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GLP-1 receptor signaling is not required for reduced body weight after RYGB in rodents

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Running Title: **GLP-1 signaling and gastric bypass**

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Abstract

Exaggerated GLP-1 and PYY secretion is thought to be a major mechanism in the reduced food intake and body weight after Roux-en-Y gastric bypass surgery. Here we use complementary pharmacological and genetic loss-of-function approaches to test the role of increased signaling by these gut hormones in high-fat diet-induced obese rodents. Chronic brain infusion of a supra-maximal dose of the selective GLP-1 receptor antagonist exendin-9-39 into the lateral cerebral ventricle significantly increased food intake and body weight in both RYGB and sham-operated rats, suggesting that, while contributing to the physiological control of food intake and body weight, central GLP-1 receptor signaling tone is not the critical mechanism uniquely responsible for the body weight lowering effects of RYGB. Central infusion of the selective Y2R-antagonist BIIE0246 had no effect in either group, suggesting that it is not critical for the effects of RYGB on body weight under the conditions tested. In a recently established mouse model of RYGB that closely mimics surgery and weight loss dynamics in humans, obese GLP-1R-deficient mice lost the same amount of body weight and fat mass and maintained similarly lower body weight compared with wildtype mice. Together, the results surprisingly provide no support for important individual roles of either gut hormone in the specific mechanisms by which RYGB rats settle at a lower body weight. It is likely that the beneficial effects of bariatric surgeries are expressed through complex mechanisms that require combination approaches for their identification.

Key Words: Roux-en-Y gastric bypass, gut hormones, brain, GLP-1R knockout, food intake, Exendin-(9-39), BIIE0246, high-fat diet

Introduction

The number of bariatric surgeries performed has steadily increased because for many obese patients it is the last hope for significant and enduring body weight loss and general improvement of health. There have been an increasing number of clinical and preclinical studies with the goal to unravel the mechanisms underlying these beneficial effects of bariatric surgeries, but there has not yet been a breakthrough. A major hypothesis is that changes in gut hormone release are crucial. Drastically increased postprandial circulating levels of GLP-1 and PYY have been demonstrated in clinical studies (18, 32, 33, 36, 38, 44, 47, 50) and in rodent models (13, 16, 37, 56) for both Roux-en-y gastric bypass and vertical sleeve gastrectomy.

GLP-1 is a powerful hormone that acts both in the periphery and brain to stimulate insulin secretion, inhibit gastric emptying, and suppress food intake (for recent reviews see (7, 12, 43)). Exogenous administration of GLP-1 or its stable analog Exendin-4, as well as PYY(3-36), has been shown in numerous preclinical and clinical studies to suppress food intake and lower body weight (e.g. (2, 4, 5, 9, 15, 19, 24, 27, 40, 57, 59), or to suppress hepatic glucose production (52), in some of them by directly acting on the brain . The stable GLP-1 receptor agonist Exendin-4 is widely used by type-2 diabetic patients to stabilize glucose levels and reduce body weight (30, 43). Although GLP-1 gains easy access to the brain (26, 29), its short half-life makes unclear whether endogenous GLP-1 from the gut reaches the brain in high enough concentrations to affect food intake and glucose homeostasis under normal conditions. The greatly increased circulating levels after gastric bypass or sleeve gastrectomy may affect the brain directly or indirectly via GLP-1 receptors on sensory vagal fibers assumed to innervate the intestinal mucosa and hepatic portal vein (42, 62). In addition, proglucagon is expressed in neurons of the solitary nucleus in the caudal brainstem, the origin of extensive local and forebrain projections of GLP-1 and GLP-2 immunoreactive fibers (61).

Although exaggerated postprandial GLP-1 and PYY responses are widely believed to play a major role in the beneficial effects of RYGB on body weight and glucose homeostasis, direct evidence has been lacking. Strong indirect evidence comes from studies in RYGB patients, with appetite and body weight negatively correlated with elevated PYY and GLP-1 concentrations (38). Furthermore, food intake increased upon treatment of RYGB patients (38) and rats after RYGB (22) with the nonspecific inhibitor of

gut hormone secretion octreotide. There have been only two attempts to mechanistically and directly test the role of endogenous GLP-1 and PYY in the weight-reducing effects of bariatric surgeries. In a study in PYY-deficient mice, the initial body weight loss after a modified gastric bypass surgery was greatly attenuated compared to wildtype mice, suggesting a role for exaggerated circulating levels of this gut peptide (14). In a study in GLP-1 receptor deficient mice, sleeve gastrectomy was similarly effective compared to wildtype mice, suggesting that exaggerated circulating GLP-1 levels are not required for VSG's effects on weight loss and improvement of glycemic control (64). This latter finding was surprising given the similarly elevated circulating levels of postprandial GLP-1 and PYY in humans (44) and rodents (13, 56).

Here we examined the potential roles of brain GLP-1 and Y2-receptor signaling in the body weight lowering effects of RYGB. We were specifically interested in mechanisms that defend the lower levels of body weight after the initial weight loss phase. Unlike calorie-restriction-induced weight loss, RYGB does not induce strong counter-regulatory responses such as increased hunger, allowing most RYGB patients to resist weight regain. We tested the hypothesis that central GLP-1 and Y2 receptor signaling contribute to the defense of the lower body weight level after RYGB. To this end, we used chronic pharmacologic blockade of central GLP-1 or Y2 receptor signaling by their respective antagonists Ex9 or BIIE0246. Having recently established a murine model of RYGB with a small gastric pouch that closely mimics surgery in humans and leads to sustained loss of body weight by selective reduction of excess fat mass (25), we then tested the effectiveness of RYGB to lower food intake and body weight in GLP-1 receptor null mice.

Materials and Methods

Animals

Rats: Male Sprague-Dawley rats initially weighing ~200 g (Harlan Industries, Indianapolis, IN) were housed individually in wire-mesh cages at a constant temperature of 21-23º C with a 12h light-dark cycle (lights on 07:00, off at 19:00). Food and water were provided ad libitum unless otherwise indicated. Animals were made obese by putting them on a two-choice diet for 8 weeks consisting of normal laboratory chow (Kcal%: Carb, 58; Fat, 13.5; Prot, 28.5, # 5001, Purina LabDiet, Richmond IN) and high-

sucrose, high-fat diet (sweet HF diet; Kcal%: Carb, 35; Fat, 45; Prot, 20, D12451, Research Diets, New Brunswick, NJ), with each of the diets containing sufficient minerals and vitamins. They were then randomly assigned to either RYGB or sham-surgery. Liquid Ensure diet (Kcal%: Carb, 64; Fat, 21.6; Prot, 14.4, Abbott Laboratories, Columbus, OH) was provided for the first 5 days after surgery or longer if needed. A lean control group without surgery was placed on a regular chow diet throughout the experiment. Four rats, two with RYGB and two with sham surgery were shipped from the Surgical Core of Harvard Medical School (Dr. Lee Kaplan).

Mice: GLP-1R deficient mice were originally generated on a 129/J, CD1, and C57BL/6J background and backcrossed six times on a pure C57BL/6J background by Dr. Daniel Drucker in Torronto, Canada (54). A breeding colony with this background was then transferred to the Pennington Biomedical Research Center, where they were maintained by sister-brother breeding. Genotyped male GLP-1R$^{-/-}$ and C57/BL6J wildtype mice were made obese on a high-fat diet (58% of energy from fat, Research Diets D12331) for 12 weeks and were 4 months old at the time of surgery. After surgery or sham-surgery, they were given medium-fat diet (27% of energy from fat, # 8626 Teklad mouse breeder diet), except for postsurgical weeks 10-16, when they were given a two-choice cafeteria diet consisting of two complete diets, one low-fat (regular chow, 4.4% energy from fat, Teklad 7001) and one high-fat (58% energy from fat, Research Diets No. D12331). The reason for switching to medium-fat diet after surgery was twofold. First, in earlier studies in both mice and rats, we noted that after RYGB animals increased preference for low-fat regular chow and that some RYGB animals showed signs of morbidity if provided only high-fat diet. Second, reduced intake of high-fat foods is an important component of post-surgical behavioral counselling in gastric bypass patients. However, we did not provide a choice of low and high-fat diets throughout the postsurgical period because it would complicate interpretation of energy expenditure data and respiratory exchange ratio.

All protocols involved in this study were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center or at Harvard University in accordance with guidelines established by the National Institutes of Health.

