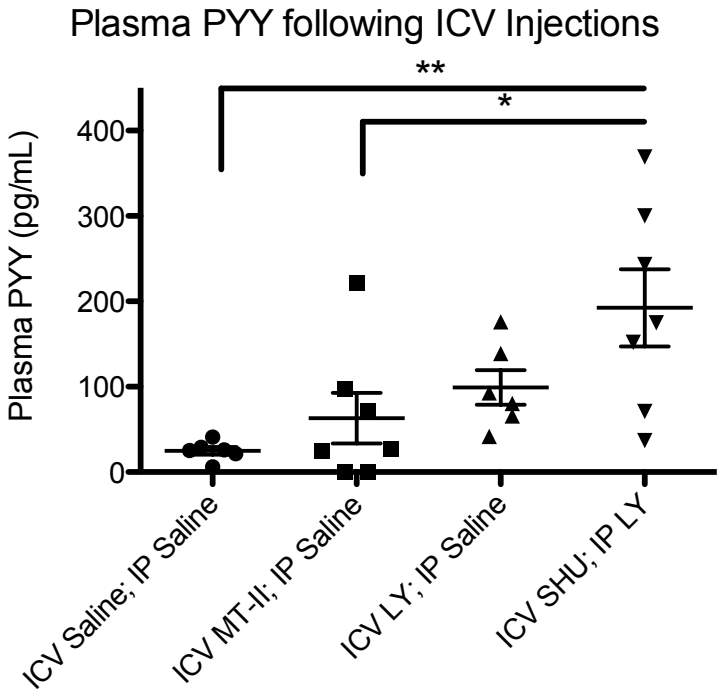

Figure S7, related to Figure 5. Effects of ICV melanocortin peptide administration on PYY release. Plasma PYY was assayed in mice given an intracerebroventricular (ICV) injection through a surgically placed guide cannula in the third ventricle immediately followed by a intraperitoneal injection (IP) with compounds as indicated. Plasma PYY was measured 10 minutes after the IP injection. Injections given via the ICV cannula included saline, 3 nmol melanotan-II (MT-II), 10 nmol LY2112688 (LY), 10 nmol SHU9119 (SHU). Injections given IP included either saline or 3 mg/kg LY2112688 (LY). Statistical significance was determined using 1-way ANOVA with Bonferroni post-test. ** $P<0.01$, * $P<0.05$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Hormone Release Assays

Hormone release from primary cultures of mouse small intestine ghrelin and GLP-1 release experiments have been described in detail previously (Engelstoft et al., 2013) and (Reimann et al., 2008). Acyl-ghrelin was measured using the Acyl-ghrelin EIA from SPI-Bio (AH Diagnostics). GLP-1 was measured according to the protocol Total GLP-1 version 2 from Meso Scale Discovery.

Culture and Stimulation of GLUTag Cells

The GLUTag cell line was grown to 75% confluence in DMEM (Invitrogen #21885) supplemented with 10% FCS, pen/strep and L-gluthamine (37oC, 10% CO2) (passage 9-16). Confluent GLUTag cells were harvested and dispersed in 12 well plates (200.000 cells/well) and incubated for two days. On the day of the experiment, cells were washed with HBSS and incubated with 500 µl of serum/L-gluthamine starved DMEM for 2 h prior to stimulation (Invitrogen #21885). 30 min prior to stimulation, the antagonist HS014 (Bachem #H-4352) was added to the appropriate wells in HBSS + 0.1 % free fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich #A0281). The remaining wells received same stimulation buffer without the antagonist. Cells were stimulated with 1, 10 or 100 nM of α-MSH (Bachem #H-1070), NDP-α-MSH (Bachem #H-1100) or LY2112688 (prepared in-house using standard peptide synthesis protocol) for 5 min (based on initial time-response data; not shown).

