Shi et al.: *Obestity* 19(11): 2137-2148, 2011

Peripheral-Specific Y2 Receptor Knockdown Protects Mice From High-Fat Diet–Induced Obesity

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Abstract

Y2 receptors, particularly those in the brain, have been implicated in neuropeptide Y (NPY)-mediated effects on energy homeostasis and bone mass. Recent evidence also indicates a role for Y2 receptors in peripheral tissues in this process by promoting adipose tissue accretion; however their effects on energy balance remain unclear. Here, we show that adult-onset conditional knockdown of Y2 receptors predominantly in peripheral tissues results in protection against diet-induced obesity accompanied by significantly reduced weight gain, marked reduction in adiposity and improvements in glucose tolerance without any adverse effect on lean mass or bone. These changes occur in association with significant increases in energy expenditure, respiratory exchange ratio, and physical activity and despite concurrent hyperphagia. On a chow diet, knockdown of peripheral Y2 receptors results in increased respiratory exchange ratio and physical activity with no effect on lean or bone mass, but decreases energy expenditure without effecting body weight or food intake. These results suggest that peripheral Y2 receptor signaling is critical in the regulation of oxidative fuel selection and physical activity and protects against the diet-induced obesity. The lack of effects on bone mass seen in this model further indicates that bone mass is primarily controlled by non-peripheral Y2 receptors. This study provides evidence that novel drugs that target peripheral rather than central Y2 receptors could provide benefits for the treatment of obesity and glucose intolerance without adverse effects on lean and bone mass, with the additional benefit of avoiding side effects often associated with pharmaceuticals that act on the central nervous system.

Introduction

Obesity, the consequence of long-term imbalance between energy intake and energy expenditure, has reached epidemic proportions and causes serious public health problems worldwide. It is a major risk factor for type 2 diabetes, cardiovascular problems and some forms of cancer (World Health Organization). Intense efforts have been put forward to improve our understanding of the molecular mechanisms regulating energy balance. However, due to its complexity, the exact mechanisms contributing to obesity remain largely elusive.

Neuropeptide Y (NPY), a 36-amino acid peptide, is widely expressed in the central and peripheral nervous systems and is an important regulator of numerous physiological processes, including energy balance (**1**,**2**). NPY is known to be a strong stimulus for food intake and body weight gain (**3**,**4**), but it also induces many neuroendocrine and metabolic changes that favor energy storage (**5**,**6**,**7**,**8**,**9**). For instance, intracerebroventricular or intrahypothalamic administration of NPY to normal rodents leads to accelerated body weight gain, massive hyperphagia, hyperleptinemia, hypercorticosteronemia, hyperinsulinemia, increased adiposity, decrease body temperature, and reduced thermogenetic capacity in brown adipose tissue (BAT (**5**,**6**,**7**,**8**,**9**)). More importantly, all of these neuroendocrine and metabolic effects of central NPY administration, notably the increased adiposity, persist even when NPY-induced hyperphagia is prevented by pair-feeding, indicating that hyperphagia is not the only mechanism by which central NPY increases adiposity (**9**,**10**).

NPY mediates its effects via G protein-coupled receptors, of which five receptors have been cloned to date: Y1, Y2, Y4, Y5, and Y6 (**11**), each showing varying distributions across central and peripheral tissues. There is clear evidence that Y2 receptors are involved in the regulation of energy homeostasis. Germline deletion of Y2 receptors leads to significant reductions in adiposity or body weight as well as attenuation of the type 2 diabetic syndrome of

ob/ob mice, in the absence of reductions in food intake (**12**,**13**). Moreover, germline Y2 receptor knockout mice gained significantly less body weight when exposed to a high-fat diet (**14**). Y2 receptors are widely expressed in central and peripheral tissues, including white and BAT and pancreas (**15**). However, compared to their central counterparts, little is known about the role of peripheral Y2 receptors in the regulation of energy homeostasis. It is important to note that peripheral Y1 receptor signalling has recently been shown to be a critical regulator of fat mass and lipid oxidation (**16**). It is thus possible that peripheral Y2 receptors could also have a potential role in the control of energy homeostasis, as indicated in a study showing that NPY acts directly on adipose tissue through Y2 receptors and mediates stress-induced obesity and the metabolic syndrome (**17**). However, the role of Y2 receptors in other tissues, particularly the liver, BAT and skeletal muscle, in the regulation of energy balance and metabolism remains unknown.

