Communication

Intact leptin receptor signalling is not required for the sustained weight loss and appetite suppression induced by Roux-en-Y gastric bypass surgery

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Abstract: Leptin is the archetypal adipokine that promotes a negative whole-body energy balance largely through its action on brain leptin receptors. As such, the sustained weight loss and food intake suppression induced by Roux-en-Y gastric bypass (RYGB) surgery have been attributed to enhancement of leptin receptor signalling. We formally revisited this idea in Zucker Fatty *fa*/*fa* rats, an established genetic model of leptin receptor deficiency, and carefully compared their body weight, food intake and oral glucose tolerance after RYGB with that of sham-operated *fa*/*fa* (obese) and sham-operated *fa*/+ (lean) rats. We found that RYGB rats sustainably lost body weight, which converged with that of lean rats and was 25.5 % lower than that of obese rats by the end of the 4 week study period. Correspondingly, daily food intake of RYGB rats was similar to that of lean rats from the second postoperative week, while it was always at least 33.9 % lower than that of obese rats. Further, oral glucose tolerance of RYGB rats was normalized at the forth postoperative week. These findings assert that leptin is not an essential mediator of the sustained weight loss and food intake suppression as well as the improved glycemic control induced by RYGB, and instead point to additional circulating and/or neural factors.

Keywords: 1. Roux-en-Y gastric bypass surgery; 2. Weight loss; 3. Food intake; 4. Oral glucose tolerance; 5. Leptin; 6. Leptin receptors; 7. Zucker Fatty *fa*/*fa* rats

1. Introduction

Bariatric surgery is presently the mainstay treatment option against morbid obesity, with numerous prospective clinical studies showing that Roux-en-Y gastric bypass (RYGB) in particular induces marked and sustained weight loss as well as long-term remission of type 2 diabetes in a majority of cases [1]. Because RYGB reduces stomach size and excludes the duodenum from contact with ingested food, physical restriction and malabsorption of nutrients, respectively, were originally thought to mainly account for its beneficial effects on energy and glucose homeostasis [2]. However, with the aid of robust rodent models of RYGB [3-5], it is becoming increasingly evident that complex molecular and cellular processes are in play postoperatively, better understanding of which may guide the development of more effective appetite suppressing and blood glucose lowering drugs than what are currently available.

Leptin is a 16-kDa endocrine protein mainly released from white adipocytes and circulates in proportion to fat mass, thereby serving as a negative feedback signal to the brain on whole-body

energy stores [6,7]. Accordingly, leptin-deficient *ob*/*ob* mice [8,9] and leptin-unresponsive *db*/*db* mice [10], which lack the intracellular signalling domain unique to leptin b receptors due to an autosomal recessive G→T point mutation in the leptin receptor gene [11], have severe, hyperphagic obesity as well as hyperglycemia. Moreover, diet-induced obesity is thought to arise from the development of central leptin resistance as a result of complex pro-inflammatory processes that directly interfere with hypothalamic leptin receptor signalling [12-15]. For these reasons, leptin supplementation to *ob*/*ob* mice normalizes their body weight, food intake and glycemic control [16,17], whereas leptin sensitizers have taken center stage in obesity drug development [18,19]. While RYGB markedly reduces circulating leptin levels [20-22], even beyond that from chronic caloric restriction-induced weight loss alone [23-28], leptin action might be enhanced postoperatively, thereby preventing the powerful counter-regulatory response to depletion of whole-body energy stores which normally pressures weight regain [6]. Indeed, the disproportionately reduced circulating leptin levels induced by RYGB may in itself paradoxically restore leptin action by reversing central leptin resistance [29]. The necessity of leptin for the beneficial outcomes of RYGB on energy and glucose homeostasis was originally tested in *ob*/*ob* mice [30,31]. The sustained weight loss and food intake suppression induced by RYGB was found to be preserved in one study [31], but not in another [30], although in both studies, RYGB failed to fully improve glycemic control [30,31]. This is consistent with the documented independent effects of leptin in beneficially regulating glucose homeostasis [32]. On the other hand, weight loss and enhanced insulin sensitivity [33], as well as improved fasting blood glucose levels and oral glucose tolerance [34] in *db*/*db* mice after RYGB appear to be largely preserved.

Zucker Fatty *fa*/*fa* rats are another genetic model of leptin receptor deficiency since they harbor an autosomal recessive A→C point mutation at position 880 of the leptin receptor gene, distinct from the *db*/*db* point mutation, which causes an inhibitory Glu→Ala amino acid substitution at position 269 in the extracellular domain common to all leptin receptor subtypes (a-f) [35-37]. As a result, Zucker Fatty *fa*/*fa* rats are obese and hyperlipidemic [38], and exhibit markedly impaired oral glucose tolerance [39], as well as severely diminished responsiveness to exogenous leptin treatment [40,41]. Zucker Diabetic Fatty *fa*/*fa* rats, on the other hand, additionally harbor a mutation that reduces insulin promoter activity in pancreatic beta cells [42], rendering them incapable of secreting adequate amounts of insulin and thus genuinely diabetic [43]. Numerous studies have been performed aimed at assessing the metabolic effects of RYGB on both Zucker Fatty *fa*/*fa* [44-48] and Zucker Diabetic Fatty *fa*/*fa* [24, 49-64] rats, but their descriptions on food intake were generally either incomplete [44-51,54,61,62,64] or, in many studies, entirely missing [24,52,53,55-60,63]. Additionally, only a few of these studies incorporated a lean control group in the form of heterozygous Zucker Fatty *fa*/+ rats [44,46,47,59], which is essential if any conclusions are to be drawn about whether RYGB normalizes glycemic control. Surprisingly, all of these studies entirely overlooked the necessity of leptin receptors in the sustained metabolic benefits induced by RYGB, which calls for their reinterpretation. We therefore directly addressed and carefully assessed if the sustained weight loss and food intake suppression as well as improved glycemic control induced by RYGB require intact leptin receptor signalling by using Zucker Fatty *fa*/*fa* rats in comparison with both sham-operated Zucker Fatty *fa*/*fa* and sham-operated Zucker Fatty *fa*/+ rats over a 4 week monitoring period. Our findings provide further evidence against leptin being an essential mediator of the two best characterized metabolic benefits induced by RYGB.