Roux-en-Y gastric bypass surgery

Rats: Under adequate isoflurane anesthesia, the stomach was first freed from all the ligaments that connect it to the liver and the spleen. Then, the focus of the surgeon turned to the gastric artery that emerges from the celiac artery. The gastric artery is in very close approximation with the gastroesophagel junction and then runs parallel to the lesser curvature of the stomach branching into one anterior and one posterior vessel that in turn give rise to several smaller branches on the anterior and posterior wall. Before transecting the stomach, the gastric artery was gently dissected off the gastric wall using a microhook. Using a bipolar cautery probe, the first anterior branch that crosses the anterior wall of the stomach, just millimeters from the gastroesophageal junction was cauterized, allowing for transection of the stomach wall with fine scissors without bleeding. After repairing the distal stomach using a running 6-0 silk suture, an end-to-end gastrojejunostomy was constructed with a 7-0 silk suture. This surgery resulted in a gastric pouch of no more than about 5% of the gastric volume, as well as Roux, biliopancreatic, and common limbs of about 20, 22, and 60 cm. Sham-surgery consisted of laparotomy and mobilization of stomach and small intestine. For analgesia, Buprenorphine, meloxicam (1-2 mg/kg, s.c.), and/or carprofen (5 mg/kg, s.c.) were administered as necessary. To overcome potential deficits in iron absorption and development of anemia, rats were administered a macromolecular dextran-iron complex (Iron Dextran injectable, catalog # 93963, 5 mg, sc; Town and Country, Ashland, OH) once a week for the first two weeks after RYGB surgery. Additional doses were administered to individual anemic animals if indicated by a hematocrit of less than 40%.

In addition to the high-fat-fed RYGB and sham-operated rats, a non-surgical, age-matched group was fed standard laboratory chow throughout the study and served as lean, never-obese, control group.

Mice: As described in detail earlier (25), surgery resulted in a small gastric pouch of less than 5% of the stomach volume, and Roux-, biliopancreatic, and common limbs of about 6, 6, and 12 cm, respecively. By taking care to preserve gastric vasculature as much as possible, the mortality rate is < 10% and the animals typically cosume significant amounts of food two days after surgery. Sham surgery consisted in cutting the jejunum followed by re-anastomoosis and by mobilizing the stomach and placing a metal clip on the greater curvature.

Measurement of body weight and body composition

Body weight was monitored daily for the first two weeks, and then was recorded weekly. Body composition was measured before introduction of the high-fat diet (23 weeks before surgery), after 20 weeks of high-fat diet (3 weeks before surgery), 6 weeks after surgery, as well as before and after the chronic pharmacological blockade, by using a Minispec LF 90 NMR Analyzer (Bruker Corporation, The Woodlands, TX). This method uses whole body magnetic resonance relaxometry in unanesthetized rodents with excellent linearity and reproducibility (34).

Experimental protocol

Rats: Eight weeks after surgery, all rats, including the chow-fed controls were equipped with ICV cannulas. After recovery from this second surgery, rats were adapted to an automated system (PhenoMaster/ LabMaster, TSE Systems, Chesterfield MO), continuously monitoring food and water intake, oxygen consumption, and locomotor activity. At this time, access of RYGB and sham-operated rats was restricted to the high-fat diet only. After adaptation with training lids, animals were monitored for a 4-6 day baseline period, at which time ALZET Minipumps (2 ml/14 days, Durect Corporation, Cupertino CA) containing either the GLP-1 receptor antagonist Ex9, or the Y2 receptor antagonist BIIE0246, or the respective vehicle were implanted under the back skin and connected to the ICV cannulae under isoflurane anesthesia. After monitoring all parameters continuously for another 14 days, the rats were transferred back to regular cages and the pumps removed.

Mice: At the time of surgery, mice were switched from high-fat (45%) to medium-fat diet (27% energy from fat). Seven weeks after surgery, body composition was measured as described above. At 12-14 weeks after surgery, the medium-fat diet was replaced with a two-choice cafeteria diet consisting of two complete diets, one low-fat (regular chow, 4.4%) and one high-fat (58% energy from fat. Intake of both components was measured daily for the first 10 days and then for 3 days every week. Body composition was measured before and after 6 weeks on this high-fat cafeteria diet.

Chronic ICV infusions

ICV cannulas (Plastics One, Roanoke, VA) were aimed at the left lateral ventricle as described in detail earlier (65). The lateral ventricle was chosen because the infusion reaches the entire brain and to avoid any inadvertent damage to the roof of the third ventricle and dorsal hypothalamus. After recovery, cannula placements were verified by monitoring the acute drinking response to ICV injection of angiotensin-2. All animals drank at least 5 ml of water within 10 minutes of ANG-2 administration.

Infusions started with the implantation and connection with ALZET minipumps loaded with either Exendin-(9-39) (Ex9) (100 µg/rat/day, dissolved in sterile saline (Twentyfirst Century Biochemicals, Inc., Marlboro, MA), BIIE0246 (100 µg/rat/day, dissolved in 40% DMSO, Tocris Bioscience, Minneapolis MN), or sterile saline as vehicle control (Veh), in separate groups of 8-10 rats. All infusions lasted for 14 days (nominally) at a rate of 0.5 µl/h, and the pumps were promptly removed at 16-18 days after the start of infusion. The dose of Ex9 was based on its effectiveness to increase food intake and body weight in both chow and high-fat diet-fed rats reported in an earlier study by Barrera et al. (6). At an average body weight of about 450 - 500 g, this amounts to about 42-46 pmol/kg/min. Because the much lower dose of 0.5 pmol/kg/min was effective in increasing food intake in mice (31), we consider our dose as clearly supra-maximal. The dose of BIIE0246 was based on earlier reports with brain injections (1, 45) and a separate experiment with single injections to test its effectiveness to prevent suppression of food intake induced by injection of PYY(3-36). To this end, male Sprague-Dawley rats (n = 5) on regular chow with fourth ventricular cannulas were food deprived overnight and received an acute injection of BIIE0246 (1 µg/3 µl in 5% DMSO, Tocris) or vehicle alone, followed 15 min later by an acute injection of PYY(3-36) (1.5 µg/3 µl in sterile saline, Tocris), or saline and food intake was measured 30 min after the last injection. One rat that did not decrease food intake after PYY(3-36) injection was not included in the analysis. Based on the effectiveness of 1 µg BIIE0246 in this acute study, we used a chronic infusion rate of 2.1 µg/30 min (100 µg/day) in the chronic infusion study.

Measurement of food intake and VO_2 consumption

Up to the time in the metabolic chambers, food intake was measured manually and taking into account spillage before and after surgery. In the chambers, food and water consumption was measured automatically, without taking spillage into account. All food intake is reported in kcal/day or kcal/12 h dark

or light period. VO_2 consumption was measured throughout the baseline and 14-day infusion period in metabolic chambers (PhenoMaster/LabMaster, TSE Systems) and expressed either per rat or per kg body mass.

Statistical analysis

All data were analyzed with appropriate two-way ANOVAs. Where appropriate, time was used as within-subjects repeated measure. Selective preplanned comparisons of individual means were made by using Bonferroni-corrected multiple comparison tests. Raw food intake data were collected in grams and multiplied by values for metabolizable energy content as provided by the supplier. Means ± SEM are reported throughout.

Results

Effects of pharmacological blockade of central GLP-1R signaling in rats

RYGB-induced effects on body weight and body composition

The effect of RYGB and sham surgery on body weight and body composition is shown in Fig. 1. RYGB rats showed a rapid initial weight loss and stayed at that significantly lower body weight level throughout the study (Fig. 1A). At the time of brain cannulation, they weighed significantly less and the fat mass and adiposity index were significantly lower compared with sham-operated controls, and similar to age-matched, chow-fed control rats (Fig. 1 B, C). Total and percent lean mass after RYGB was similar to chow-fed lean controls, and significantly lower compared with sham-operated rats (Fig. 1 D, E).

Effects of chronic ICV Ex9 infusion on body weight and body composition

In RYGB rats, infusion of the GLP-1 receptor antagonist Exendin-9 resulted in a slow but steady body weight increase which became significant compared with saline infusion around day 12 (Fig. 2A). At the end of the infusion period on day 16, Ex9 infused RYGB rats had gained significantly ($t[9] = 3.56$, $p < 0.01$) more body weight compared with vehicle infused RYGB rats (Fig. 2B). However, sham-operated rats gained body weight more rapidly with Ex9 compared to vehicle infusion, with the first significant difference

on day 6 (t[9] = 3.38, p<0.01), and more rapidly than RYGB rats with Ex9 infusion. Analysis of body weight gain from pump implantation to day 16 revealed significant main effects of infusion (F[1,41] = 58.48, p <0.0001) and surgery (F[2,41] = 6.00, p < 0.005) but no significant interaction (Fig. 2 B). Follow-up comparisons showed that Ex9 significantly increased weight gain vs. vehicle in all groups. Vehicle infusion did not differentially affect body weight gain in the three groups, although RYGB rats gained the least.