In addition to effects on energy metabolism, germline Y2 receptor deficiency in mice has also been demonstrated to increase cortical and trabecular bone mass, notably due to the deletion of hypothalamic Y2 receptors, since adult-onset selective deletion of hypothalamic Y2 receptors results in a similar bone anabolic phenotype to that seen in mice with germline Y2 receptor deletion (**18**). Moreover, hypothalamic NPY neuron-specific Y2 receptor deletion leads to increased adiposity and a slight but significant increase in trabecular bone volume, but not of cortical bone volume (**19**), indicating that Y2 receptors on other hypothalamic neurons besides those expressing NPY are involved in regulating bone. It is also possible that peripheral Y2 receptors may play a role in the regulation of bone mass, particularly since peripheral Y1 receptors have been shown to be involved in this process (**20**), but little is known about the function of peripheral Y2 receptors in the regulation of bone homeostasis.

Given the clear involvement of Y2 receptors—particularly those expressed in the central nervous system—in the regulation of energy balance and bone accretion, it is of critical importance to understand the potential roles of peripheral Y2 receptors in these processes to provide specific evidence for the development of novel antiobesity or antiosteoporotic drugs based on strategies targeting different systems. To this end, we generated a conditional tissue-specific mouse model: Mx1Cre;Y2lox/lox, in which Y2 receptor expression was knocked down predominantly in peripheral tissues of adult mice with little impact on central Y2 receptor expression and—due to the adult-onset inducibility of gene deletion—with little interference from development effects of gene deletion. Because of the limited efficacy and/or safety concerns of currently available antiobesity drugs, the results from the present study offers exciting possibilities for developing specific and effective pharmocological agents for obesity that reduce body weight and blood glucose without the safety concerns of agents that act on the central nervous system.

Methods and Procedures

Animals

Animal experiments were approved by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee (Ethics No. HH #08/01) and were conducted in accordance with relevant guidelines and regulations. Mice were housed under conditions of controlled temperature (22 °C) with a 12-h light, 12-h dark cycle (lights on at 0700 h). Mice were fed a normal chow diet *ad libitum* (6% calories from fat, 23% calories from protein, 66% calories from carbohydrate, 5% calories from crude fiber, 2.6 kcal/kg; Gordon's Speciality Stock Feeds, Yanderra, Australia) unless otherwise stated. A subset of conditional Y2 receptor knockdown and control mice at 8 weeks of age was fed a high-fat diet (23% calories from fat, 19.4% calories from protein, 48.2% calories from carbohydrate, 4.7% calories from crude fiber, 4.7% calories from acid detergent fiber, 4.78 kcal/kg; Gordon's Specialty Feeds, Glen Forrest, Australia). Water was available *ad libitum*. Generation of the conditional Y2lox/lox mouse, in which the entire coding sequence of the Y2 receptor gene is flanked by LoxP sites ensuring that not even truncated versions of the receptor can be produced after cre-recombinase treatment, was previously described (**16**,**21**).

Generation of the peripheral conditional Y2 receptor knockdown mouse model

All mice used in these studies were on a mixed C57Bl6/129SVJ background. Y2 receptor peripheral conditional knockdown mice were generated by crossing Y2lox/lox mice onto transgenic mice expressing Cre-recombinase under the control of the interferon-α–responsive Mx1 promoter. This Mx1-activated Cre-mediated gene deletion, induced by intraperitoneal injection of polyinosinic-polycytidylic acid (Poly I:C), has been shown to be partial and to

preferentially target tissues in the periphery, with almost no effect on gene expression in the brain (**16**,**22**). In our model, deletion of the Y2 receptor gene was induced in mice at 12 weeks of age via intraperitoneal injection of Poly I:C (300 μg/injection; Sigma-Aldrich. St Louis, MO) three times at 2-day intervals as previously described (**16**,**22**). Commencement of induction of gene deletion was defined as the day of the first Poly I:C injection.