2. Materials and Methods

Animals

Two cohorts of male Zucker Fatty *fa*/*fa* and Zucker Fatty *fa*/+ rats were purchased from Charles River, France, aged 6 weeks. The first cohort comprised 12 Zucker Fatty *fa*/*fa* rats and 7 Zucker Fatty *fa*/+ rats whereas the second cohort comprised 16 Zucker Fatty *fa*/*fa* rats and 5 Zucker Fatty *fa*/+ rats and were used in a previous study [46]. Because housing and treatment conditions were identical for both cohorts, they were merged for the purposes of this study. Animals were individually housed under ambient humidity and temperature of 22 °C in a 12-hour light/dark cycle with free access to tap water and Purina 5008 Lab diet (Purina Mills, USA, 16.7 % of calories from fat) unless otherwise stated.

Surgeries

At 12 weeks of age, Zucker Fatty *fa*/*fa* rats were randomly allocated to RYGB (n = 16) or sham (n = 12) surgeries with the latter forming the "obese" group. Twelve Zucker Fatty *fa*/+ rats also underwent sham surgeries forming the "lean" group (Figure 1). Surgical anesthesia was induced and maintained in 6-hour fasted rats with an isoflurane/oxygen mixture after subcutaneous administration of 5 mg/kg carprofen as analgesia. The abdomen was opened using a midline laparotomy and closed after surgeries using continuous suturing. For the sham procedure, the small bowel and gastro-esophageal junction were mobilized, and a gastrostomy on the anterior wall of the stomach and a jejunostomy with subsequent closure were performed. For the RYGB procedure, the jejunum was transected 16 cm aboral to the pylorus to create the biliopancreatic limb. The stomach was divided 3 mm below the gastro-esophageal junction to create a small pouch and the stomach remnant was subsequently closed. The aboral jejunum was anastomosed end-to-side to the small pouch. At the level of the lower jejunum, a 7-mm side-to-side jejuno-jejunostomy between the biliopancreatic limb and the alimentary limb was performed creating a common channel of~25 cm in length.

Upon recovery from surgeries, rats were placed on a liquid diet for 6 days postoperatively and then returned to their previous solid diet. Food intake was measured daily from postoperative day 6 while body weight was measured daily throughout the 27 monitoring period. All experiments were in compliance with the guidelines on animal welfare of the European Union and reviewed and approved by the Animal Care Committee of the local government of Unterfranken, Bavaria, Germany (License 55.2-2531.01-72/12).

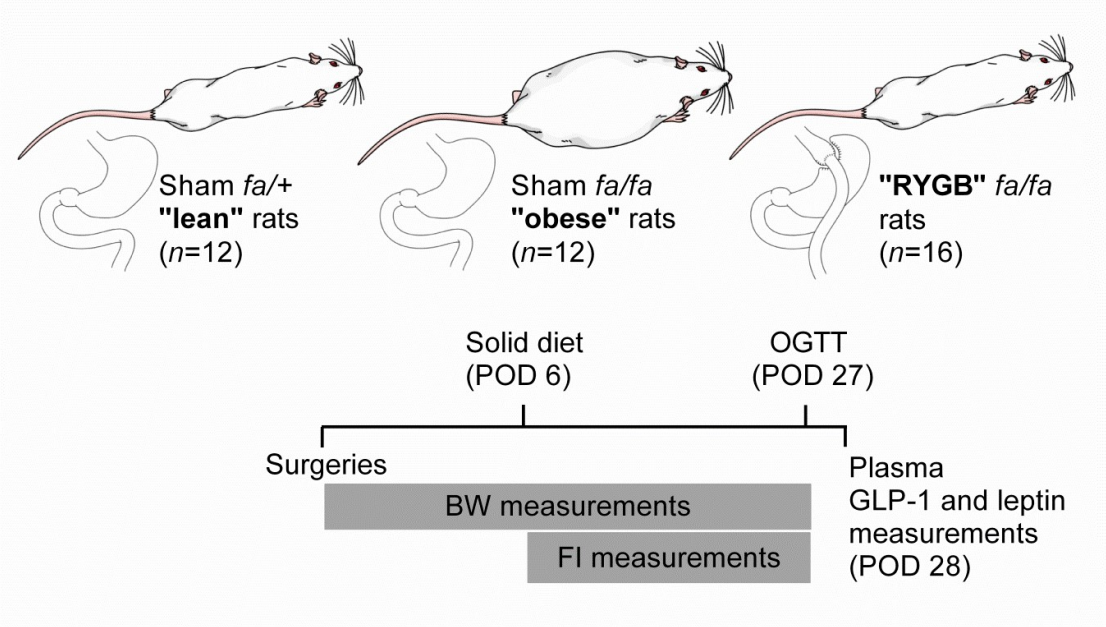

Figure 1. Schematic of experimental design

POD: postoperative day; BW: body weight; FI: food intake; OGTT: oral glucose tolerance test.