Analysis of changes in body composition was based on measurements at 8.7 ± 1.0 days before and 8.8 + 1.0 days after metabolic chamber housing, an average total period of 38 days. For fat mass gain, it revealed significant main effects of infusion (F[1,37] = 33.07, p < 0.0001) and surgery (F[2,37] = 9.50, p < 0.001), but no significant interaction (Fig. 2 C). Direct comparisons showed that Ex9 significantly increased fat mass gain vs. vehicle in RYGB and sham-operated rats (RYGB: +18.6 g, t = 3.54, p <0.01; sham: 18.96 g, t = 4.02, p < 0.01), but not in chow-fed rats (+10.8 g, t = 2.37, n.s.). As shown in Fig. 2 D, the adiposity index was similarly affected, except that only the main effect of infusion was significant (F[1,37] = 42.79, p < 0.0001) and that in follow-up comparisons, the adiposity-promoting effect of Ex9 was significant in RYGB (t = 4.65, p< 0.001), sham (t = 3.61, p < 0.01), and chow-fed rats (t = 2.96, p < 0.05. Finally, analysis of lean mass revealed only a significant main effect of surgery, reflecting the significantly lower lean mass gain in RYGB rats compared to sham-operated and chow-fed rats (Fig. 2 E).

Effects of Ex9 on food and water intake and feed efficiency

Analysis of food intake data assessed during the baseline and infusion periods in the metabolic chambers revealed a significant main effect of infusion (F[3,82] = 11.02, p <0.0001), and surgery (F[2,82] = 4.77, p , 0.05) (Fig. 3 A). Follow-up multiple comparisons showed that in both RYGB and sham-operated rats, but not in chow-fed controls, food intake was significantly higher under conditions of GLP-1 blockade compared with the same rats before infusion and with vehicle infused rats. The increase of food intake in RYGB rats was of similar magnitude as in sham-operated rats. As expected, RYGB rats consumed about 12 % less calories compared with sham-operated rats during baseline and vehicle infusion.

Focusing on the dark period food intake, ANOVA applied to only RYGB and sham-operated rats revealed a significant main effect of surgery ($F[1,54] = 15.76$, $p < 0.001$) and a marginally significant effect of infusion ($F[3,54] = 2.63$, $p = 0.06$). Follow-up tests showed RYGB rats generally consuming less calories (~20%) during baseline and vehicle infusion compared with sham-operated rats, and significantly higher intake with Ex9 vs. saline in RYGB rats (Fig. 3 B). Focusing on light period intake, there was a significant main effect of infusion $F[3,54] = 6.48$, $p < 0.001$), with both RYGB and sham rats, but not chow rats, showing significant Ex9 induced increases. In the absence of Ex9, food intake was not different between RYGB and Sham rats.

Feed efficiency calculated as weight gain per calorie eaten in the 11-day period from day 4-14 after the start of infusion showed that RYGB rats are slightly less efficient under saline conditions, but that sham-operated, RYGB, and chow-fed lean rats significantly increase feed efficiency under the influence of Ex9 (Fig. 3 D).

Finally, Ex9 infusion resulted in small increases in water intake that seemed secondary to the increased food intake (not shown).

Effects of Ex9 on energy expenditure, RER, and locomotor activity

Analysis of VO_2 consumption in mL/rat during the baseline infusion periods in the metabolic chambers for all three groups (RYGB, Sham, and Chow) revealed a significant main effect of surgery ($F[2,82] =$ 5.59, p, 0.01), but not infusion ($F[3,96] = 1.93$, n.s.) or interaction ($F[6,82] = 0.72$, n.s.) (Fig. 4A). The main effect of surgery was due to generally lower VO_2 consumption in RYGB rats compared to sham-operated and chow-fed rats. Expression of VO_2 consumption per kg total body mass (Fig. 4B), revealed a significant main effect of surgery ($F[2,82] = 7.13$, $p < 0.005$), which was due to higher energy expenditure of chow-fed rats with Ex9 infusions. If the ANOVA only included RYGB and sham-operated rats, the main effect of surgery was no longer significant ($F[1,54) = 0.28$, n.s.). Thus, VO_2 consumption was not significantly different between RYGB and sham-operated rats. Finally, if energy expenditure was corrected for lean body mass, it was slightly higher in RYGB vs. sham-operated rats, but the main effect of surgery did not reach statistical significance (data not shown).

As expected, the respiratory exchange ratio was significantly higher for chow-fed lean rats as reflected by the significant main effect of surgery ($F[2,82] = 23.10$, p ,0.0001; ANOVA across all 3 groups) (Fig. 4C). There was also a marginally significant main effect of infusion ($F[3,82] = 3.14$, $p < 0.05$), reflecting the consistently higher RER during Ex9 vs. vehicle infusion in all 3 groups. However, ANOVA across only RYGB and sham groups yielded no significant main effect of surgery ($F[1,54] = 1.8$, n.s.), revealing that there was no difference in RER between RYGB and sham-operated rats.

Locomotor activity was generally higher in chow-fed controls as indicated by the highly significant main effect of surgery ($F[2,82] = 5.32$, $p < 0.01$) (Fig. 4D). However, ANOVA applied only to RYGB and sham-operated rats did not reveal a significant main effect of surgery ($F[1,54] = 0.73$, n.s.), and there was no significant effect of Ex9 on locomotor activity.

Effects of pharmacological blockade of central Y2R-signaling in rats with BIIE0246

In contrast to Ex9, chronic infusion of BIIE0246 had no significant effects on body weight compared to vehicle infusion (Fig. 5 A). There was a slight decrease of body weight over the 14 day infusion period in both RYGB and sham-operated rats. To verify potency of BIIE0246, the Y2 receptor blocker or vehicle was injected ICV 15 min before injection of PYY(3-36) or saline in a separate group of rats (Fig. 5B). Two way ANOVA yielded significant effects of pretreatment (blocker vs. vehicle, $F[1,12] = 10.48$, $p < 0.01$), post-treatment (PYY(3-36) vs. vehicle, $F[1,12] = 13.26$, $p < 0.010$), and a significant interaction ($F[1,12] = 8.83$, $p < 0.05$), meaning that the food intake suppressing effect of PYY(3-36) was almost completely abolished by BIIE0246, without having an effect of its own, confirming efficacy of BIIE0246 to acutely block the anorexic effects of ICV PYY(3-36).

Effect of RYGB on body weight and composition in GLP-1R null and wildtype mice

Our mouse model of RYGB closely matches human surgery in the size of the gastric pouch and relative lengths of the three limbs (25), and produces sustained body weight loss in wildtype animals (Fig. 6A). Here we show that RYGB-induced weight loss was almost identical in whole body GLP-1 receptor knockout mice (Fig. 6B). Maximal body weight loss of 30-35% was reached after about 3 weeks and was sustained for 8 weeks after surgery in both genotypes (Fig. 6C). Weight loss was entirely accounted for

by fat mass loss in both genotypes, so that fat mass of RYGB animals was not different from chow-fed lean controls (Fig. 6D,E).

Exposure to a high-fat diet did not reveal a differential response in GLP-1R$^{-/-}$ compared with wildtype mice. Sham operated mice of both genotypes significantly gained body weight during the 6 weeks of high-fat feeding, but this weight gain was significantly less in GLP-1R$^{-/-}$ mice (Fig. 7 A,B). In contrast, RYGB mice of both genotypes did not gain any weight; they initially even lost some weight. Because some GLP-1R$^{-/-}$ mice exhibited reduced food intake and body weight and showed signs of morbidity on a single high-fat diet, we offered them a two-choice diet composed of regular chow and high-fat pellets. In addition to measuring total food intake this allowed us to determine a preference ratio for the high-fat diet. Compared to regular chow as the only diet, access to this choice diet significantly increased total calorie intake in RYGB and sham operated mice of both genotypes, but the increase was smaller in knockout mice (Fig. 7 D). On the choice diet, while sham operated mice showed a high preference for the high-fat pellets, RYGB mice consumed just as many calories from low (regular chow) compared to high-fat (Fig. 7 C,E). There was no effect of genotype on this RYGB-induced loss of high-fat preference.