RNA extraction and quantitative real-time PCR

At 8 weeks after commencement of induction of gene deletion, various tissues and organs (the hypothalamic region of the brain, liver, skeletal muscle (quadriceps femoris), spleen, pancreas, epididymal white adipose tissue (WAT), inguinal WAT, retroperitoneal WAT, mesenteric WAT and BAT) from saline-injected control and Poly I:C-injected Mx1Cre;Y2lox/lox mice fed on either a chow diet or high-fat diet were collected and total RNA was isolated using Trizol Reagent (Sigma, St Louis, MO) following the manufacturer's protocol. The quality and concentration of total RNA was measured by a spectrophotometer (Nanodrop 1000; NanoDrop Technologies, Thermo Scientific, Wilmington, DE). One microgram of total RNA was reverse transcribed into complementary DNA using Superscript III First-Strand Synthesis System (Invitrogen, Mount Waverley, Australia). Quantitative real-time PCR using primers for the Y2 receptor gene (Forward: 5′-caccaaatcggacctgct-3′, Reverse: 5′-agaaccagttcactctcac ttgg-3′) was carried out on a LightCycler (Light-Cycler 480 Real-Time PCR system; Roche Diagnostic, Rotkreus, Switzerland) using SensiMix Probe (Bioline (Aust) Pty Ltd, Alexandria, Australia) following the manufacturer's instructions. Expression of a housekeeping gene, ribosomal protein L19 (RPL19), was carried out in the same manner and was used to normalize mRNA expression level of the Y2 receptor.

Determination of food intake and body weight

Male mice (8–12 per group) were housed individually throughout the whole experiment and body weight was measured twice a week at the same time of day. At 12 weeks of age, spontaneous daily food intake was measured over 3 consecutive days. Actual food intake was calculated as the weight of pellets taken from the food hopper minus the weight of food spilled in the cage. The weight of spilled food per day was determined as the 24-h increase in weight of the cage bedding, after removing all feces and air-drying to eliminate weight changes due to urine and water bottle drips. Repeated measures ANOVA was carried out. At 13 weeks of age, fasting-induced food intake was carried out after fasting for 24 h at 0900 h, then determining food intake as described above at 2, 8, 24, 48, and 72 h after reintroduction of food. Food intake was also measured at 4 h after refeeding in high fat-fed mice. Body weight was tracked at the same time each day before and up to 72 h after the 24-h fast.

Rectal temperature measurements

At 14–15 weeks of age, body temperature was measured at 0830–0900 h with a rectal thermometer (Physitemp Instruments, Clifton, NJ). Temperature readings were taken within 10 s of removing the mouse from its cage. Repeat readings were taken from each mouse on 3 consecutive days and analyzed by repeated measures ANOVA as described below.

Glucose and insulin tolerance tests

At 17 weeks of age, mice were fasted for 16 h before intraperitoneal injection of a 10% D-glucose solution (1.0 g/kg body weight). Blood samples were obtained from the tail tip at the indicated times, and glucose levels were measured using a glucometer (AccuCheck II; Roche, Castle Hill, Australia). Insulin tolerance test was carried out to determine insulin-induced hypoglycemia in mice at 18 weeks of age. Briefly, the mice were injected with insulin (1 IU/kg body weight) intraperitoneally to induce hypoglycemia after being fasted for 6 h (starting time point was regarded as 0), then blood samples were collected from the tail tip at the indicated times, glucose levels were measured using a glucometer as shown in the results.

Indirect calorimetry studies

Oxygen consumption rate (VO_2) and carbon dioxide output (VCO_2) were measured using an open circuit eight-chamber indirect calorimeter (Oxymax series; Columbus Instruments, Columbus, OH) with airflow of 0.6 l/min. Studies were commenced after 24 h of acclimatization to the metabolic chamber (20 × 10 × 12.5 cm). VO_2 and VCO_2 were measured in individual mice at 27-min intervals over a 24-h period under a consistent environmental temperature (22 °C). The respiratory exchange ratio (RER) was calculated as the quotient of VCO_2/VO_2, with pure carbohydrate oxidation resulting in a value of 1 and pure fat oxidation resulting in a value of 0.7

(**23**,**24**). Energy expenditure was calculated as calorific value (CV) × *V*O2, where CV is 3.815 + 1.232 × RER (**25**). During the calorimetry study, mice had *ad libitum* access to food and water.

Determination of physical activity
During calorimetry, relative physical activity of individually-housed mice over 24 h was evaluated using an OPTO-M3 sensor system from Columbus Instruments (Oxymax Series; Columbus Instruments). Ambulatory counts were recorded as sequential beam breaks on an infrared grid. Cumulative ambulatory counts of X, Y and Z directions were summed for 1-h intervals.