Metabolic Measurements

An oral glucose tolerance test (OGTT) was performed at the beginning of the dark cycle on postoperative day 27 in all rats. In order to reduce the stress associated with oral gavage, overnight-fasted rats were trained to consume 10 mL/kg body weight of a 25 % glucose solution within 10 minutes on 2 occasions prior to the OGTT. For blood glucose measurements during the OGTT, a small tail vein incision was made in in 8-hour fasted rats and a drop of blood was directly applied onto a glucometer (Breeze 2® glucometer, Bayer, Zurich, Switzerland) at baseline and 15, 30, 60 and 120 minutes after glucose ingestion. A further 100µL of tail vein blood was collected at each time-point into tubes containing EDTA, and plasma was isolated by centrifugation at 8000 rpm for 10 minutes at 4 °C for future plasma insulin measurements using the Ultrasensitive Rat Insulin ELISA kit (Mercodia AB, Sweden 10-1251-10). Homeostatic model of insulin resistance (HOMA-IR) [65] was calculated by the dividing the product of fasting plasma insulin (in µU/L) and blood glucose (in nmol/L) levels by 22.5. Matsuda–DeFronzo insulin sensitivity index (ISI-M) [66] was calculated based on the results of the OGTT as follows:

$$\text{ISI-M} = 10,000/(G_0 \times I_0 \times G_{mean} \times I_{mean})^{1/2}$$

Where G and I represents blood glucose (in mmol/dL) and plasma insulin (in mU/L) levels, respectively, and '0' and 'mean' indicates fasting value and mean value during the OGTT, respectively.

Tissue Harvesting

At the 28th postoperative day, overnight-fasted animals were sacrificed 45 minutes after a fixed meal of 3 g Purina 5008 diet by isoflurane overdose. Trunk blood was collected into tubes containing EDTA and a dipeptidyl peptidase-4 inhibitor, and plasma was isolated by centrifugation at 8000 rpm for 10 minutes at 4 °C for future plasma GLP-1 and leptin measurements using a Rat GLP-1 ELISA kit (EMD Millipore, #EZGLP1T-36 K) a Rat Leptin ELISA kit (abcam, ab100773), respectively. Epididymal white adipose tissue (eWAT) and retroperitoneal white adipose tissue (rWAT) were dissected, weighed and summed to provide a measure of visceral WAT (vWAT) [67]. Percentage body weight of vWAT was estimated by dividing calculated vWAT weights by body weight.

Statistics

Statistical analysis was performed using GraphPad PRISM Version 7®. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. A one-way analysis of variance (ANOVA) with Sidak's post hoc test was used to test differences between groups. A Pearson correlation was performed on plasma GLP-1 data vs. average daily food intake and body weight using two-tailed unpaired *t*-test. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Intact leptin receptor signalling is not required for the sustained weight loss induced by RYGB

To formally assess the necessity of intact leptin receptor signalling in the sustained negative energy balance induced by RYGB, we performed a detailed analysis of body weight trajectories in Zucker Fatty *fa/fa* and Zucker Fatty *fa*/+ rats subjected to either RYGB or sham surgeries (Figure 1).

Importantly, RYGB and obese rats had similar body weights at baseline (443.7 ± 2.8 g vs. 442.3 ± 6.7 g, respectively; p=0.99), which was significantly higher than that of lean rats (348.8 ± 8.1 g; p<0.0001 for both comparisons) (Figure 2a) and serves to confirm the state of leptin receptor deficiency in the former 2 groups. From postoperative day 3 onwards, both obese and lean rats progressively gained body weight while RYGB rats lost body weight until postoperative day 6, which then largley stabilized for the remainder of the 27 day monitoring period (Figure 2a). This meant that body weights for RYGB rats were statistically indistinguishable from lean rats from postoperative day 18 onwards and converged by the end of the 27 day monitoring period (414.7 ± 12.5g vs. 405.0 ± 10.7g, respectively; p=0.99) (Figure 2a).

To gain a clearer impression of the magnitude of sustained weight loss induced by RYGB, body weights were expressed relative to baseline for the rats in each group during the 27 day monitoring period (Figure 2b). This revealed that peak weight loss for RYGB rats at postoperative day 6 was at -9.7 ± 0.6 % and stabilized at -6.5 ± 2.8 % by postoperative day 27 (Figure 2b). Consistent with the leptin receptor-deficient state of obese rats, they gained body weight at a greater rate than lean rats, diverging signficantly at postoperative day 15 and reaching + 25.9 ± 0.5 % vs. + 16.1 ± 1.6 % by postoperative day 27, respectively (Figure 2b).

Because the magnitude of sustained weight loss induced by RYGB was less than the 30-40% typically observed in the clinical setting [1], we also factored in the high rate of weight gain in Zucker Fatty *fa/fa* rats (Figure 2c). This revealed that relative to obese rats at the same time-point, body weights of RYGB rats at postoperative day 27 were 25.5 ± 2.2 % lower, converging with that of lean rats (27.3 ± 1.9 % lower; p=0.81) (Figure 2c).

At study end on postoperative day 28, rats were sacrificed and vWAT was dissected and weighed. This revealed that vWAT of obese rats weighed significantly more than that of lean and RYGB rats (28.4 ± 1.2g vs. 8.0 ± 0.6g and 18.6 ± 1.0g, respectively; p<0.0001 for both comparisons) (Figure 2d). Interestingly, Sidak post-hoc test also reavealed that the vWAT of RYGB rats weighed significantly more than that of lean rats (p<0.0001), suggesting that lean mass of the latter group is greater. When expressed relative to body weight, vWAT of obese rats was significantly higher than that of lean and RYGB rats (5.2 ± 0.25% vs 1.8 ± 0.05% and 4.1 ±0.2%, respectively; p<0.0001 and p<0.01 for obese vs lean and obese vs RYGB, respectively), and remained significantly higher for RYGB rats compared with lean rats (p<0.0001) (Figure 2e). This was also reflected both quantitatively and statistically in plasma leptin levels, which were the highest for obese rats (3.1 ± 0.11 μg/mL) followed by RYGB rats (1.9 ± 0.10 μg/mL) and then by lean rats (0.8 ± 0.07 μg/mL) (Figure 2f).

