Original Article

MOLECULAR METABOLISM

Combined loss of GLP-1R and Y2R does not alter progression of high-fat diet-induced obesity or response to RYGB surgery in mice

Brandon B. Boland [1,3], Michael B. Mumphrey [2,3], Zheng Hao [2], R. Leigh Townsend [2], Benji Gill [1], Stephanie Oldham [1], Sarah Will [1], Christopher D. Morrison [2], Sangho Yu [2], Heike Münzberg [2], Christopher J. Rhodes [1], James L. Trevaskis [1,4], Hans-Rudolf Berthoud [2,*,4]

ABSTRACT

Objective: Understanding the mechanisms underlying the remarkable beneficial effects of gastric bypass surgery is important for the development of non-surgical therapies or less invasive surgeries in the fight against obesity and metabolic disease. Although the intestinal L-cell hormones glucagon-like peptide-1 (GLP-1) and peptide tyrosine–tyrosine (PYY) have attracted the most attention, direct tests in humans and rodents with pharmacological blockade or genetic deletion of either the GLP1-receptor (GLP1R) or the Y2-receptor (Y2R) were unable to confirm their critical roles in the beneficial effects gastric bypass surgery on body weight and glucose homeostasis. However, new awareness of the power of combinatorial therapies in the treatment of metabolic disease would suggest that combined blockade of more than one signaling pathway may be necessary to reverse the beneficial effects of bariatric surgery.

Methods: The metabolic effects of high-fat diet and the ability of Roux-en-Y gastric bypass surgery to lower food intake and body weight, as well as improve glucose handling, was tested in GLP1R and Y2R-double knockout (GLP1RKO/Y2RKO) and C57BL6J wildtype (WT) mice.

Results: GLP1RKO/Y2RKO and WT mice responded similarly for up to 20 weeks on high-fat diet and 16 weeks after RYGB. There were no significant differences in loss of body and liver weight, fat mass, reduced food intake, relative increase in energy expenditure, improved fasting insulin, glucose tolerance, and insulin tolerance between WT and GLP1RKO/Y2RKO mice after RYGB.

Conclusions: Combined loss of GLP1R and Y2R-signaling was not able to negate or attenuate the beneficial effects of RYGB on body weight and glucose homeostasis in mice, suggesting that a larger number of signaling pathways is involved or that the critical pathway has not yet been identified.

Keywords Bariatric surgery; PYY; GLP-1; Diabetes; Glucose tolerance; Insulin tolerance

1. INTRODUCTION

It is difficult to sustain the beneficial effects of behavioral and lifestyle modifications and low-calorie meal replacement strategies, as the normal biological adaptations, such as increased hunger and decreased metabolism, tend to counteract the weight loss and result in relapse [1]. It is estimated that more than eighty percent of patients engaging in such non-invasive conventional weight loss therapies regain all lost body weight within a few years [2]. More sustained effects are achieved with pharmacotherapy, particularly if combined with these conventional approaches [3], but the prospect that children and adolescents with obesity have to take drugs for decades is of concern.

On the other hand, bariatric surgery, particularly vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB), results in large and sustained weight loss and the concomitant remission or prevention of T2DM, cardiovascular disease, fatty liver disease, sleep apnea, and certain cancers, in a majority of patients [4]. For many reasons, it is not possible to treat all obese patients with surgery; however, understanding the molecular mechanisms by which surgery confers its remarkable beneficial effects would go a long way in designing surgeries with a better efficiency/invasiveness ratio or non-surgical obesity treatments.

In the search for molecular mechanisms underlying the beneficial effects of bariatric surgeries on body weight and metabolic health, favorable changes in gut hormone signaling have been prime

[1]Cardiovascular, Renal and Metabolic Diseases, MedImmune LLC, Gaithersburg, MD, USA [2]Pennington Biomedical Research Center, Baton Rouge, LA, USA

[3] These authors contributed equally to this work.
[4] Co-senior authors.

*Corresponding author. Neurobiology of Nutrition & Metabolism, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, USA. Fax: +1 225 763 0260. E-mail: berthohr@pbrc.edu (H.-R. Berthoud).

Received March 13, 2019 • Revision received April 30, 2019 • Accepted May 4, 2019 • Available online 9 May 2019

https://doi.org/10.1016/j.molmet.2019.05.004

candidates [5,6]. GLP-1 and PYY, both secreted from the intestinal L-cells have attracted the most attention, because their postprandial circulating levels are greatly increased after both VSG and RYGB [7,8] and because they have powerful physiological actions on gut, pancreas, and brain that result in reduced food intake and lower body weight [9]. However, in direct tests of their importance for successful bariatric surgeries, systemic disruption of GLP-1 signaling with Exendin-9,39 in obese diabetic and non-diabetic human subjects [10,11] and with GLP-1R deletion in mice did not change the effectiveness of RYGB to lower body weight, food intake, and improve glycemic control [12,13]. Support for a role of PYY comes from the observation that the body weight-lowering effect of surgery seen in wildtype mice was reduced in mice with PYY-deficiency [14]. However, interpretation of that study is complicated, as duodenal bypass surgery with a side-to-side anastomosis of the duodenum to the gastric greater curvature, not RYGB or VSG was performed, and the preparation was only viable for 10 days after surgery. Pharmacological blockade of Y2-receptor signaling for 2 weeks in the brain of rats with previous RYGB was unable to increase food intake and body weight, suggesting that PYY-signaling in the brain is not required for the beneficial effects of RYGB [13]. Furthermore, RYGB was similarly effective in lowering body weight and adiposity in Y2R knockout mice [15].

One of the reasons for these mostly negative and unexpected outcomes with single pathway germline knockout models could be compensatory adaptive changes in other pathways. It is plausible that, when knocking out the GLP-1 signaling pathway, the PYY-signaling pathway takes over some of the functions and vice versa [16]. This view is somewhat supported by the demonstration that subthreshold doses of GLP-1 and PYY(3—36) produce substantial decreases in food intake in humans, when combined [17]. Therefore, we hypothesized that deleting both signaling pathways simultaneously in GLP1R/Y2R-double knockout mice would significantly attenuate or abolish the effects of RYGB on food intake, body weight, and glycemic control.

2. MATERIALS AND METHODS

2.1. Experimental animals

Animal studies conducted at MedImmune (USA) adhered to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at MedImmune in line with the AstraZeneca Animal Welfare and Bioethics policies. Studies conducted at the Pennington Biomedical Research Center adhered to protocols approved by the Pennington Center IACUC.