Bone densitometry and body composition analysis
Whole body bone mineral content, bone mineral density, fat and lean mass were measured in isoflurane-anesthetized mice, ventral side down, at 15 weeks of age using dual-energy X-ray absorptiometry (DXA, Lunar PIXImus2 mouse densitometer; GE Medical Systems, Madison, WI) using an analysis program provided by the manufacturer. The head and the tail of the animal were excluded from the analysis of body composition. Whole femoral bone mineral content and bone mineral density were also measured in excised left femora. Femora were scanned with tibiae attached and the knee joint in flexion to ninety degrees to ensure consistent placement and scan of the sagittal profile.

Tissue collection and analysis
At the completion of the study, mice of 19–20 weeks of age were killed by cervical dislocation between 1200–1500 h, trunk blood was collected, allowed to clot at room temperature, centrifuged, and the resultant serum were stored at −20 °C for subsequent analysis. Brains were removed and immediately frozen on dry ice. WAT depots (right inguinal, right retroperitoneal, right epididymal, and mesenteric), BAT, pancreas, liver, spleen, kidney, heart and testis were collected, weighed, and some tissues (brain, epididymal WAT, inguinal WAT, retroperitoneal WAT, mesenteric WAT, liver, spleen, pancreas and BAT as well as a sample of quadriceps femoris skeletal muscle) were stored at −80 °C for further analysis. The weights of WAT depots were summed together and expressed as summed WAT weight.

Statistical analysis
One- or two-way ANOVA or repeated measures ANOVA was used to determine the significance of treatment effects and interactions (GraphPad Prism 5, version 5.0a; GraphPad Software, San Diego, CA). When there was a significant overall effect or interaction effect, Bonferroni *post hoc* tests were performed to identify differences among means. For all statistical analyses, a P value <0.05 was considered to be statistically significant.

Results

Peripheral Y2 receptor gene knockdown
Peripheral-specific Y2 receptor knockdown was achieved with a mouse model in which conditional deletion of the floxed gene was induced in the adult animal by intraperitoneal injection of Poly I:C, which activates the interferon-α–responsive Mx1 promoter to drive Cre-recombinase expression predominantly in peripheral tissues (**22**). To determine the efficiency and specificity of Y2 receptor gene deletion, RNA was isolated from various peripheral tissues and the hypothalamic region of the brain and subjected to quantitative real-time PCR analysis. Poly I:C-injection resulted in a marked and significant reduction of Y2 mRNA expression in epididymal WAT, liver, spleen and skeletal muscle relative to the expression levels seen in saline-injected control mice when fed with a chow diet (**Figure 1a**). There is no detectable Y2 mRNA in inguinal WAT, retroperitoneal WAT or mesenteric WAT (**Figure 1b**) from either saline-or poly I:C-injected mice fed with a chow diet, consistent with a previous study showing that Y2 receptor expression was not detected in subcutaneous fat in mice fed on a chow diet (**17**). In contrast, no significant change in Y2 mRNA expression was observed neither in the hypothalamic region of the brain nor in BAT of Poly I:C-injected Y2 receptor knockdown mice compared to that of saline-injected control mice, in keeping with a previous study showing poor Mx1-activated Cre-mediated gene deletion in the brain (**16**,**22**). Interestingly, there was no detectable Y2 receptor mRNA expression in pancreas from either saline- or Poly I:C-injected mice, consistent with previous report showing lack of Y2 receptor expression in this tissue (**26**). Interestingly, when

fed on a high-fat diet, Y2 mRNA expression in liver and spleen was downregulated to undetectable levels in both saline- and poly IC-injected mice, while expression of Y2mRNA in the brain and muscle remained present (**Figure 1c**). Furthermore, Poly I:C treatment effectively reduced Y2 mRNA expression to undetectable levels in all WAT depots studied (**Figure 1c,d**). These data confirm that effective Y2 receptor knockdown occurs predominantly in peripheral tissues relative to the brain, both under chow- and high fat-fed conditions. More importantly, the effectiveness of this strategy for conditional Y2 receptor knockdown in peripheral tissues was demonstrated by the induction of significant physiological effects, particularly on a high-fat diet, as described below. Poly I:C was injected into wild types as well as into Mx1Cre;Y2lox/lox mice to control for possible effects of Poly I:C injection *per se*. There was no significant effect of Poly I:C injection on any of the parameters studied in this work (data not shown), indicating that peripheral Y2 receptor knockdown, not Poly I:C itself, is responsible for the changes observed.