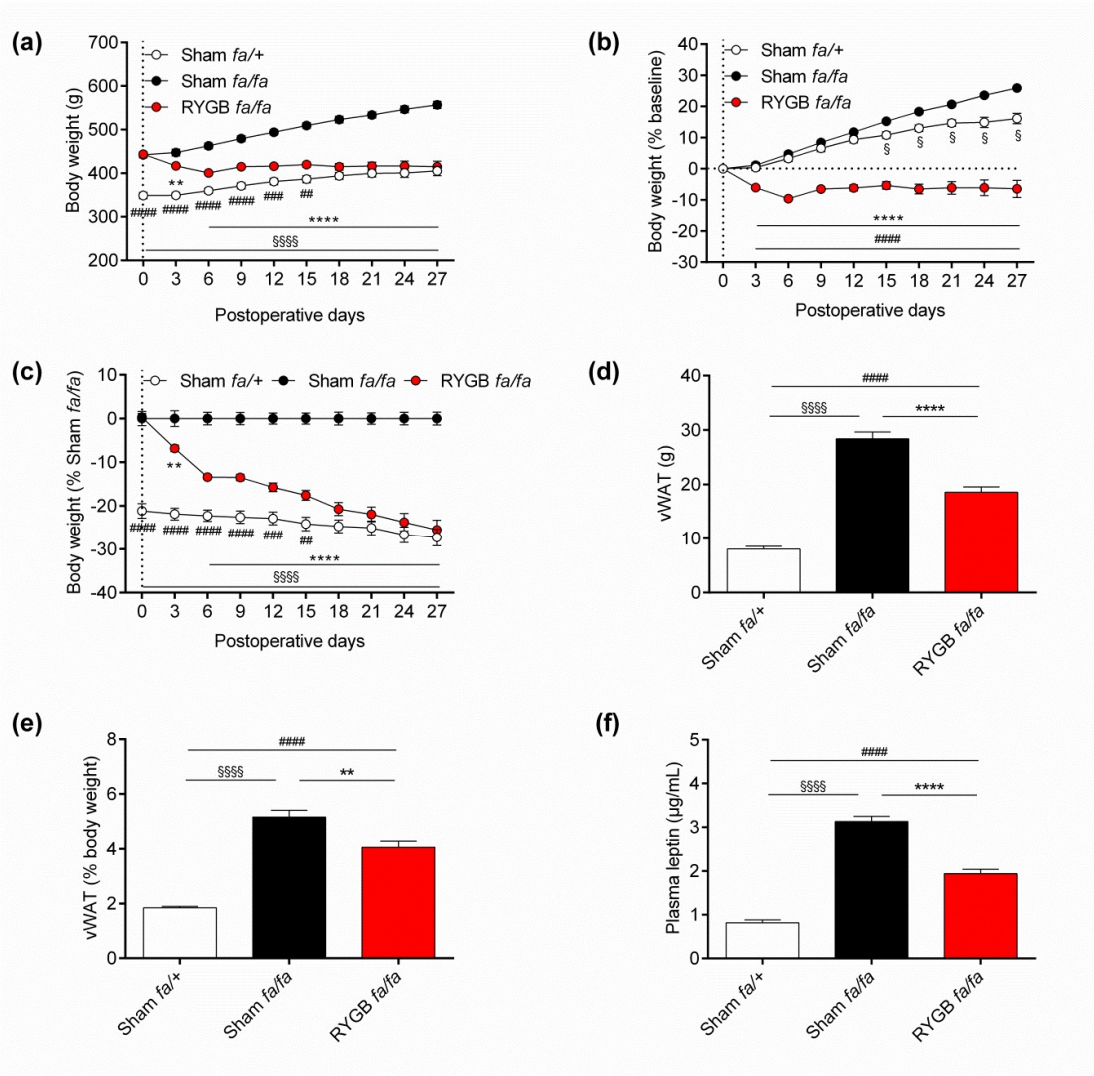

Figure 2. Intact leptin receptor signalling is not required for the sustained weight loss induced by RYGB

(**a**) Absolute body weight in grams (g), (**b**) percentage (%) body weight change relative to baseline and (**c**) % body weight of sham-operated *fa/fa* rats in sham-operated *fa*/+ rats (n=12), sham-operated *fa/fa* rats (n=12) and RYGB-operated *fa/fa* rats (n=16) during the 27 day monitoring period. (**d**) Absolute vWAT (eWAT + rWAT) weight, (**e**) % vWAT of body weight and (**f**) fasting plasma leptin concentrations in the rats from (**a-c**) at the time of sacrifice on postoperative day 28. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Sidak post-hoc test. $^{§§§§}p<0.0001$ for sham-operated *fa*/+ vs. sham-operated *fa/fa* rats, $^{**}p<0.01$ and $^{****}p<0.0001$ for RYGB-operated *fa/fa* vs. sham-operated *fa/fa* rats and $^{\#\#\#\#}p<0.0001$, $^{\#\#\#}p<0.001$ and $^{\#\#}p<0.01$ for RYGB-operated *fa/fa* vs. sham-operated *fa*/+ rats.

3.2. Intact leptin receptor signalling is not required for the sustained food intake suppression induced by RYGB

Next, to formally assess the necessity of intact leptin receptor signalling in the sustained appetite suppression induced by RYGB, we performed a detailed analysis of food intake in the rats from each group when they were reintroduced to solid diets at postoperative day 6 (Figure 1).

Average daily food intake for RYGB rats was lower than that of lean rats from postoperative days 7-9 (17.6 ± 0.7 kcal vs. 24.7 ± 0.9 kcal per day, respectively; $p<0.0001$) to postoperative days 13-15 (20.9 ± 0.7 kcal vs. 24.3 ± 0.5 kcal per day, respecitvely; $p<0.01$), but from postoperative days 16-18 onwards was statistically indistinguishable (Figure 3a). This is possibly due to the extra time needed for healing of the reconfigured gastrointestinal tract in RYGB rats (Figure 1d and Figure 1e). Compared with obese rats, however, RYGB rats consumed significantly less food at every time interval during the entire recording period ($p<0.0001$), so that food intake suppression was sustained until completion of the study at postoperative days 25-27 (21.5 ± 1.8 kcal vs. 32.5 ± 0.9 kcal per day, respectively; $p<0.0001$). This equated to 33.9 ± 5.5% lower food intake for RYGB rats compared with obese rats (Figure 3b). Accordingly, cummulative food intake over the recording period for RYGB rats was significantly lower than that for obese and lean rats (424.8 ± 23.8 kcal vs. 676.8 ± 13.6 kcal and 504.4 ± 12.2 kcal, respectively; $p<0.0001$ for both comparisons) (Figure 3c).