Double GLP1RKO:Y2RKO mice were derived from crosses of GLP1RKO:Y2R heterozygous breeding pairs. Initially, Y2R-deficient mice (Y2RKO) were generated by CRISPR/Cas9 technology as described previously [15]. GLP-1R-deficient mice (GLP1RKO) were originally obtained from the laboratory of Dr Daniel Drucker [18]. GLP1RKO mice were bred with heterozygous mice to generate GLP1R heterozygotes either wildtype or heterozygous for Y2R. Double heterozygous mice were bred to yield GLP1RKO mice heterozygous for Y2R. It was these mice that were interbred at The Jackson Laboratory (Bar Harbor, ME, USA) to generate GLP1RKO and GLP1RKO/Y2RKO used in these studies. Wildtype controls and Y2RKO mice were obtained from a parallel Y2R heterozygous breeding colony [15]. Genotyping was performed at Transnetyx (Cordova, TN) from samples collected from mice generated at The Jackson Laboratory, using previous published PCR protocols [15] for the *Npy2r* allele and Baggio et al. for *Glp1r* [18]. All mouse lines were backcrossed onto C57BL6J genetic background and were housed in standard caging at 22 °C in a 12-h light: 12-h dark cycle at standard temperature and humidity conditions with *ad libitum* access to water and food (except where noted).

2.2. High-fat diet studies

2.2.1. Animals and diets

Male C57BL6/J mice (WT), Y2RKO, GLP1RKO, and GLP1RKO/Y2RKO mice were used for these studies. They arrived at 10—12 weeks of age and, following one-week of acclimatization, baseline characteristics were recorded, including %HbA1c, and used to randomize the mice. Mice were then single-housed and transitioned to a 60% HF diet (# 12492, Research Diets) to yield 4 groups: WT, Y2RKO, GLP1RKO, and GLP1RKO/Y2RKO. Body weight was tracked weekly for 3 months. Six-hour fasting insulin and glucose were tracked at monthly intervals, as was body composition. At month 3, an intraperitoneal glucose tolerance test (1.5 g/kg) was performed following 6 h of fasting. One week later, a mixed-meal tolerance test (10 μL Ensure®/g) following 6 h of fasting; acetaminophen was added to the Ensure® mixture to allow assessment of gastric emptying (10 g/L). One week later, an additional mixed meal tolerance test was performed to assess baseline and meal-stimulated peptide release. Three days later, animals were sacrificed, and the proximal duodenum was removed and snap frozen for subsequent gene expression analysis.

2.2.2. Plasma parameters

For the 6-h fasting blood glucose values in all cohorts and the blood glucose values indicated for the ipGTTs and ITTs, a glucometer was used (Ascensia Breeze, Bayer, Germany) with tail-vein blood. For all other tolerance tests, plasma glucose was determined via colorimetric glucose oxidase kit (Cayman Chemical, Ann Arbor, MI). Six-hour fasting plasma insulin was determined via ELISA (MSD, Rockville, MD). The % HbA1c was determined colorimetrically from whole blood (Crystal Chem, Elk Grove Village, IL). For determination of circulating metabolic plasma peptide levels, the animals were 6-h fasted, retro-orbitally (RO) bled, gavaged with 10 μL/g Ensure®, and then RO bled again at minute 30. The Milliplex MAP mouse metabolic hormone magnetic bead panel was used to assess all peptides (Kenilworth, NJ), except for GLP-2, which was determined using the ALPCO mouse GLP-2 ELISA (Salem, NH) and total GLP-1, which was determined using a commercial ELISA (EZGLP1T-36K assay, Millipore Sigma, Burlington, MA). Plasma acetaminophen was determined at minute 30 using a colorimetric kit (Cambridge Life Sciences, UK).

2.2.3. Gene expression analysis

Duodenal RNA was isolated using TRIzol reagent (ThermoFischer, Waltham, MA). cDNA was synthesized using the Superscript III First-Strand Synthesis System (ThermoFischer). qRT-PCR analysis was performed using Taqman Gene Expression probe/primer sets (ThermoFischer) for *Npy1r*, *Npy2r*,*Glp1r*, *Glp2r*, *Cckar*, *Cckbr*, *Gipr*, *Cck*, *Pyy*, *Gcg*, *Gip*, and *Ppia*. Results are shown as the target gene expression relative to *Ppia* expression and normalized to WT expression using the $2^{(-\Delta\Delta Ct)}$ method.

2.2.4. Tolerance tests and gastric emptying

Intraperitoneal glucose tolerance tests were performed at either month 3 or 4. Mice were 6-h fasted and injected intraperitoneally with 1.5 g/kg glucose in saline. Blood glucose was determined at 0, 15, 30, 60, 120, and 180 min for cohort 1; 0, 15, 30, 60, and 120 min for cohort 2; and 0, 15, 30, 60, 90, and 120 min for cohort 3.

Mixed-meal tolerance tests and simultaneous assessment of gastric emptying were performed at month 3. Mice were 6-h fasted for 6 h

and gavaged with 10 μL Ensure®/g containing 10 g/L acetaminophen (Sigma, St. Louis, MO). Blood glucose was determined at baseline and at 30 min.

2.3. RYGB studies

2.3.1. Animals and diets

Male mice homozygous for both the Y2R and GLP-1R deletions, GLP1RKO/Y2RKO mice, and age-matched C57BL6/J wildtype mice exposed to high-fat diet (60%, # 12492, Research Diets) from 6 weeks of age were shipped to the Pennington Biomedical Research Center at the age of 14 weeks. They were housed individually and switched to a two-choice diet consisting of high-fat diet and regular chow (Purina LabDiet). Baseline measurements of food intake were carried out after 4 weeks of adaptation and before surgeries were performed.

2.3.2. RYGB, sham surgery, and weight matching

RYGB was carried out according to a protocol described previously [19]. Briefly, in a jejuno-gastric anastomosis, the cut end of the mid jejunum was connected with a very small gastric pouch and the other end of the cut jejunum was anastomosed to the lower jejunum, resulting in a 5–6 cm long Roux limb, a 9–11 cm long biliopancreatic limb, and a 20–25 cm long common limb. Sham surgery consisted of laparotomy only, without transection of jejunum and stomach. Mice weight-matched (WM) to the RYGB group were restricted to about 50–70% of the caloric intake of the RYGB group. Pre-weighed amounts of food (Kcal: ~93% high-fat, ~7% chow) were given once per day during the light period.

Three of the double-knockout mice undergoing RYGB died, two after 1 week and one after 4 weeks. No autopsy was performed, and there was no apparent reason for their death, except for signs of an inflammation and infection. One additional double-knockout mouse in the weight-matched group died for apparently the same reasons. These mice were not included in any of the analyses. None of the double-knockout with sham surgery and none of the WT mice had any complications.

2.3.3. Measurement of body weight, body composition, food intake, and energy expenditure

Body weight was measured every 2–3 days for RYGB and sham mice, and every day for WM mice. Body composition was measured before and every 2 weeks (±4 days) after surgery with a Minispec LF 90 NMR Analyzer (Bruker Corporation, The Woodlands, TX). Adiposity index was defined as fat mass divided by lean mass.

Food intake was measured for 5 days before and 10 days after surgery. Total food intake in kcal was derived from intake of high-fat diet (5.24 kcal/g) and regular chow diet (3.02 kcal/g) and by taking spillage into account. Chow preference was calculated as the percentage of total food intake in kcals obtained from regular chow diet.