Discussion

There is considerable evidence that both GLP-1 and PYY physiologically inhibit food intake [e.g. (7, 8, 17, 19, 57)]. Because both of these gut hormones are hyper-secreted after RYGB and sleeve gastrectomy, they are prime candidates for the weight lowering effects of these two types of bariatric surgeries [e.g. (14, 38)]. To test the hypothesis that either exaggerated GLP-1 or Y2 receptor signaling in the brain is responsible for the maintenance of a low body weight level after RYGB, we chronically infused the respective antagonists Ex9 or BIIE0246 into the brain over a period of 2 weeks. Infusion of Ex9 but not BIIE0246 significantly increased body weight and adiposity of RYGB rats by increasing food intake and feed efficiency, suggesting that central GLP-1 receptor signaling but not Y2 receptor signaling contributes to the lower weight level defended in rats after RYGB. Although it is not clear how Ex9 significantly increased feeding efficiency in the absence of significantly reduced energy expenditure, a similar observation was made in rats with knockdown of brainstem proglucagon (6) and in rats with acute peripheral treatment with Ex9 (3). We speculate that it may be due to a combination of changes in energy

intake, absorption, and partitioning, together with non-significant changes in energy expenditure. As weight gain with Ex9 infusion did not slow down at the end of the two week infusion, RYGB rats may well have reached pre-surgical body weight levels if blockade would have continued.

However, interpretation of our findings is complicated because of the same or even greater anabolic response to Ex9 infusion in sham-operated obese and chow-fed normal rats. This outcome suggests that central GLP-1 signaling is involved in normal physiological body weight control, but is not uniquely involved in the effects on body weight of RYGB. Ideally, for demonstrations of the effectiveness of a compound to specifically antagonize a mechanism that suppresses food intake, a dose is chosen that by itself does not significantly increase food intake (55). However, choosing a lower dose of Ex9 that would produce a smaller or no anabolic effect in sham-operated rats could be expected to produce even less of an effect in RYGB rats, and it is questionable whether a dose could be found that increases body weight in RYGB rats more than in sham-operated rats. On the other hand, considering the exaggerated circulating postprandial GLP-1 levels after RYGB, we cannot rule out that a higher dose of Ex9 may have been more effective in increasing food intake and reversing suppressed body weight levels after RYGB. However, we consider the dose of 100 µg/day/rat as supra-maximal, because a more than 100-fold lower dose (per body weight) still significantly changed food intake and glucose tolerance in mice (31). On a per animal basis, our dose of Ex9 was more than 1000-fold higher and in all likelihood blocked even the presumptively high levels of central GLP-1 receptor signaling after RYGB in the rat. While only a full dose-response study could provide definitive answers, this is not practical for this type of long-term infusion with a costly peptide.

Because we infused the blockers 3-5 month after surgery, we cannot rule out different outcomes at earlier time points, particularly the initial acute weight loss phase. Given that in human subjects, GLP-1 and PYY levels were elevated and associated with appetite and weight loss as soon as 2 days after RYGB (38), blockade during this phase in our paradigm would be expected to prevent or reduce initial weight loss. However, the complicating factor with similar anabolic effects in control rats would remain. We were particularly interested in the mechanisms defending the relatively stable lower body weight level after surgery because this is potentially the most remarkable feature of bariatric surgeries. After voluntary or forced calorie-restriction, strong counter-regulatory responses such as heightened hunger and reduced

energy expenditure make it difficult to stay at the lower body weight level for most dieters (48). These counter-regulatory responses seem to be absent or masked after RYGB. The possibility that central GLP-1 receptor signaling is involved in this process is intriguing and should be further investigated.

By infusing the blockers centrally, we were not able to affect peripheral GLP-1 and Y2 receptor signaling. Particularly GLP-1 receptor signaling via vagal afferents has been implicated in the control of food intake (28, 35, 46, 51, 58). It is possible that peripheral infusions would have provided clearer results, perhaps with a larger anabolic effect in RYGB compared with sham-operated rats. This is suggested by a study with acute subcutaneous injections of Ex9 in RYGB and sham-operated rats at about 6 weeks after surgery, where Ex9 significantly increased two hour food intake in RYGB but not sham-operated rats (3). We chose to infuse the blockers centrally mainly for economic reasons, but also because of the clear implication of the central GLP-1 signaling system in food intake and energy homeostasis (6, 63) and recent demonstrations of GLP-1 receptor mediated effects on food intake and food reward in areas outside the classical homeostatic areas of hypothalamus and brainstem, in the mesolimbic dopamine system (4, 20, 21). A corollary of central infusion of antagonists is that our experiment does not distinguish between GLP-1 and PYY originating from the gut and from respective neurons in the brain. If GLP-1 from the gut does not reach the brain in sufficient concentrations even after RYGB, the observed effect must be due to antagonism of neuronal GLP-1. It would thus be interesting to carry out gastric bypass in animals with selective loss of neuronal GLP-1.

In contrast to the effect of Ex9, BIIE0246 did not increase body weight of RYGB and sham-operated rats, suggesting that under the conditions of our paradigm, central Y2 receptor signaling does not play a role in the effects of RYGB on body weight. Absence of any effect of the blocker on body weight of sham-operated obese and chow-fed lean controls questioned its efficacy, prompting us to test the ability of BIIE0246 to antagonize PYY(3-36) induced suppression of food intake. We found pretreatment with BIIE0246 abolished the acute food intake reducing effects of fourth ventricular PYY(3-36). However, it is possible that the drug lost efficacy over the two weeks in the minipumps at body temperature. The lack of effect of Y2 receptor blockade in RYGB rats is in contrast to findings with a modified gastrointestinal bypass model in PYY-deficient mice (14). In that study, wildtype, but not mice with modified gastrointestinal bypass weighed significantly less 10 days after surgery compared with sham-operated

mice, supporting a role for PYY in the acute effects of gastric bypass on body weight. There are many experimental differences that could explain the apparent discrepancy. As for GLP-1, PYY signaling through the Y2 receptor has both a peripheral and central component (2, 8, 9, 49, 53, 58). However, in contrast to the GLP-1 receptor antagonist Ex9, the Y2 receptor antagonist BIIE0246 does not easily cross the blood brain barrier (11), and may not reach Y2 receptors on arcuate nucleus neurons in sufficient concentrations. Chronic infusions into the lateral ventricle are highly likely to penetrate to all areas of the brain, including critical neurons in the basomedial hypothalamus (2).

Given these limitations with semi-chronic central blockade of GLP-1 and Y2-receptors we then took advantage of an available GLP-1 receptor-deficient mouse generated by the laboratory of Daniel Drucker (54) and a murine model of RYGB recently developed in our laboratory (25). Unlike other models, our mouse RYGB model is characterized by a very small gastric pouch without using a metal clip, a very low mortality rate of < 10%, and a rapid postsurgical recovery, allowing animals to ingest solid food as soon as 1-3 days after surgery (25).

RYGB was just as effective in reducing body weight and fat mass in GLP-1 receptor deficient compared with wildtype mice, suggesting that signaling through this receptor, whether centrally or peripherally, is not required for the beneficial effects on body weight of RYGB. The same conclusion was recently reached in a mouse model of vertical sleeve gastrectomy (64), a surgical intervention that is drastically different from RYGB, but produces equal weight loss and similarly elevated levels of postprandial circulating GLP-1 levels in rodents and humans (13, 44). Therefore, surprisingly, GLP-1R signaling alone does not seem to be required for the beneficial effects of these two weight loss surgeries and other mechanisms that operate independently or in cooperation with GLP-1 signaling must be responsible. An inherent potential problem of germline knockout models is the presence of compensatory developmental changes. This could account for the unexpected outcome of these two types of surgery. It is conceivable that other mechanisms involved in the control of food intake and regulation of energy balance at least partially take over the role of GLP-1 signaling. Such redundant mechanisms may include signaling through the GLP-2 receptor, known to play a role not just in intestinal plasticity (41), but also in the neural control of appetite and energy balance (23, 39, 60). In fact, the capacity of acute ICV injections of GLP-1 to suppress food intake is greatly enhanced if the GLP-1 receptor is blocked by co-injection of

Ex-9 and in GLP-1R$^{-/-}$ mice (39), and plastic adaptations in the interaction between GLP-1R and GLP-2R signaling could be the reason that no overt perturbations of appetite and energy balance is observed in GLP-1R$^{-/-}$ mice (54). It is also possible that the contribution of critical sites of GLP-1 signaling were masked by the whole body knockout approach. Inducible and targeted knockout strategies will be necessary to rule out these possibilities of false negatives. Another limitation of our study is the absence of functional verification of GLP-1R knockout by testing known effects of GLP-1R agonist administration. However, homozygous knockout was verified by genotyping the mice before surgery and was previously demonstrated to result in functional loss of GLP-1R signaling (54) . Finally, we cannot rule out the possibility that GLP-1R-deficiency was unable to change the outcome of RYGB because GLP-1 secretion was not increased in our particular mouse model. However, this is highly unlikely in view of the overwhelming evidence of such GLP-1 hypersecretion across many rodent models and the human literature involving both RYGB and sleeve gastrectomy.