To relate the sustained food intake suppression and weight loss in RYGB rats to a gut-derived satiety factor, we also measured postprandial plasma levels of the anorexigenic gut hormone GLP-1, which are well known to be increased by RYGB [68], at postoperative day 28. Consistent with previous findings in Zucker Fatty *fa/fa* rats [45,46], RYGB rats had markedly higher plasma levels of GLP-1 compared with obese and lean rats (262.0 ± 19.4 pg/mL vs. 112.3 ± 9.7 pg/mL and 122.8 ± 16.2 ng/mL, respectively; $p<0.0001$ for both comparisons) (Figure 3d). Further, fasting plasma GLP-1 levels in Zucker Fatty *fa/fa* rats negatively correlated with food intake at postoperatve days 25-27 ($r=-0.5$, $p<0.01$) (Figure 3e) as well as body weight at postoperative day 27 ($r=-0.63$, $p<0.001$) (Figure 3f).

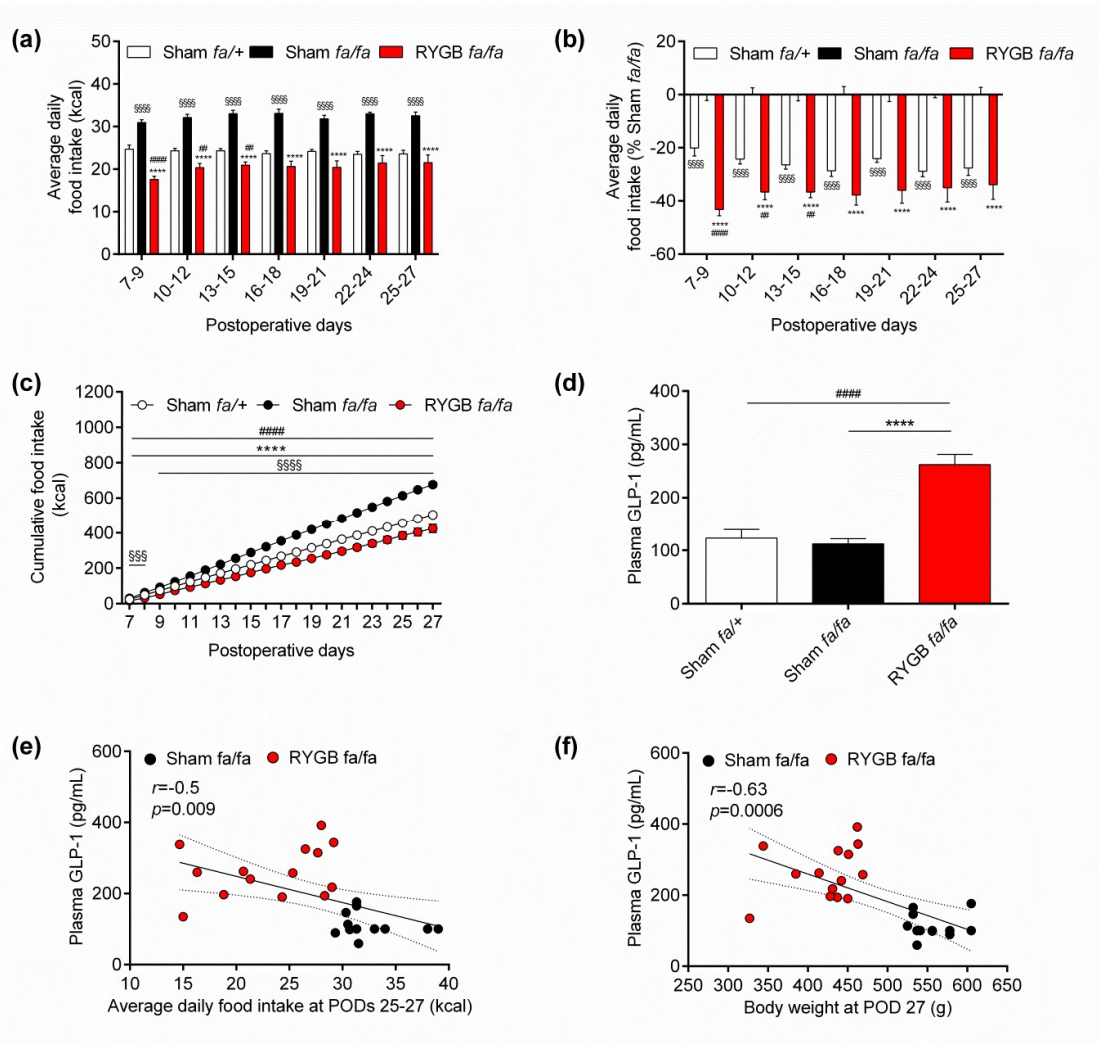

Figure 3. Intact leptin receptor signalling is not required for the sustained food intake suppression induced by RYGB

(**a**) Average daily food intake in kcal, (**b**) percentage (%) average daily food intake relative to sham-operated *fa/fa* rats and (**c**) cumulative food intake in sham-operated *fa*/+ rats (n=12), sham-operated *fa/fa* rats (n=12) and RYGB-operated *fa/fa* rats (n=16) during the indicated recording period. (**d**) Plasma GLP-1 concentrations and (**e**) its correlation with food intake and (**f**) body weight in the rats from (**a-c**) at the indicated times. Data in (**a-d**) are presented as mean ± SEM. Solid lines in (**e** and **f**) are the least square fit of data and interrupted lines are 95% confidence intervals. Statistical significance was determined in (**a-d**) by one-way ANOVA with Sidak post-hoc test and in (**e** and **f**) by two-tailed, unpaired t-test. $^{\S\S\S\S}p<0.0001$ for sham-operated *fa*/+ vs. sham-operated *fa/fa* rats, $^{****}p<0.0001$ for RYGB-operated *fa/fa* vs. sham-operated *fa/fa* rats and $^{\#\#\#\#}p<0.0001$ and $^{\#\#}p<0.01$ for RYGB-operated *fa/fa* vs. sham-operated *fa*/+ rats.