Energy expenditure, RER, and locomotor activity were measured at 5–7 weeks after surgery in metabolic chambers (Phenomaster/Labmaster, TSE Systems, Germany). All mice were first adapted to eating food from hanging baskets in training cages for 4–6 days. Mice that had difficulty eating from hanging baskets were floor-fed. Energy expenditure was measured at two ambient temperatures, normal room temperature at 23 °C for 3 days and near thermoneutrality at 29 °C for 3 days. Mice were adapted for at least one day to each condition before taking measurements. Energy expenditure is reported as both unadjusted in kcal/mouse and adjusted for lean and total body mass. Locomotor activity was measured in numbers of beam breaks in the X and Y planes (7 mm spatial resolution, 10 ms temporal resolution).

2.3.4. Glucose and insulin tolerance tests, fasting insulin and leptin

Glucose tolerance was assessed at 3–4 weeks after surgery by injecting α-D-glucose (1.5 mg/kg, 30% w/v in sterile saline, i.p.) and measuring blood glucose from the tail vein before and at 15, 30, 60, and 120 min after injection, with glucose strips and a glucometer (Onetouch Ultra Strips and Onetouch Ultra Glucometer, LifeScan INC, Milpitas, CA). Glucose tolerance tests were conducted between 09:00 and noon, after 3–5 h of food deprivation.

Insulin tolerance was assessed at 9–10 weeks after surgery by injecting insulin (0.6 U/kg in sterile saline, i.p., Novolin R, Novo Nordisk, Bagsvaerd, Denmark) and measuring blood glucose as above.

2.3.5. Final plasma and tissue harvest

16 weeks after surgery, mice were food deprived for 3–5 h and euthanized by decapitation. A few drops of trunk blood were collected, and blood glucose was immediately tested using glucose strips as above. An additional 500 μL of trunk blood was collected, treated with 83.5 μL EDTA (Sigma, St. Louis, MO) and a protease inhibitor cocktail (1.5 μL of each of the following: Protease inhibitor, Sigma, St. Louis, MO; DPP-IV inhibitor, EMD Millipore, St. Charles, MO; Prefabloc SC, Roche, Indianapolis, IN), and immediately centrifuged at 4 °C and 3000 RPM for 10 min to separate the plasma from the whole blood. Plasma aliquots were frozen in liquid nitrogen and stored at −80 °C prior to processing. Plasma was subjected to ELISA for measurement of insulin and other hormone concentrations (MADKMAG-71K Milliplex map mouse adipokine magnetic bead panel – endocrine multiplex assay, EMD Millipore, St. Charles, MO).

2.4. Statistical analysis

For high-fat diet growth curves, data normality was determined by the D'Agnostino and Pearson test. Parametric data were analyzed by one-way or two-way ANOVA followed by Tukey's test. Data are presented as mean ± SEM. All data were analyzed using GraphPad Prism software version 7.02 (San Diego, CA). Statistical significance was set at $p \leq 0.05$.

Differential changes in weight, fat mass, and adiposity index between RYGB and sham were analyzed with ANOVA, followed by Students *t*-tests and considered significant at $p < 0.05$. Food intake, energy expenditure, locomotor activity, RER, insulin and glucose tolerance AUC, and fasting plasma assays were analyzed with two-way ANOVA using treatment group and genotype as between subject variables. Benjamini-Hochberg corrected pairwise *t*-tests with false discovery rate set at 0.05 were used for specific group comparisons. All data are reported as mean ± SEM.

3. RESULTS

3.1. Response to high-fat diet

High-fat diet-induced body weight gain, and body composition was similar in WT, GLP1R-KO, Y2R-KO, and GLP1RKO/Y2RKO mice over the 15 weeks of exposure (Figure 1A–C). Both fasting glucose and insulin increased with time of exposure to high-fat diet in all groups, with only minor differences, most notably lower fasting insulin levels at 15 weeks in GLP1RKO and GLP1R/Y2R-double knockout compared with WT mice (Figure 1D,E). Glucose tolerance at 10 weeks tended to be more impaired in the GLP1RKO and GLP1RKO/Y2RKO, but less impaired in Y2RKO compared to WT mice (Figure 1G). However, when

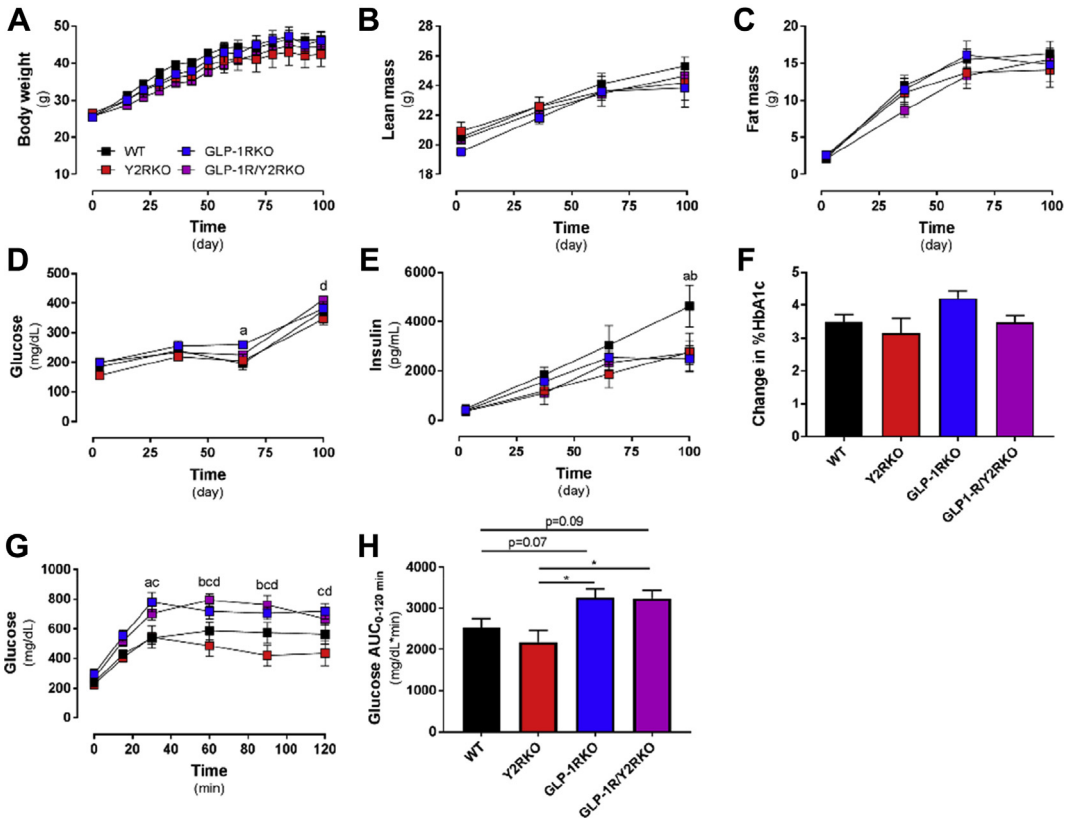

Figure 1: Physiological parameters of male WT ($n = 11$), Y2RKO ($n = 7$), GLP1RKO ($n = 11$), and GLP1RKO/Y2RKO mice ($n = 11$) on 60% HF diet. A–C: Absolute body weight (A), lean mass (B) and fat mass (C). D, E: 6-h fasting plasma glucose (D) and insulin (E). F: Overall change in % HbA1c during the study period. G, H: Intraperitoneal glucose tolerance test (1.5 g/kg, G) and the associated AUC (H). $N \geq 7$ for all experiments. [a] $p \leq 0.05$ WT vs GLP1RKO, [b] $p \leq 0.05$ WT vs GLP1RKO/Y2RKO, [c] $p \leq 0.05$ Y2RKO vs GLP1RKO, [d] $p \leq 0.05$ Y2RKO vs GLP1RKO/Y2RKO, [*] $p \leq 0.05$ vs indicated groups.