Taken together, the present findings from two complementary approaches surprisingly do not support a major role of GLP-1R in the effects of RYGB on body weight control. However, the results do not rule out a role for the GLP-1R in other beneficial effects of RYGB such as weight loss-independent improvements in glycemic control. In addition, our study also does not support an important role for central Y2 receptor-signaling, at least under the limited conditions tested and without a semi-chronic positive control for the effectiveness of the Y2R antagonist.

Perspective:

Given the complexity of gut-brain signaling (10), it is likely that a number of pathways are involved, operating synergistically and in a fashion reflecting the dynamic changes taking place over time in the gut and other peripheral organs. Interrupting only one signaling mechanism as done here may thus not reveal its real contribution to the overall effect. This argument is supported by recent studies highlighting the redundancy of mechanisms in the control of food intake and energy balance – the demonstration that combinations of drugs often have a larger effect than each of the individual drugs has alone. Therefore, it might be important to target more than one suspected pathway in interventional studies to obtain significant results.

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Author Contributions

Study concept and design (HRB, JY, HM, CDM), acquisition of data (NS, MBM, ZH, RLT, LMP), analysis and interpretation of data (MBM, HRB, CDM, HM), generation and breeding of knockout mice (DJD, JY).

Disclosures

The authors have nothing to disclose.

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Figure legends

Fig. 1 Effect of RYGB surgery in high-fat diet-induced obese rats on body weight and body composition. **A:** Male Sprague-Dawley rats were maintained on high-fat diet throughout the study and body composition was assessed before and after surgery. In addition, an age-matched, non-surgical control group was fed regular low-fat chow throughout. Body weight of RYGB rats rapidly decreased within the first two weeks and remained at that reduced level throughout the observation period, closely matching body weight of chow-fed controls. **B-E:** Effects of RYGB on total fat mass (B), relative fat mass (adiposity, C), total lean mass (D), and relative lean mass (E) in sham-operated (light gray bars), RYGB rats (dark gray bars) and chow-fed, unoperated, age-matched lean controls (white bars). Note that RYGB completely reversed excess high-fat diet-induced fat mass, and lean mass gain to levels found in chow-fed rats. Mean ± SEM of 15-20 rats/group. Bars that do not share the same letters denote significant differences at $p < 0.05$ (based on ANOVA followed by Bonferroni-adjusted multiple comparisons).

Fig. 2 Effect of chronic pharmacological blockade of central GLP-1 receptor signaling on body weight and composition in rats after RYGB or sham-operation. **A:** Two to four months after RYGB or sham-

surgery, Exendin-(9-39) (Ex9), 100µg/rat/day for 14 d) or vehicle (Veh, 0.5 µl/h) was infused ICV into the lateral ventricle. Ex9 significantly increased body weight gain in all 3 groups compared to their respective vehicle controls. Mean ± SEM of 7-8 rats/group. * p ,0.05, RYGB/Ex9 vs. RYGB/Veh and Sham/Ex9 vs. Sham/Veh. **B-E:** Change of body weight (B), fat mass (C), adiposity (D), and lean mass (E), as measured by NMR before and after infusion. Means ± SEM of 7-8 rats/group. * $p < 0.05$, Ex9 vs. vehicle. $^{\#}$ $p < 0.05$, RYGB vs. Sham.

Fig. 3 Effects of chronic pharmacological blockade of central GLP-1 receptor signaling on food intake and feeding efficiency of rats 2-4 months after RYGB surgery. **A:** Average daily food intake during pre-infusion baseline period (white bars) and days 4-14 of infusion with either Ex9 (dark gray) or vehicle (light gray). **B,C:** Average dark (B) and light (C) period food intake. **D:** Feed efficiency calculated during days 4-14 of Ex9 or vehicle infusion. Note that Ex9 significantly increases 24 h and 12 h light period food intake in RYGB and Sham rats and feed efficiency in all three groups. Mean + SEM of 6-8 rats/group. * $p < 0.05$, Ex9 vs. vehicle.

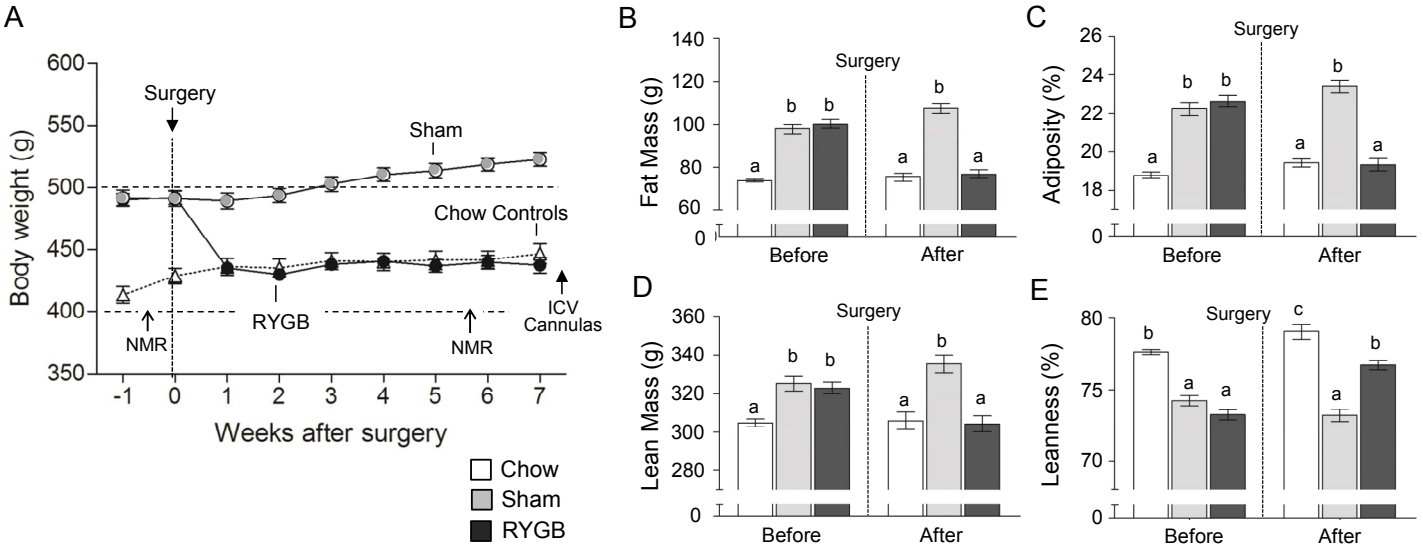
Fig. 4 Effects of chronic pharmacological blockade of central GLP-1 receptor signaling on energy expenditure, respiratory exchange ratio, and locomotor activity of rats 2-4 months after RYGB surgery. **A:** Average daily energy expenditure during pre-infusion baseline period (white bars) and days 4-14 of infusion with either Ex9 (dark gray) or vehicle (light gray). **B:** Energy expenditure corrected for total body mass. **C:** Respiratory exchange ratio. Note that chow-fed rats show the expected higher RER compared with the high-fat-fed RYGB and sham rats. **D:** Locomotor activity. Mean ± SEM of 6-8 rats/group.