Protein Extraction and Western Blotting

After stimulation, the cells were placed on ice, washed in cold HBSS, and incubated with 200 µl/well protein extraction buffer (RIPA buffer, Millipore #20-188) supplemented with Mini complete protease inhibitor (Roche #5892953001) for 30 min. The lysates were centrifuged for 10 min (10.000 g, 4 °C) and the supernatant was used for determination of protein content using BCA protein assay (Pierce #23227). Samples (20 µg) and SeeBlue marker (Invitrogen #LC5625) were loaded on 10% Bis-Tris Precast gels (Invitrogen #WG1202) (30–45 min at 150 V and 30–45 min at 180 V). Gels were transferred to membranes, which were washed, blocked (1 h) and incubated overnight (4 °C) with primary antibody. Membranes were washed (3 × 10 min) and secondary antibody was added to the membranes (1 h at room temperature). Immobilon Western Chemiluminescent HRP Substrate (Millipore #WBKLS0500) was used when developing blots.

Perfused Small Intestine

Non-fasted male C57BL/6J mice (11 weeks) and Wistar rats (250-300g) were anesthetized with a subcutaneous injection of a mixture of Hypnorm/Midazolam. The procedure was performed as previously described (de Heer and Holst, 2007) with modifications to accommodate intestinal perfusion. Briefly, the small intestine was perfused through the superior mesenteric artery for inflow of

perfusion buffer. The venous effluent was collected for 1 min periods via a catheter inserted into the portal vein. Experiments were performed on either proximal or distal small intestine in separate experiments. The remaining small intestine and the colon were excluded from perfusion.

The perfusion buffer consisted of a modified Krebs Ringer bicarbonate buffer containing 0.1% BSA (Merck KGaA, Darmstadt, Germany), 5% Dextran T-70 (Sigma Aldrich, St. Louis, MO, USA), 3.5 mM glucose, and 5 mM pyruvate, fumarate, and glutamate each. The perfusion medium was gassed with a 95% O2/5% CO2 mixture to achieve pH 7.4 and maintained at 37°C during the entire experiment. The flow rate was kept constant at 5ml/min (rats) or 2.5 ml/min (mice). Infusion of test substances was performed through a sidearm syringe pump in 5-10-min periods separated by 20 min rest periods for measuring basal level. Test substances included LY2112688, αMSH and NDP-αMSH. Bombesin (10nM; Bachem, Bubendorf, Switzerland) was infused at the end in all experiments as our internal control. The effluent was analyzed for GLP-1 concentrations using an in-house developed radioimmunoassay. GLP-1 was measured with an antibody (antiserum #2135) directed against a mid-sequence of GLP-1, cross-reacting with all forms of GLP-1 including major proglucagon fragment (Orskov C, Holst JJ, Poulsen SS, Kirkegaard P 1987 Pancreatic and intestinal processing of proglucagon in man. Diabetologia 30:874-881). Synthetic GLP-1 7-36 amide (Bachem H-6795-GMP, 4081700) were used as standard and 125I-labeled GLP-1 7-36 amide (a gift from Novo Nordisk A/S, Bagsvaerd, Denmark) as tracer.

Colonic Transit Measurement *In Vitro*

Measurement of fecal pellet movement down the entire colon isolated from WT mice, involved recording photographically the position of pellets (at t=0 min). Tissue was then placed in aerated KH buffer (at 37˚C) with drug (1 μM a-MSH, 100 nM LY2112688 or 10 μM PSN) or vehicle for 20 min and the colons then re-photographed. The distances of remaining pellets from the rectum were measured and colonic transit was calculated as the mean distance travelled relative to the total colon length (as % colonic transit).

SUPPLEMENTAL REFERENCES

de Heer, J., and Holst, J.J. (2007). Sulfonylurea compounds uncouple the glucose dependence of the insulinotropic effect of glucagon-like peptide 1. Diabetes 56, 438-443.

Engelstoft, S.M., Park, W., Sakata, I., Kristensen, L.V., Husted, A.S., Osborne-Lawrence, S., Piper, P.K., Walker, A.K., Pederson, M.H., Nohr, M.K., et al. (2013). Seven transmembrane G protein-coupled receptor repertoire of gastrin ghrelin cells. Molecular Metabolism 2, 376-392.

Reimann, F., Habib, A.M., Tolhurst, G., Parker, H.E., Rogers, G.J., and Gribble, F.M. (2008). Glucose sensing in L cells: a primary cell study. Cell Metab 8, 532-539.