looking at the area under the curve, only the differences between Y2RKO and GLP1RKO/Y2RKO as well as Y2RKO and GLP1RKO were statistically significant (Figure 1H). There were no significant differences in hemoglobin A1c levels between any groups (Figure 1F). Similar observations were made with a mixed meal tolerance test. Thirty minute blood glucose concentrations were similar between WT and Y2RKO mice, but significantly higher in GLP1RKO and GLP1RKO/Y2RKO mice (Supplementary Figures S1a and b). There were no significant group differences in gastric emptying (Supplementary Figure S1c).

With few exceptions, fasting plasma hormone levels were similar among the 4 groups (Supplementary Table 1). Plasma levels of GLP-1 were higher in GLP1RKO/Y2RKO mice, GLP-2 were significantly higher in the Y2RKO and GLP1RKO compared to the GLP1RKO/Y2RKO mice, and ghrelin levels were significantly lower in Y2RKO and GLP1RKO compared to WT and GLP1RKO/Y2RKO mice. Postprandial plasma hormone levels at 30 min following a mixed meal test were also mostly similar (Supplementary Table 1). Plasma amylin concentrations were significantly higher in GLP1RKO compared with double-knockout mice, and glucagon concentrations were higher in GLP1RKO mice compared with Y2RKO and GLP1RKO/Y2RKO mice.

Gene expression analysis of proximal intestine confirmed complete absence of *Npy2r* expression in Y2RKO and *Glp1r* expression in GLP1RKO mice (Supplementary Table 2). In none of the three strains of knockout mice was there compensatory up- or down-regulation of other relevant genes, except for a significant upregulation of *Glp2r* in double knockout mice.

3.2. Response to RYGB

3.2.1. Body weight and body composition

Body weight was similarly affected by RYGB and sham surgery in WT and GLP1RKO/Y2RKO mice (Figure 2A,B). RYGB in these continuously high-fat fed mice resulted in a rapid weight loss followed by a small weight regain, with a nadir of 20% weight loss around 2–6 weeks after surgery. Body weight loss compared to presurgical levels was not significant between genotypes at any time point up to 16 weeks. Sham surgery resulted in a small and transient weight loss, followed by continued weight gain. Compared to their respective sham, mice with RYGB of both genotypes lost similar amounts of body weight over 16 weeks.

Weight loss was primarily due to loss of fat mass but not lean mass (Figure 2C–E). Compared to calorie resricted, weight-matched controls, RYGB produced greater fat mass loss but less lean mass loss, resulting in a significantly lower adiposity index. However these effects were not significantly different in WT and GLP1RKO/Y2RKO mice. Plasma leptin levels at termination generally reflected body composition, except that the lower leptin levels in GLP1RKO/Y2RKO mice with RYGB compared with sham were not significantly different because of 2 mice with very high leptin levels (Figure 2F).

Weights of individual fat pads generally confirmed overall body fat analysis (Supplementary Figure S2). RYGB resulted in significantly lower perirenal, mesenteric, and inguinal fat pad weight in both WT and GLP1RKO/Y2RKO mice compared with sham surgery. While the lower weight of retroperitoneal fat after RYGB was also significant in

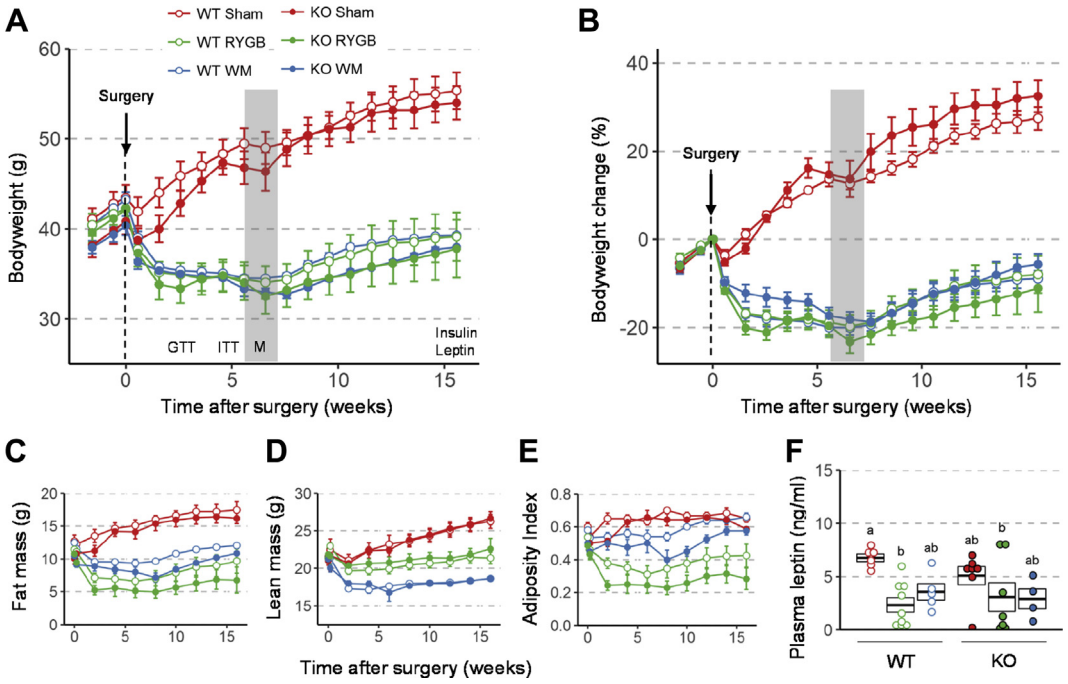

Figure 2: A, B: Effect of RYGB, sham surgery, or weight matching to RYGB by caloric restriction (WM) on body weight and body composition of GLP1RKO/Y2RKO and WT mice. A, B: Effect on absolute and percent body weight over the 16 week observation period. Timing of glucose tolerance test (GTT), insulin tolerance test (ITT), metabolic chamber exposure (M, gray bars) are shown in A. C–E: Effect on fat mass (C), lean mass (D), and adiposity index (E) over the 18 week observation period. F: Plasma leptin levels at termination of the study. RYGB, KO/WT mice, $n = 7/9$; Sham, $n = 6/7$; WM, $n = 4/5$. Data in A–E are shown as means ± SEM. Data in F are presented as individual data points over a box showing means ± SEM. Groups that do not share the same letters are significantly different from each other ($p < 0.05$, pairwise *t*-tests with Benjamini-Hochberg correction, FDR = 0.05, following ANOVA).

double-knockout mice, it did not reach significance in WT mice. RYGB did not significantly lower gonadal fat pad weight in either genotype. Liver weight was significantly lower in RYGB and weight-matched compared to sham mice, and this effect was the same in WT and GLP1RKO/Y2RKO mice (Supplementary Figure S3a). Heart and kidney weight was similar in RYGB and sham but lower in weight-matched mice of both genotypes (Supplementary Figures S3b and c). As reported previously, the weight of both small intestine and colon was significantly higher in RYGB compared to both sham and weight-matched mice, and this effect was significantly enhanced in GLP1RKO/Y2RKO mice (Supplementary Figures S3d and e).