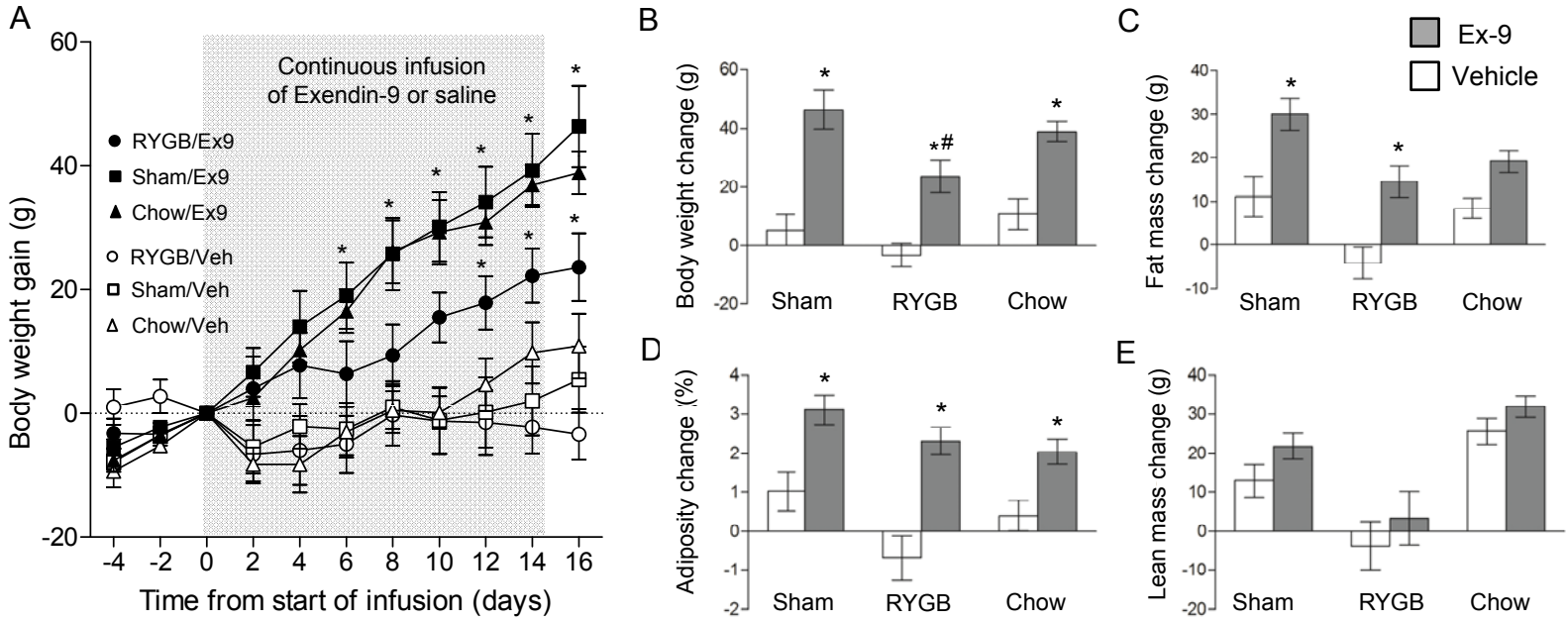
Fig. 5 Effects of chronic pharmacological blockade of PYY/Y2 receptor signaling on body weight. **A:** Five months after RYGB or sham-surgery, BIIE0246 (BIIE, 100µg/rat/day for 14 d) or vehicle (Veh, 0.5 µl/h) was infused ICV into the lateral ventricle. Note that vehicle-treated groups are the same as in Fig. 2 and that BIIE did not have any significant effects on body weight in either surgical group. Mean ± SEM of 6-8 rats/group. **B:** ICV infusion of PYY(3-36) significantly suppresses 30 min food intake in overnight food

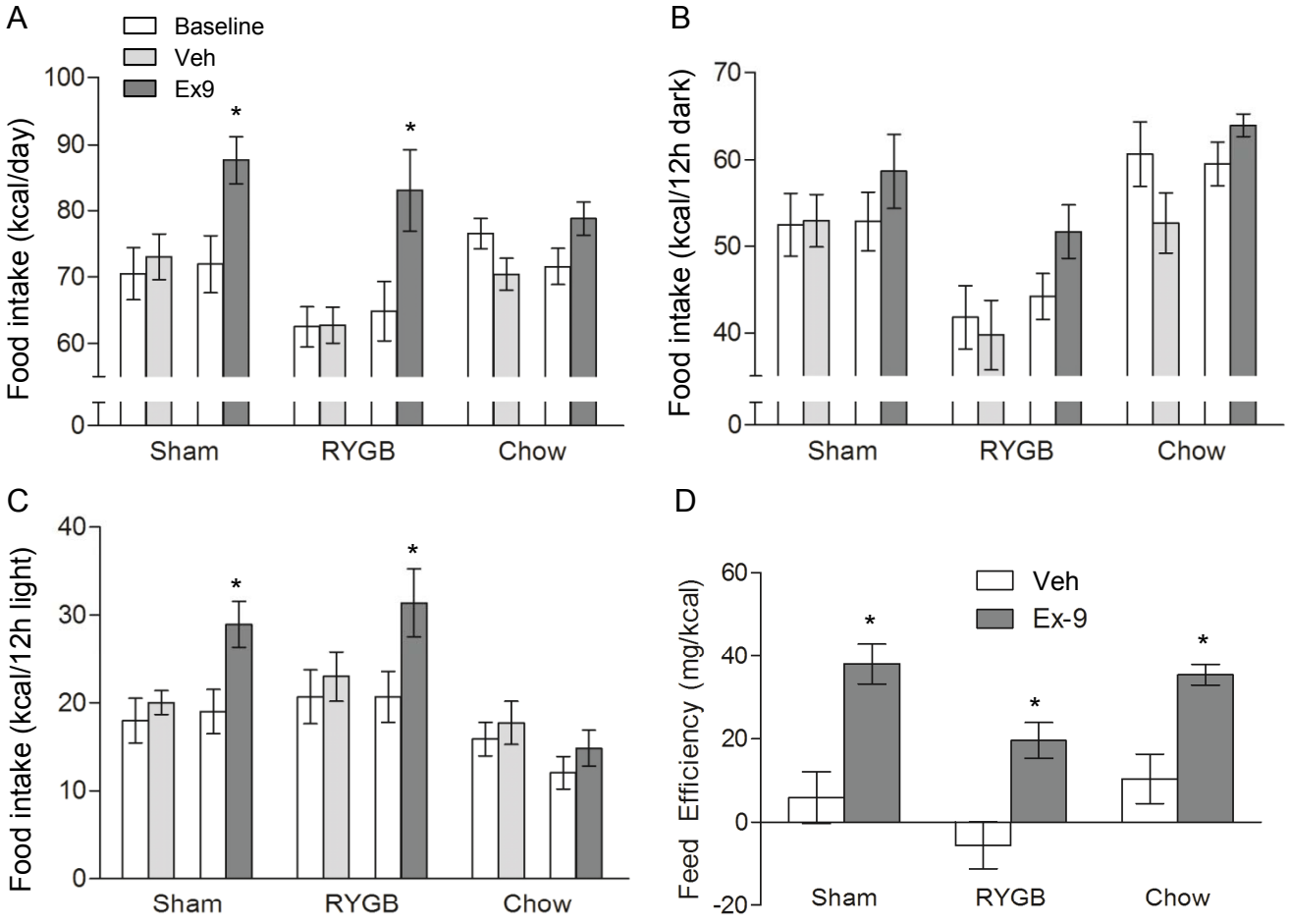
695 deprived rats, and co-infusion of BIIE0246 completely antagonizes the effect of PYY(3-36), demonstrating
696 short-term potency of the antagonist. Mean ± SEM of 4 rats.