3.3. Intact leptin receptor signalling is not required for the improved glycemic control induced by RYGB

Finally, to assess the importance of intact letpin receptor signalling in the improved glycemic control induced by RYGB an OGTT was performed at postoperative day 27 (Figure 1).

Fasting blood glucose levels were slightly but significantly higher for obese compared with lean rats (105.8 ± 8.6 mg/dL vs 80.8 ± 3.3 mg/dL, respectively; p=0.01), whereas they were statistically indistinguishable for RYGB rats (92.9 ± 3.7 mg/dL) compared with both lean (p=0.23) and obese (p=0.27) rats (Figure 4a).

During the OGTT, blood glucose levels peaked for lean rats at 123.5 ± 5.1 mg/dL by 15 minutes and then steadily declined to 94.6 ± 2.9 mg/dL by 120 minutes (Figure 4a). For obese rats, blood glucose levels peaked at 211.4 ± 15.2 mg/dL by 30 minutes and remained elevated at 197.7 ± 15.3 mg/dL by 60 minutes before eventually declining to 108.0 ± 4.7 mg/dL by 120 minutes. The blood glucose excursion curve for RYGB rats during the OGTT was markedly different from both lean and obese rats peaking at 186.3 ± 12.1 mg/dL by 15 minutes, then rapidly dropping to 133.6 ± 8.0 mg/dL by 30 minutes before declining below baseline values to 84.7 ± 3.8 mg/dL at 60 minutes and then returning to near baseline values of 91.9 ± 3.6 mg/dL at 120 minutes (Figure 4a). The associated area under the curve analysis illustrates how RYGB rats have markedly improved oral glucose tolerance compared with obese rats (p<0.0001) being similar to that of lean rats (Figure 4b).

Concerning plasma insulin levels, obese rats were hyperinsulinemic at baseline (1.2 ± 0.2 nmol/L), whereas both lean and RYGB rats had signficantly lower plasma insulin levels at baseline (0.14 ± 0.01 nmol/L and 0.39 ± 0.07 nmol/L; p<0.0001 for both comparisons), which were statistically indistinguishable (p=0.12) (Figure 4c). During the OGTT, plasma insulin levels only slightly increased for lean rats peaking at 0.3 ± 0.1 nmol/L by 15 minutes and then steadily declined to 0.1 ± 0.02 nmol/L by 120 minutes. For obese rats, plasma insulin levels peaked at 1.9 ± 0.4 nmol/L by 30 minutes and remained elevated at 1.6 ± 0.3 mg/dL by 60 minutes before gradually declining to 0.9 ± 0.1 nmol/L by 120 minutes (Figure 4c). Again, the plasma insulin curve for RYGB rats during the OGTT was qualitatively different from both lean and obese rats with plasma insulin levels peaking at 2.6 ± 0.3 nmol/L by 15 minutes but remaining elevated at 2.4 ± 0.3 nmol/L by 60 minutes before rapidly declining to near baseline levels of 0.48 ± 0.07 nmol/L by 120 minutes (Figure 4c). This is possibly due to the increased glucose-induced release of GLP-1, which is also an incretin, in RYGB rats. The associated area under the curve analysis suggests that RYGB rats might have improved insulin sensitivity compared with obese rats since their integrated plasma insulin levels throughout the OGTT were similar (Figure 4d) despite having markedly lower integrated blood glucose levels (Figure 4b). However, while HOMA-IR indices, as an indicator of insulin resistanace [65], were normalized in RYGB rats (Figure 4e), ISI-M indices, as an indicator of insulin sensitivity [66], were significantly higher for lean rats compared with both obese and RYGB rats (p<0.0001 for both comparisons), which were statistically indistinguishable from each other (p=0.67) (Figure 4f).