3.2.2. Food intake and food choice

Baseline total daily food intake and food choice was not different among the 4 groups (Figure 3). In response to RYGB food intake was suppressed for about 10 days and then approached pre-surgical levels (Figure 3A). Sham surgery suppressed food intake to a lesser degree, so that food intake during the first 5 days was significantly lower in RYGB vs Sham mice of both genotypes (Figure 3B). Chow-preference was significantly higher for the first 5 days after RYGB compared to sham surgery in both WT and GLP1RKO/Y2RKO mice (Figure 3C,D).

3.2.3. Energy expenditure, RER, and locomotor activity

Total daily energy expenditure measured in metabolic chambers 6–7 weeks after surgery confirmed previous findings in WT mice. Measured at near thermoneutrality, RYGB mice had similar energy expenditure compared to sham mice, but significantly higher energy expenditure compared to weight-matched mice, and this effect was similar in both genotypes (Figure 4A). Effects on energy expenditure were reflected by similar effect on RER (Figure 4B). RYGB mice had similar RER compared to sham mice, but significantly higher RER compared to weight-matched mice, and this effect was similar in both genotypes. There were no significant differences in locomotor activity between surgery between any group (Figure 4C).

The metabolic chamber data obtained at room temperature were generally similar to the ones obtained at thermoneutrality, but some of the differences in energy expenditure and RER did not reach statistical significance (Supplementary Figure S4).

3.2.4. Glycemic control parameters

Intraperitoneal glucose tolerance measured at 3 weeks and insulin tolerance measured at 5 weeks after surgery were similarly affected by RYGB in both genotypes (Figure 5A,B). Glucose tolerance AUC was significantly lower in RYGB and the ability of insulin to suppress blood glucose was significantly enhanced in RYGB compared with sham mice in both WT and GLP1RKO/Y2RKO mice. However, across all groups, glucose tolerance was significantly lower in GLP1RKO/Y2RKO compared with WT mice (main effect of genotype on AUC: $F(1,32) = 21.75$, $p < 0.001$). At termination, fasting blood glucose was not significantly different between any group (Figure 5C). However, fasting insulin (Figure 5D) and HOMA-IR (Figure 5E) were significantly higher in sham compared to RYGB in both WT and GLP1RKO/Y2RKO mice. Importantly, RYGB-induced improvements in any glycemic control parameter were completely accounted for by weight-matching in both WT and GLP1RKO/Y2RKO mice.

4. DISCUSSION

Based on the powerful effects of GLP-1 and PYY to lower food intake and body weight and improve glucose homeostasis, and their greatly

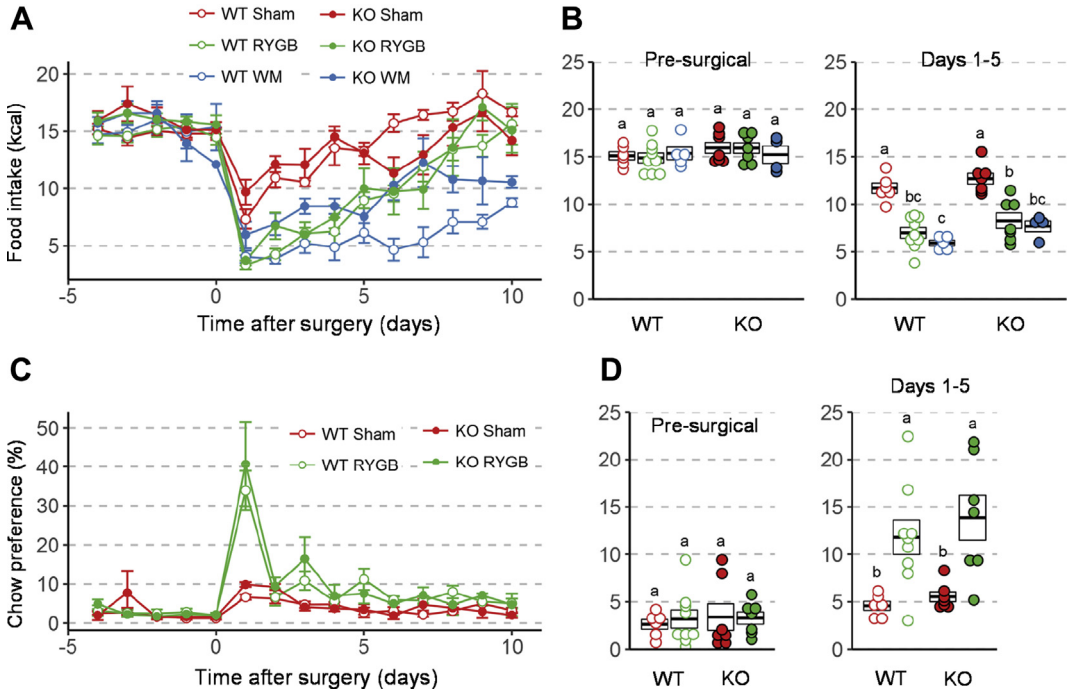

Figure 3: Effect of RYGB, sham surgery, or weight matching to RYGB by caloric restriction (WM) on food intake and diet preference in GLP1RKO/Y2RKO and WT mice (RYGB, KO/WT mice, $n = 7/9$; Sham, $n = 6/7$; WM, $n = 4/5$). All mice were on a two choice diet consisting of high-fat (60%) and regular (10%) chow. A: Daily food intake before and for 10 days after surgery. B: Average daily food intake over the pre-surgical (5 days before surgery) and the early recovery period (first 5 days after surgery). C: Daily chow preference before and for 10 days after surgery. Preference is expressed as percent of calories obtained from chow diet. WM mice were given a fixed percent of calories from chow so their data is not shown. D: Average daily chow preference over the same periods as in (B). Data in A and C are presented as means ± SEM. Data in B and D are presented as individual data points over a box showing means ± SEM. Data that do not share the same letters are significantly different from each other ($p < 0.05$, pairwise *t*-tests with Benjamini-Hochberg correction, FDR = 0.05, following ANOVA).