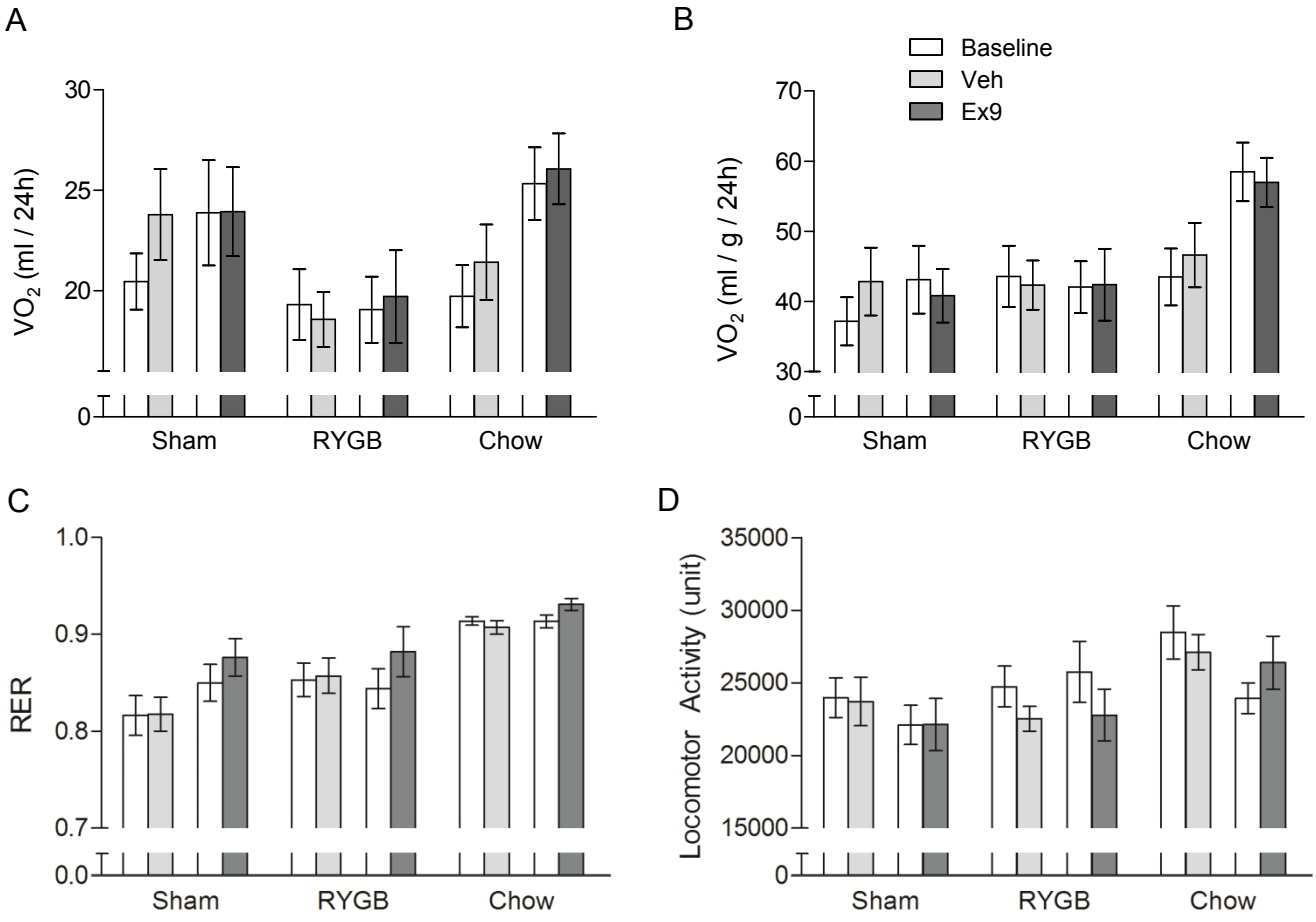
Fig. 6 RYGB-induced reduction in weight loss and food intake is similar in GLP-1 receptor-deficient and wildtype mice. **A,B:** Body weight of wildtype (A) and GLP-1R$^{-/-}$ mice (B) made obese with high-fat diet for 12 weeks and subjected to either RYGB or sham-surgery. High-fat diet was replaced with medium fat diet after surgery. Note that the RYGB-induced weight-loss curves are almost identical for the two genotypes. **C:** Percent weight loss at 8 weeks after surgery. Note that percent weight loss after RYGB was not significantly different between the two genotypes but significantly greater than after sham-operation. The small weight loss in sham-operated animals is due to the switch from high to medium fat after surgery. **D, E:** Fat mass (D) and lean mass (E) of lean (chow-fed), sham-operated (high-fat obese), and RYGB rats at 7 weeks after surgery. Note that all excess fat mass but no lean mass is lost after RYGB. Means ± SEM (n=8-10 mice).

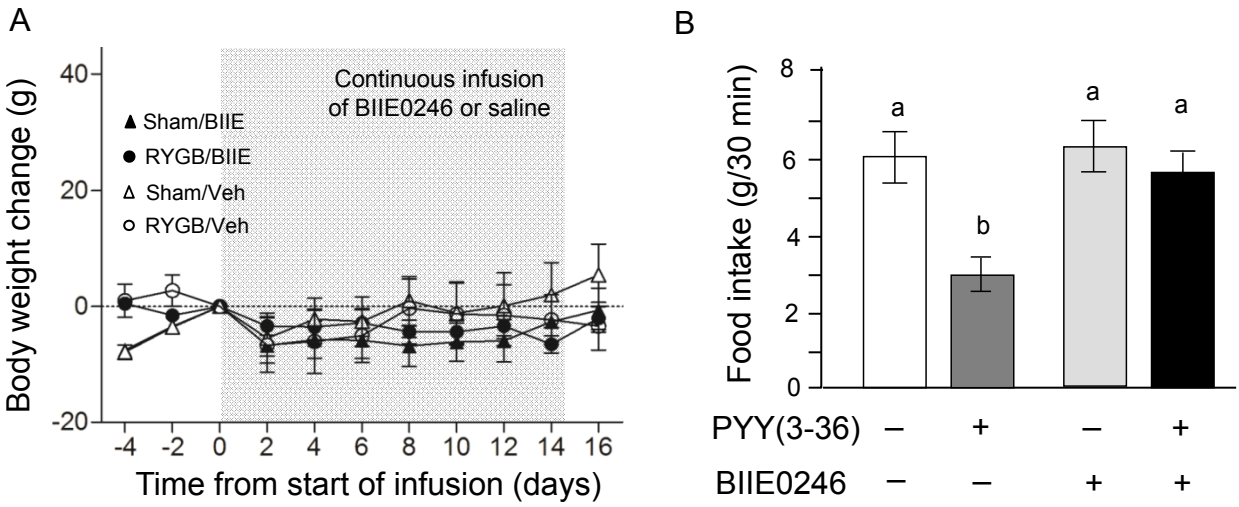
Fig. 7 Food Intake and body weight response of wildtype (GLP-1R$^{+/+}$) and GLP-1R$^{-/-}$ mice with RYGB or sham surgery to high-fat diet exposure. Mice were switched from regular chow diet to a two-choice diet consisting of regular chow (low-fat) and high-fat. **A,B:** Sham but not RYGB mice of both genotypes significantly increased body weight during 6 weeks of high-fat choice diet (* $p < 0.05$ vs. sham, # $p < 0.05$ vs.GLP-1R$^{+/+}$). **C-E:** Mice of all groups increased food intake on the high-fat choice diet (D), but while sham mice consumed significantly more of the high-fat, RYGB mice consumed equal amounts of low-fat and high-fat (E). Bars that do not share the same letter are significantly different from each other. Thus, RYGB mice of both phenotypes had a significantly lower preference ratio for high-fat (C), * $p < 0.05$, vs. sham). Means + SEM, n = 7 – 8).