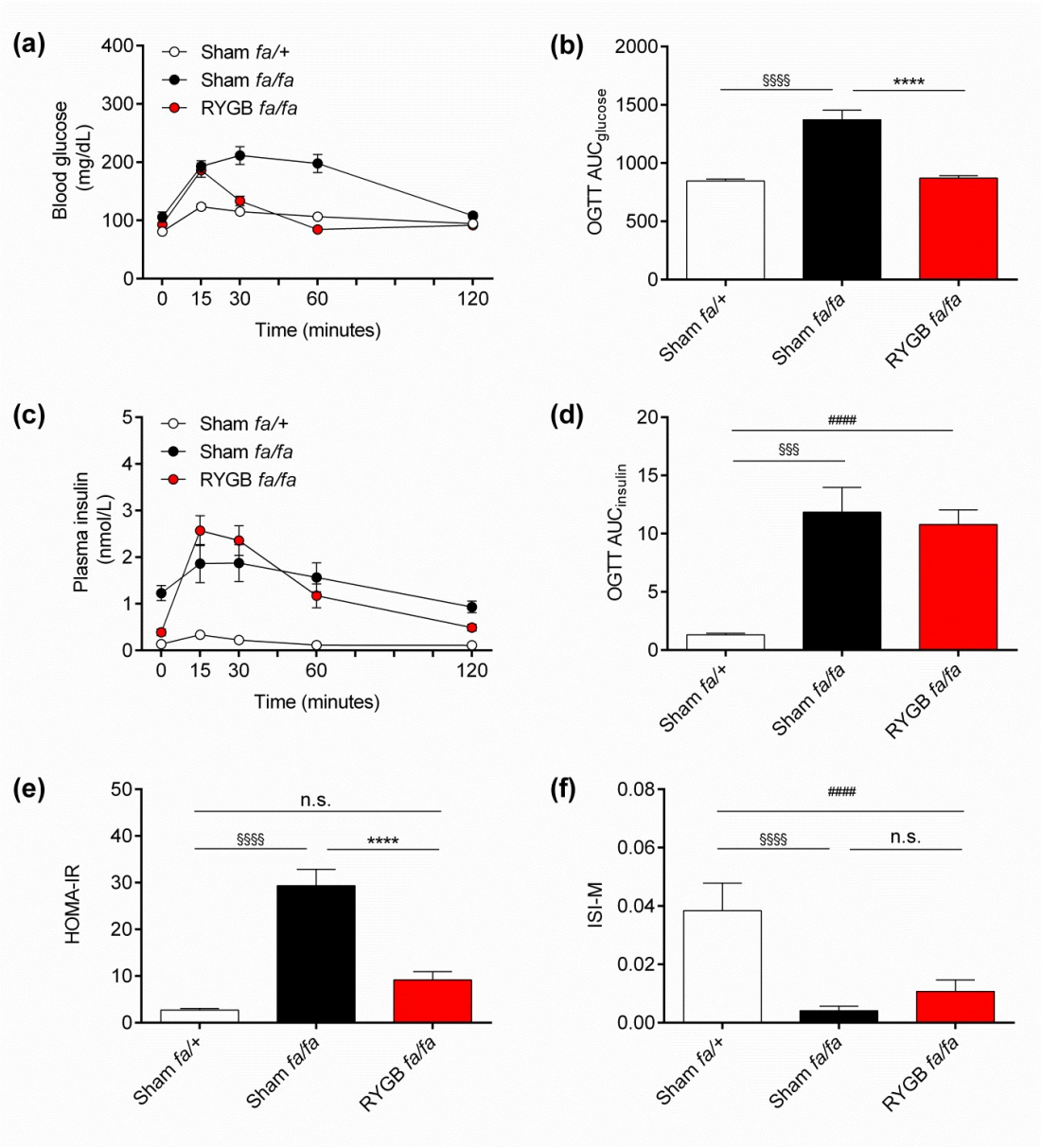

Figure 4. Intact leptin receptor signalling is not required for the improved glycemic control induced by RYGB

(**a**) Blood glucose, (**b**) the associated area under the curve (AUC), (**c**) plasma insulin and (**d**) the associated AUC during an oral glucose tolerance test in sham-operated *fa*/+ rats (n=12), sham-operated *fa*/*fa* rats (n=12) and RYGB-operated *fa*/*fa* rats (n=16) at postoperative day 27. (**e**) HOMA-IR and (**f**) ISI-M indices calculated from the rats in (**a-d**). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Sidak post-hoc test. §§§§p<0.0001 and §§§p<0.001 for sham-operated *fa*/+ vs. sham-operated *fa*/*fa* rats, ****p<0.0001 for RYGB-operated *fa*/*fa* vs. sham-operated *fa*/*fa* rats and ####p<0.0001 for RYGB-operated *fa*/*fa* vs. sham-operated *fa*/+ rats.

4. Discussion

Zucker Fatty *fa*/*fa* rats harbor an autosomal recessive point mutation in the leptin receptor gene that negatively affects the extracellular domain common to all leptin receptor subtypes (a-f) [35-37], making them an established genetic model of leptin receptor deficiency. We formally assessed using these rats if enhanced endogenous leptin action is required for the sustained weight loss and food intake suppression as well as the improved glycemic control characteristic of RYGB. We found that RYGB-operated Zucker *fa*/*fa* rats had a 9.7 % reduction in body weight that was stabilized at -6.5 % by the end of the 27 day monitoring period, and that food intake at this late time-point was 33.9 % lower than that of sham-operated counterparts, normalizing to that of sham-operated Zucker Fatty *fa*/+ rats. By incorporating this important lean control group, we could further show that RYGB normalizes oral glucose tolerance independently of leptin receptor signalling.

The first studies aimed at assessing the necessity of leptin in the positive outcomes of RYGB on energy and glucose homeostasis utilized leptin-deficient *ob*/*ob* mice [30,31]. Our findings contrast with those of Hao et al [30] who found that RYGB failed to induce sustained weight loss and food intake suppression as well as improved insulin sensitivity during the first 6 postoperative weeks in *ob*/*ob* mice. Our findings do, however, align with those of Mokadem et al [31] who found that RYGB induced sustained weight loss of 25-33% in *ob*/*ob* mice by the sixth postoperative week, and reduced average food intake by 23%, although unlike in their study, we could show that bodyweights of RYGB-operated animals converged with that of lean controls. Another difference with Mokadem et al [31] is that RYGB failed to improve oral glucose tolerance in *ob*/*ob* mice to the level of lean wild-type mice whereas we could show normalized oral glucose tolerance in our RYGB-operated rats. The reasons for these discrepancies are unclear, but could be due to species differences or the degree of diminished leptin action between *ob*/*ob* mouse (absolute) and Zucker Fatty *fa*/*fa* rat (severely diminished) models. We do note, however, that while Zucker Fatty *fa*/*fa* rats reduce food intake upon central leptin administration at pharmacological doses [40,41], they fail to do so to peripherally administered leptin [41]. Therefore, it is unlikely that physiologically relevant (peripherally-derived) leptin action could be enhanced by RYGB in Zucker Fatty *fa*/*fa* rats. In this regard, our findings align with those from a study in leptin-unresponsive *db*/*db* mice, which lack functional leptin b receptors due to an autosomal recessive point mutation in the leptin receptor gene [11], in which the sustained weight loss induced by RYGB was preserved [33].