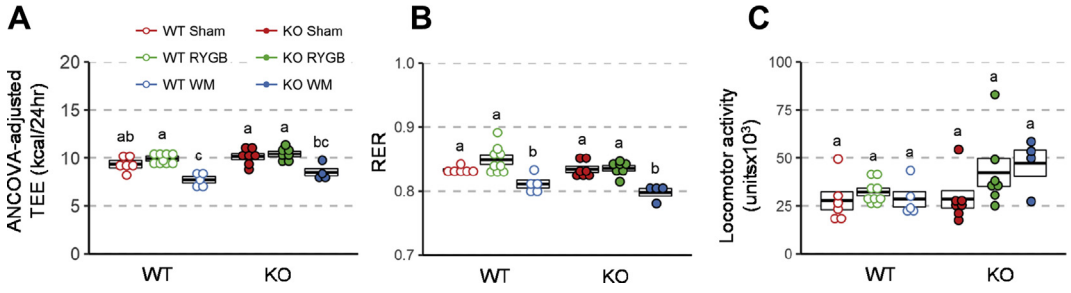

Figure 4: Effect of RYGB, sham surgery, or weight matching to RYGB by caloric restriction (WM) on ANCOVA adjusted energy expenditure using body weight as a covariate (A), respiratory exchange ratio (B), and locomotor activity (C) in GLP1RKO/Y2RKO and WT mice measured at thermoneutrality (29 °C) in metabolic cages. (RYGB, KO/WT mice, $n = 7/9$; Sham, $n = 6/7$; WM, $n = 4,5$). Data are expressed as individual data points over a box showing means ± SEM. Data that do not share the same letters are significantly different from each other ($p < 0.05$, pairwise *t*-tests with Benjamini-Hochberg correction, FDR = 0.05, following ANOVA).

increased postprandial levels after RYGB, we hypothesized that double deletion of their relevant mediating receptors, GLP-1R and Y2R would result in excess weight gain on a high-fat diet and reduced effectiveness of RYGB to reverse obesity. However, contrary to expectations, double-knockout mice behaved like WT mice with very few and only minor exceptions. Except for a non-significant tendency to display lower intraperitoneal glucose tolerance in non-surgical (Figure 1H) and a significantly lower intraperitoneal glucose tolerance in all three surgical groups (Figure 5A), GLP1RKO/Y2RKO mice responded almost exactly the same as WT mice to high fat diet and to RYGB. On high-fat diet, both WT and GLP1RKO/Y2RKO mice gained the same amount of body weight and fat mass and showed similar fasting levels of glucose, insulin, and HbA1c, and RYGB had similar beneficial effects in both genotypes. Specifically, RYGB induced similar weight loss and fat mass loss, had similar effects on food intake and energy expenditure, and improved insulin tolerance, fasting insulin, and HOMA-IR similarly.

We and others have previously shown that high-fat diet-induced obese mice with simple GLP1R deficiency also respond normally to RYGB [12,13] and VSG [20]. Furthermore, clinical studies in weight-matched diabetic and non-diabetic subjects that had undergone RYGB, short-term infusion of the selective GLP-1 receptor antagonist Exendin 9-39 was unable to reverse the beneficial effects of the surgery on glucose homeostasis [10,11]. These findings suggest that blocking the GLP-1 signaling pathway alone is not sufficient to prevent the beneficial effects of bariatric surgeries, and that blocking more than one signaling pathway may be necessary [21]. This view is indirectly

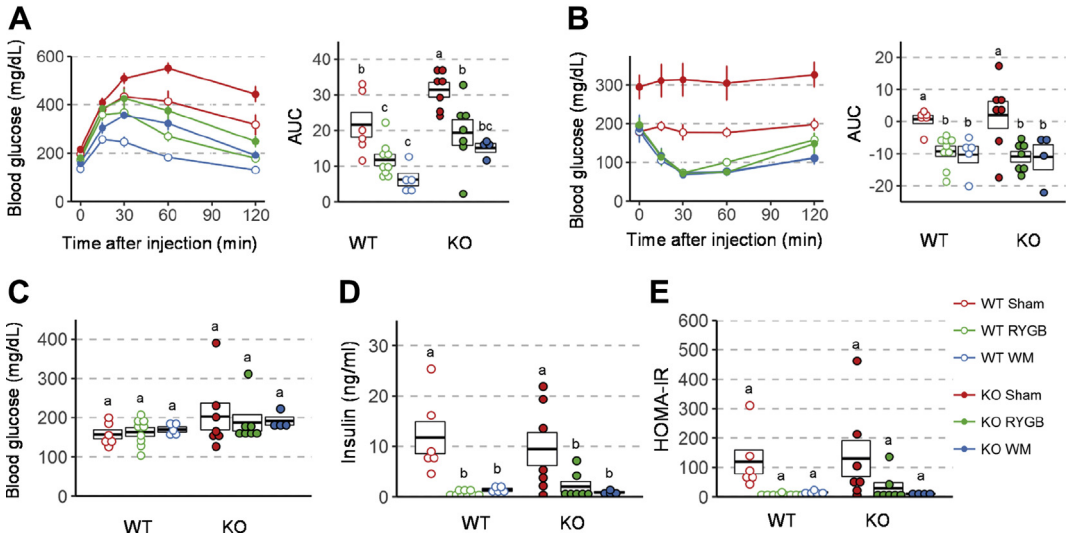

Figure 5: Effect of RYGB, sham surgery, or weight matching to RYGB by caloric restriction (WM) on glycemic control in GLP1RKO/Y2RKO and WT mice. A: Glucose tolerance time course and area under the curve. B: Insulin tolerance time course and area under the curve. C–E: Fasting blood glucose (C), plasma insulin (D), and HOMA-IR (E). (RYGB, KO/WT mice, $n = 7/9$; Sham, $n = 6/7$; WM, $n = 5/4$). Data are presented as means ± SEM, or as individual data points over a box showing means ± SEM. Data that do not share the same letters are significantly different from each other ($p < 0.05$, pairwise t-tests with Benjamini-Hochberg correction, FDR = 0.05, following ANOVA).

supported by two previous studies. First, acute octreotide administration was able to rescue RYGB-induced food intake suppression in rats [22]. As a general inhibitor of gut hormone release, octreotide can be expected to reduce signaling through more than one gut hormone receptor. Second, the observation that subthreshold doses of GLP-1 and PYY, which do not have effects when administered separately, significantly reduce food intake when given in combination in humans [17] could also be taken as evidence for a synergistic effect that needs to be considered in pharmacological blocking or knockout studies. The use of combinations of drugs and other treatments in the fight against obesity and metabolic disease has recently drawn a lot of attention [23] and was our rationale for generating the GLP1RKO/Y2RKO double knockout mouse. However, even this model responded similarly to RYGB, suggesting that combined blocking of GLP1R and Y2R signaling is still not sufficient to attenuate the beneficial effects of RYGB and that other signaling pathways must be or become more important.