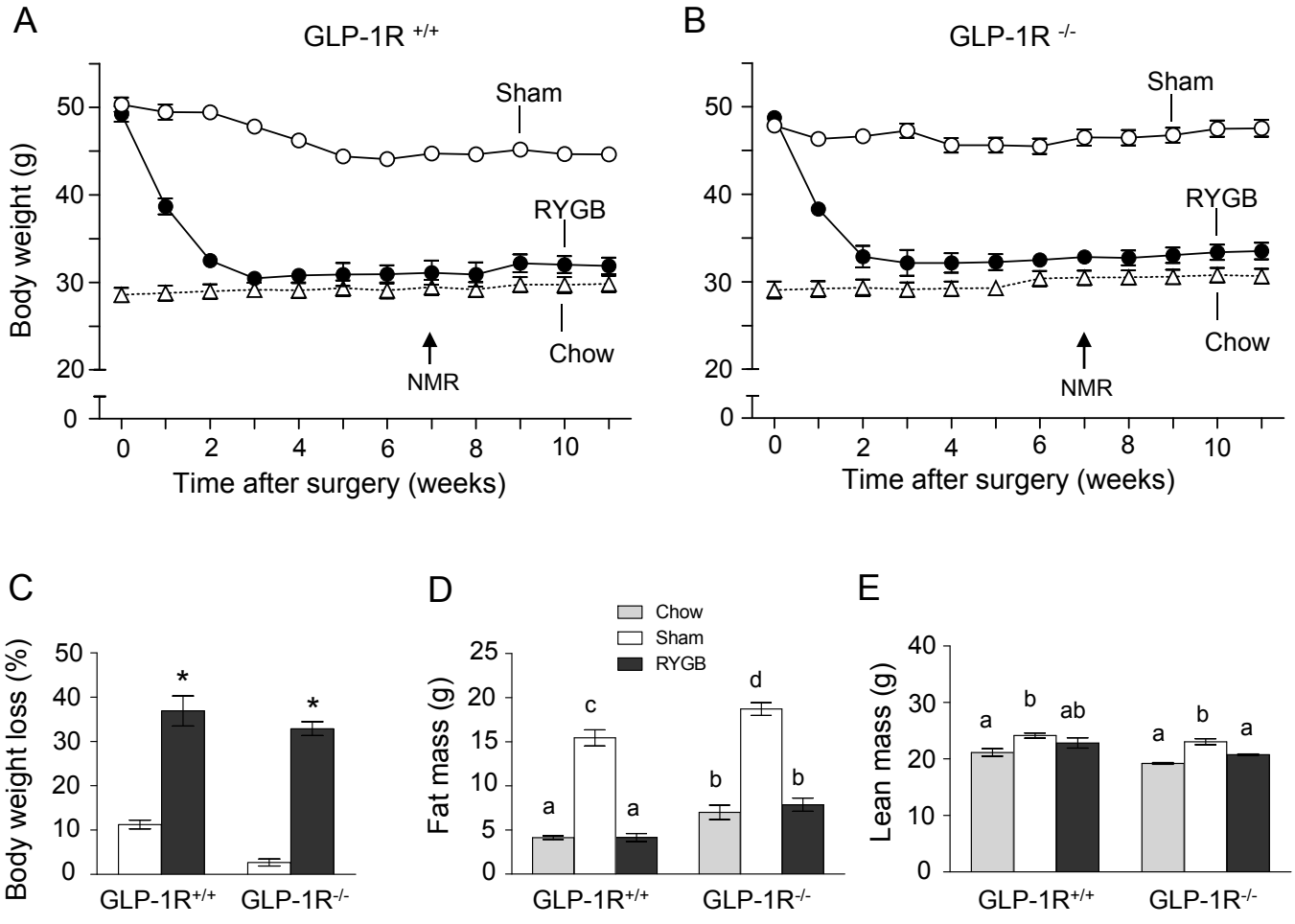

A
Body weight (g)
600
550
500
450
400
350
Surgery
Sham
Chow Controls
RYGB
NMR
NMR
ICV
Cannulas
-1
0
1
2
3
4
5
6
7
Weeks after surgery
Chow
Sham
RYGB
B
Fat Mass (g)
140
120
100
80
60
0
Surgery
a
b
b
a
b
a
Before
After
C
Adiposity (%)
26
24
22
20
18
0
Surgery
a
b
b
a
b
a
Before
After
D
Lean Mass (g)
360
340
320
300
280
0
Surgery
a
b
b
a
b
a
Before
After
E
Leanness (%)
80
75
70
0
Surgery
b
a
a
c
a
b
Before
After

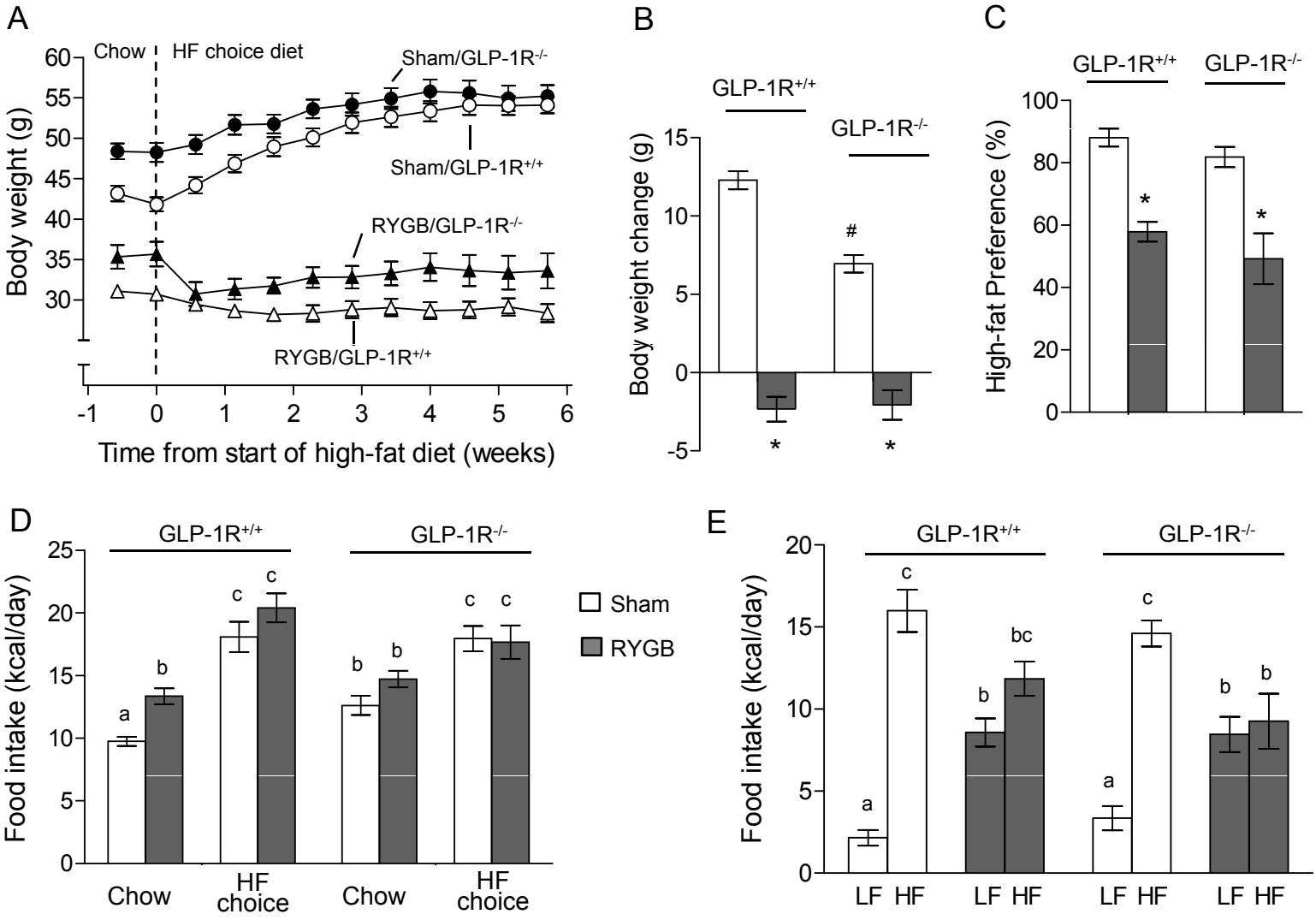

A
Continuous infusion
of Exendin-9 or saline
RYGB/Ex9
Sham/Ex9
Chow/Ex9
RYGB/Veh
Sham/Veh
Chow/Veh
Body weight gain (g)
Time from start of infusion (days)
B
Body weight change (g)
Sham
RYGB
Chow
C
Ex-9
Vehicle
Fat mass change (g)
D
Adiposity change (%)
E
Lean mass change (g)


A
Baseline
Veh
Ex9
Food intake (kcal/day)
100
90
80
70
60
0
*
*
Sham
RYGB
Chow
B
Food intake (kcal/12h dark)
70
60
50
40
0
Sham
RYGB
Chow
C
Food intake (kcal/12h light)
40
30
20
10
0
*
*
Sham
RYGB
Chow
D
Veh
Ex-9
Feed Efficiency (mg/kcal)
60
40
20
0
-20
*
*
*
Sham
RYGB
Chow


A
B
C
D
Baseline
Veh
Ex9
VO_2 (ml / 24h)
VO_2 (ml / g / 24h)
RER
Locomotor Activity (unit)
Sham
RYGB
Chow
0
20
25
30
30
40
50
60
70
0.0
0.7
0.8
0.9
1.0
15000
20000
25000
30000
35000


A
Body weight change (g)
40
20
0
-20
-4
-2
0
2
4
6
8
10
12
14
16
Time from start of infusion (days)
Continuous infusion
of BIIE0246 or saline
Sham/BIIE
RYGB/BIIE
Sham/Veh
RYGB/Veh
B
Food intake (g/30 min)
8
6
4
2
0
a
b
a
a
PYY(3-36) – + – +
BIIE0246 – – + +


A
GLP-1R +/+
Sham
RYGB
Chow
NMR
Body weight (g)
Time after surgery (weeks)
B
GLP-1R -/-
Sham
RYGB
Chow
NMR
Time after surgery (weeks)
C
Body weight loss (%)
GLP-1R+/+
GLP-1R-/-
D
Chow
Sham
RYGB
Fat mass (g)
GLP-1R+/+
GLP-1R-/-
E
Lean mass (g)
GLP-1R+/+
GLP-1R-/-


A
Chow
HF choice diet
Sham/GLP-1R-/-
Sham/GLP-1R+/+
RYGB/GLP-1R-/-
RYGB/GLP-1R+/+
Body weight (g)
Time from start of high-fat diet (weeks)
B
GLP-1R+/+
GLP-1R-/-
Body weight change (g)
C
GLP-1R+/+
GLP-1R-/-
High-fat Preference (%)
D
GLP-1R+/+
GLP-1R-/-
Sham
RYGB
Food intake (kcal/day)
Chow
HF choice
Chow
HF choice
E
GLP-1R+/+
GLP-1R-/-
Food intake (kcal/day)
LF HF
LF HF
LF HF
LF HF