Because RYGB is technically more feasible to execute in rats than in mice, numerous studies have been performed aimed at assessing its metabolic effects in Zucker Fatty *fa*/*fa* [44-48] and Zucker Diabetic Fatty *fa*/*fa* [24, 49-64] rats. These studies, however, like those in *db*/*db* mice [33,34], were not directly aimed at assessing the necessity of leptin receptors on the sustained weight loss and food intake suppression as well as the improved glycemic control induced by RYGB, which is most likely why their details of food intake were either generally incomplete [44-51,54,61,62,64] or entirely missing [24,33,34,52,53,55-60,63], and their study conclusions were unrelated to leptin. Our findings contrast with the studies showing a lack of sustained weight loss induced by RYGB [24,48,51,54,57,58,61,63], but are in line with the majority that do [45-47,49,50,52,55,59,62,64]. Further, our findings contrast with the study showing a lack of sustained food intake suppression induced by RYGB [48], but are consistent with those showing suppression of average food intake over the postoperative monitoring period [46,49,50,54,61,62,64]. Our study therefore extends previous work by showing a clear normalization of food intake induced by RYGB that is sustained at a late postoperative time-point when body weight is also normalized by the procedure.

With regards to improvements in glucose homeostasis induced by RYGB, we found a reduction of plasma insulin levels and HOMA-IR indices which is consistent with the studies on Zucker Fatty *fa*/*fa* or Zucker Diabetic Fatty *fa*/*fa* rats showing a reduction in blood glucose and/or plasma insulin levels [24,45,47-52,54,55,59,62,64]. Further, the improved oral glucose tolerance in our RYGB-operated rats is in line with previous studies in which it was evaluated [45,46,57,62]. However, while ISI-M, as an indicator of insulin sensitivity [*63*], was not higher in RYGB-operated

compared with sham-operated Zucker Fatty *fa/fa* rats, a previous study on Zucker Diabetic Fatty *fa/fa* rats using hyperinsulinaemic-hyperglyecamic clamp showed that RYGB increases peripheral insulin sensitivity [57]. The discrepancies between rat studies can possibly be attributed to the differences in postoperative diets employed and monitoring periods as well as the RYGB model, which has varied considerably between laboratories since their inception [69].

If leptin is not an essential mediator of the sustained weight loss, food intake suppression and improved glycemic control induced by RYGB, then additional circulating and/or neural factors may be involved. We indeed confirmed that plasma levels of the anorexigenic and incretin gut hormone GLP-1 are increased by RYGB and could show that it negatively correlated with food intake and body weight at the time-point it was measured. However, numerous studies have shown that, like leptin, GLP-1 is also not an essential mediator of the sustained weight loss and food intake suppression as well as the improved glycemic control induced by RYGB [70-74]. Therefore, other hormones and/or gut-derived microbiota products could be involved in mediating these cardinal metabolic benefits, which will be an important line of future investigation. Notably, Zucker Fatty *fa/fa* or Zucker Diabetic Fatty *fa/fa* rats may be the ideal model for such investigations, as food intake suppression in diet-induced obese mice and rats tends to diminish [4,5,50,71,75,76] or are even absent [30,61,74] after RYGB.

Strengths of our study include the well-powered group sizes allowing for robust statistical comparisons to be performed, as well as the incorporation of a lean control group. Another study strength is the detailed reporting of food intake absent in previous studies with Zucker Fatty *fa/fa* or Zucker Fatty Diabetic *fa/fa* rats. A limitation of our study is that despite achieving the degree of food intake suppression typically observed in the laboratory setting in patients after RYGB [73], the 30-40% weight loss characteristic of the procedure [77] wasn't. However, when factoring in the rapid weight gain of sham-operated Zucker Fatty *fa/fa* rats, RYGB-operated rats weighed 25.5 ± 2.2 % less which resembles the clinical outcome. Also, we did not directly compare the effects of RYGB on energy and glucose homeostasis with a diet-induced obese group in which endogenous leptin action could be restored or enhanced as with the previous studies utilizing *ob/ob* mice [30,31]. Indeed, average daily food intake and hypothalamic leptin receptor signaling is normalized by RYGB in diet-induced obese Sprague Dawley rats, associated with normalized hypothalamic levels of protein tyrosine phosphatase 1B [78], a major inducer of central leptin resistance [12]. Nevertheless, we recently showed in diet-induced obese Wistar rats under identical housing and surgical conditions as the rats in the present study, a similar degree of weight loss maintenance (-6.1 ± 1.3 %) induced by RYGB as well as lower body weight compared with sham-operated counterparts (-23.7 ± 2.1 %) [75]. Further, others have shown a similar 13% weight loss 11 days after RYGB in diet-induced obese Sprague Dawley rats compared with Zucker Diabetic Fatty *fa/fa* rats [50], as well as similar body weight trajectories over a lengthier 4 week monitoring period [51,61], also suggesting a redundant role for leptin in the effects of RYGB on energy homeostasis. Interestingly, while one clinical study showed that sensitivity to exogenous leptin (as determined by changes in body weight over 8 weeks of treatment) is not restored by RYGB at 18 months postoperatively [79], another study showed that endogenous leptin sensitivity (as determined by serum IGF-binding protein 2/leptin ratios) is eventually normalized at 12 months postoperatively [80]. Future studies with leptin antibodies or a leptin receptor antagonist will be needed to determine the causal role of enhanced leptin action in mediating the distinct metabolic benefits induced by RYGB in the clinical setting.

In summary, we have presented further evidence against leptin being an essential mediator of the sustained weight loss, appetite suppression and improved glycemic control induced by RYGB, which instead points to additional circulating and/or neural factors. Our findings are thus consistent with the majority of previous studies in Zucker Fatty *fa/fa* and Zucker Diabetic Fatty *fa/fa* rats as models of leptin receptor deficiency [45-47,49,50,52,55,59,62,64], and place their findings in a new light.

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