Could it be that GLP-1R and Y2R are not the critical receptors for GLP-1 and PYY to exert their beneficial effects after RYGB? Although GLP-1 and GLP-2 selectively signal through their respective receptors, GLP-2 co-secreted on an equimolar basis from the intestinal L-cell and similarly elevated after RYGB [24,25] could also be involved in its beneficial effects. Since GLP-2 has been shown to stimulate intestinal growth and blood flow [26,27], it may be required for the hypertrophic effects of RYGB [24]. There is ample evidence for hypertrophy and L-cell proliferation of the Roux and common limbs in rats [24,28,29] and as shown here in mice, the small and large intestine weights were significantly higher in mice with RYGB compared to sham surgery or weight-matching in both WT and GLP1RKO/Y2RKO mice, while all other organs weighed either less or the same in mice with RYGB. Although we have not histologically analyzed the different gut segments as we did in rats [29], it seems clear that the hypertrophic response was fully intact or even exaggerated in our DK mice and GLP-2 receptor signaling may have played a role. Furthermore, the *Glp2r* gene was significantly upregulated more than 2-fold in the duodenum of double-knockout mice, suggesting that the GLP-2R signaling pathway was upregulated and may have contributed to the greater hypertrophic response in DK mice. Gastric bypass surgery in GLP-2R- and GLP1R/GLP2R-deficient mice will be necessary to further investigate this possibility. Interestingly, there is no measurable hypertrophy of the small intestine after VSG in mice [30], and loss of GLP-2R does not attenuate the weight loss and improvement in glucose control after VSG [31].

Although the preferred receptor for PYY is the Y2R, and pharmacological studies have demonstrated that the beneficial effects of endogenous PYY on food intake, body weight, and glycemic control are mediated by the Y2R, PYY also has a low affinity for the Y1- and Y5-receptors. It could thus be that signaling through these receptors plays a role in the beneficial effects of RYGB. This possibility is supported by observations in PYY-deficient mice subjected to duodenal bypass surgery, which did not lose as much body weight in the first 10 days after surgery compared with WT mice [14]. To further address issues of potential multiple receptor usage by GLP-1 and PYY, the effects of RYGB in GLP-1/PYY double knockout mice should be investigated. In addition, future gene knockouts combined with gastric bypass surgeries should be ideally conducted postnatally to eliminate potential developmental compensations and complications.

As in previous studies using mouse models of RYGB and high-fat diet [15,32–34], food intake of both WT and GLP1RKO/Y2RKO mice returned to near presurgical levels around 2 weeks after RYGB. However, when taking in consideration the increased fecal energy loss after RYGB, energy available for metabolism was found to be significantly decreased by about 10–15% [34]. Although we did not measure fecal energy loss in our study, it is likely to be very similar since our surgical model and diet were almost identical. Thus, weight loss in this mouse model of RYGB is driven by both reduced energy intake and increased energy expenditure [34]. In humans, the main driver of weight loss appears to be reduced energy intake, as most studies did not find significant increases of energy expenditure (see [35] for recent review). Given that our double deletion was directed at two major satiety signaling pathways, it could be argued that the negative outcome was due to food intake reduction not being the main driver in the mouse model. While we cannot completely rule out this possibility,

we should have seen at least an attenuated weight loss in the GLP1RKO/Y2RKO mice after RYGB. Long-term clinical studies with combined administration of GLP1 and PYY antagonists would be necessary to provide a more definitive answer.

5. CONCLUSIONS

Unexpectedly, combined loss of GLP-1R and Y2R signaling in male and female mice had only minor effects on body weight, fat mass, and glucose homeostasis under both low and high-fat diet conditions. Furthermore, RYGB in diet-induced obese male mice was just as effective in lowering body weight and adiposity, and improving glucose tolerance and insulin sensitivity. Future experiments with inducible and tissue-specific knockout strategies will be necessary to rule out the potential role of adaptive compensatory mechanisms.

ACKNOWLEDGMENTS

We thank Donna Goldsteen, Krystal Nacel and the Laboratory Animal Resource staff (MedImmune, Gaithersburg, MD) as well as Timothy Mixson and Brooke Hillman and the Comparative Biology staff at PBRC for their assistance with animal husbandry and care.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.05.004.

FUNDING

These studies were partly conducted at and funded by MedImmune, LLC. Investigators at PBRC were funded by National Institutes of Health Grants DK047348 (HRB), DK105032 (CDM), DK092587 (HM), and P30 GM118430 (COBRE/NORC Behavioral and Physiological Cores).

CONFLICT OF INTEREST

BBB, BG, SO, SW, CJR, and JLT are, or were at the time these studies were performed, employees and/or stockholders of MedImmune/AstraZeneca.

AUTHOR CONTRIBUTIONS

Project conception: BBB, JLT, HRB; experimental design and interpretation: BBB, JLT, MM, HRB; data acquisition and analysis: BBB, MM, BG, SO, SW, JLT, HRB; writing and editing the manuscript: HRB, BBB, MM, CJR, JLT, CDM, SY, HM.

REFERENCES

[1] Maclean, P.S., Bergouignan, A., Cornier, M.A., Jackman, M.R., 2011. Biology's response to dieting: the impetus for weight regain. American Journal of Physiology — Regulatory, Integrative and Comparative Physiology 301:R581—R600.

[2] Kraschnewski, J.L., Boan, J., Esposito, J., Sherwood, N.E., Lehman, E.B., Kephart, D.K., et al., 2010. Long-term weight loss maintenance in the United States. International Journal of Obesity 34:1644—1654.

[3] Wadden, T.A., Walsh, O.A., Berkowitz, R.I., Chao, A.M., Alamuddin, N., Gruber, K., et al., 2019. Intensive behavioral therapy for obesity combined with Liraglutide 3.0 mg: a randomized controlled trial. Obesity 27:75—86.

[4] Sjostrom, L., 2013. Review of the key results from the Swedish Obese Subjects (SOS) trial — a prospective controlled intervention study of bariatric surgery. Journal of Internal Medicine 273:219—234.

[5] le Roux, C.W., Aylwin, S.J., Batterham, R.L., Borg, C.M., Coyle, F., Prasad, V., et al., 2006. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Annals of Surgery 243:108—114.

[6] Svane, M.S., Bojsen-Moller, K.N., Madsbad, S., Holst, J.J., 2015. Updates in weight loss surgery and gastrointestinal peptides. Current Opinion in Endocrinology Diabetes and Obesity 22:21—28.

[7] Korner, J., Bessler, M., Inabnet, W., Taveras, C., Holst, J.J., 2007. Exaggerated glucagon-like peptide-1 and blunted glucose-dependent insulinotropic peptide secretion are associated with Roux-en-Y gastric bypass but not adjustable gastric banding. Surgery for Obesity and Related Diseases 3:597—601.

[8] Peterli, R., Steinert, R.E., Woelnerhanssen, B., Peters, T., Christoffel-Courtin, C., Gass, M., et al., 2012. Metabolic and hormonal changes after laparoscopic Roux-en-Y gastric bypass and sleeve gastrectomy: a randomized, prospective trial. Obesity Surgery 22:740—748.

[9] Drucker, D.J., 2018. Mechanisms of action and therapeutic application of glucagon-like peptide-1. Cell Metabolism 27:740—756.

[10] Shah, M., Law, J.H., Micheletto, F., Sathananthan, M., Dalla Man, C., Cobelli, C., et al., 2014. Contribution of endogenous glucagon-like peptide 1 to glucose metabolism after Roux-en-Y gastric bypass. Diabetes 63:483—493.

[11] Vetter, M.L., Wadden, T.A., Teff, K.L., Khan, Z.F., Carvajal, R., Ritter, S., et al., 2015. GLP-1 plays a limited role in improved glycemia shortly after Roux-en-Y gastric bypass: a comparison with intensive lifestyle modification. Diabetes 64:434—446.

[12] Mokadem, M., Zechner, J.F., Margolskee, R.F., Drucker, D.J., Aguirre, V., 2014. Effects of Roux-en-Y gastric bypass on energy and glucose homeostasis are preserved in two mouse models of functional glucagon-like peptide-1 deficiency. Molecular Metabolism 3:191—201.

[13] Ye, J., Hao, Z., Mumphrey, M.B., Townsend, R.L., Patterson, L.M., Stylopoulos, N., et al., 2014. GLP-1 receptor signaling is not required for reduced body weight after RYGB in rodents. American Journal of Physiology — Regulatory, Integrative and Comparative Physiology 306:R352—R362.

[14] Chandarana, K., Gelegen, C., Karra, E., Choudhury, A.I., Drew, M.E., Fauveau, V., et al., 2011. Diet and gastrointestinal bypass-induced weight loss: the roles of ghrelin and peptide YY. Diabetes 60:810—818.

[15] Boland, B., Mumphrey, M.B., Hao, Z., Gill, B., Townsend, R.L., Yu, S., et al., 2019. The PYY/Y2R-deficient mouse responds normally to high-fat diet and gastric bypass surgery. Nutrients 11.

[16] Svane, M.S., Jorgensen, N.B., Bojsen-Moller, K.N., Dirksen, C., Nielsen, S., Kristiansen, V.B., et al., 2016. Peptide YY and glucagon-like peptide-1 contribute to decreased food intake after Roux-en-Y gastric bypass surgery. International Journal of Obesity 40:1699—1706.

[17] Schmidt, J.B., Gregersen, N.T., Pedersen, S.D., Arentoft, J.L., Ritz, C., Schwartz, T.W., et al., 2014. Effects of PYY3-36 and GLP-1 on energy intake, energy expenditure, and appetite in overweight men. American Journal of Physiology. Endocrinology and Metabolism 306:E1248—E1256.

[18] Baggio, L.L., Huang, Q., Cao, X., Drucker, D.J., 2008. An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis. Gastroenterology 134:1137—1147.

[19] Hao, Z., Zhao, Z., Berthoud, H.R., Ye, J., 2013. Development and verification of a mouse model for Roux-en-Y gastric bypass surgery with a small gastric pouch. PLoS One 8:e52922.

[20] Wilson-Perez, H.E., Chambers, A.P., Ryan, K.K., Li, B., Sandoval, D.A., Stoffers, D., et al., 2013. Vertical sleeve gastrectomy is effective in two genetic mouse models of glucagon-like Peptide 1 receptor deficiency. Diabetes 62:2380—2385.

[21] Vidal, J., de Hollanda, A., Jimenez, A., 2016. GLP-1 is not the key mediator of the health benefits of metabolic surgery. Surgery for Obesity and Related Diseases 12:1225—1229.

[22] Fenske, W.K., Bueter, M., Miras, A.D., Ghatei, M.A., Bloom, S.R., le Roux, C.W., 2012. Exogenous peptide YY3-36 and Exendin-4 further decrease

food intake, whereas octreotide increases food intake in rats after Roux-en-Y gastric bypass. International Journal of Obesity 36:379–384.

[23] Clemmensen, C., Finan, B., Muller, T.D., DiMarchi, R.D., Tschop, M.H., Hofmann, S.M., 2019. Emerging hormonal-based combination pharmacotherapies for the treatment of metabolic diseases. Nature Reviews Endocrinology 15:90–104.

[24] le Roux, C.W., Borg, C., Wallis, K., Vincent, R.P., Bueter, M., Goodlad, R., et al., 2010. Gut hypertrophy after gastric bypass is associated with increased glucagon-like peptide 2 and intestinal crypt cell proliferation. Annals of Surgery 252:50–56.

[25] Taqi, E., Wallace, L.E., de Heuvel, E., Chelikani, P.K., Zheng, H., Berthoud, H.R., et al., 2010. The influence of nutrients, biliary-pancreatic secretions, and systemic trophic hormones on intestinal adaptation in a Roux-en-Y bypass model. Journal of Pediatric Surgery 45:987–995.

[26] Bremholm, L., Hornum, M., Henriksen, B.M., Larsen, S., Holst, J.J., 2009. Glucagon-like peptide-2 increases mesenteric blood flow in humans. Scandinavian Journal of Gastroenterology 44:314–319.

[27] Drucker, D.J., Erlich, P., Asa, S.L., Brubaker, P.L., 1996. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proceedings of the National Academy of Sciences of the United States of America 93:7911–7916.

[28] Hansen, C.F., Bueter, M., Theis, N., Lutz, T., Paulsen, S., Dalboge, L.S., et al., 2013. Hypertrophy Dependent Doubling of L-Cells in Roux-en-Y Gastric Bypass Operated Rats. PLoS One 8:e65696.

[29] Mumphrey, M.B., Patterson, L.M., Zheng, H., Berthoud, H.R., 2013. Roux-en-Y gastric bypass surgery increases number but not density of CCK-, GLP-1-, 5-HT-, and neurotensin-expressing enteroendocrine cells in rats. Neurogastroenterology and Motility 25:e70–e79.

[30] Wismann, P., Barkholt, P., Secher, T., Vrang, N., Hansen, H.B., Jeppesen, P.B., et al., 2017. The endogenous preproglucagon system is not essential for gut growth homeostasis in mice. Molecular Metabolism 6:681–692.

[31] Patel, A., Yusta, B., Matthews, D., Charron, M.J., Seeley, R.J., Drucker, D.J., 2018. GLP-2 receptor signaling controls circulating bile acid levels but not glucose homeostasis in Gcgr(−/−) mice and is dispensable for the metabolic benefits ensuing after vertical sleeve gastrectomy. Molecular Metabolism 16: 45–54.

[32] Hao, Z., Leigh Townsend, R., Mumphrey, M.B., Gettys, T.W., Yu, S., Munzberg, H., et al., 2018. Roux-en-Y gastric bypass surgery-induced weight loss and metabolic improvements are similar in TGR5-deficient and wildtype mice. Obesity Surgery 28:3227–3236.

[33] Hao, Z., Mumphrey, M.B., Townsend, R.L., Morrison, C.D., Munzberg, H., Ye, J., et al., 2016. Body composition, food intake, and energy expenditure in a murine model of Roux-en-Y gastric bypass surgery. Obesity Surgery 26: 2173–2182.

[34] Liou, A.P., Paziuk, M., Luevano Jr., J.M., Machineni, S., Turnbaugh, P.J., Kaplan, L.M., 2013. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Science Translational Medicine 5, 178ra141.

[35] Munzberg, H., Laque, A., Yu, S., Rezai-Zadeh, K., Berthoud, H.R., 2015. Appetite and body weight regulation after bariatric surgery. Obesity Reviews 16(Suppl. 1):77–90.