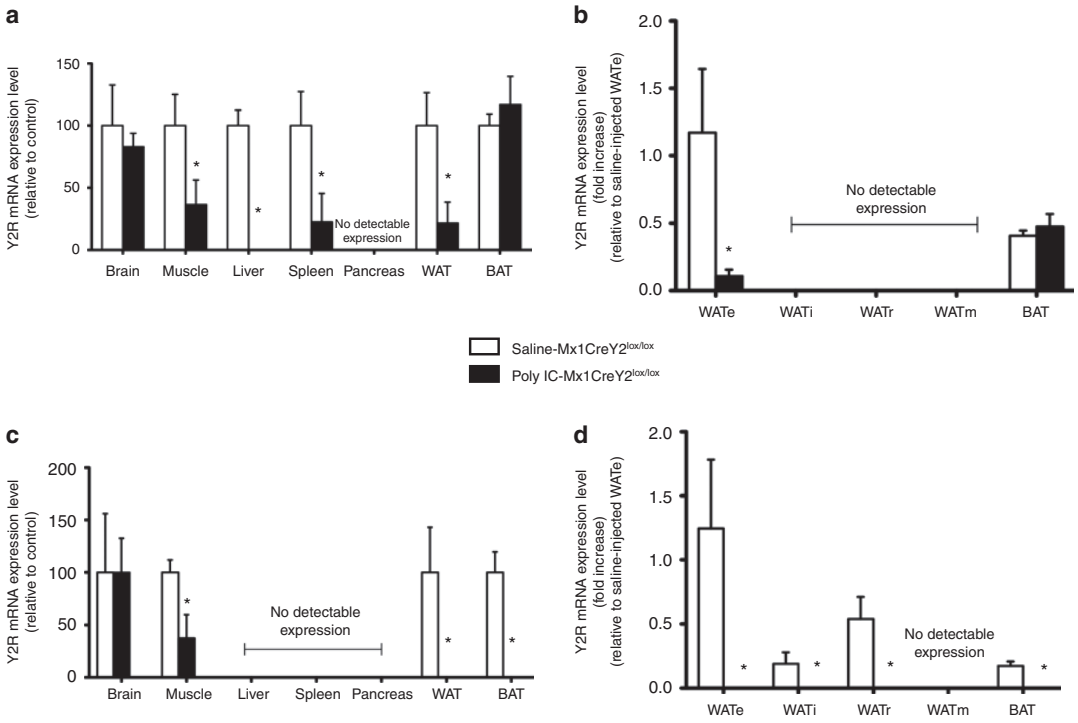

Figure 1 Confirmation of Y2 receptor knockdown in various peripheral tissues from saline- or polyinosinic-polycytidylic acid (Poly I:C)-injected mice fed on a chow diet or high-fat diet. Y2 receptor mRNA expression levels in the hypothalamic region of the brain, skeletal muscle, liver, spleen, pancreas and brown adipose tissue (BAT) of conditional Y2 receptor knockdown (Poly I:C-Mx1CreY2$^{lox/lox}$) vs. control mice (saline-Mx1CreY2$^{lox/lox}$, regarded as 100%) fed on (**a**) a chow diet or (**c**) a high-fat diet. Y2 mRNA expression level in various white adipose tissues relative to that in epididymal white adipose tissue (WAT) of saline-injected mice fed on (**b**) a chow diet or (**d**) a high-fat diet. Data are mean ± s.e.m. of four male mice per group. *$P < 0.05$ vs. saline-injected control mice. WATe, epididymal WAT; WATi, inguinal WAT; WATm, mesenteric WAT; WATr, retroperitoneal WAT.

Effect of conditional peripheral Y2 receptor knockdown on energy metabolism on a normal chow diet

Body weight gain over an 8-week period was not significantly different in Poly I:C-injected Mx1Cre;Y2lox/lox mice compared to saline-injected Mx1Cre;Y2lox/lox control mice (**Figure 2a**). Twenty four-hour spontaneous food intake (**Figure 2b**), food intake in response to 24-h fasting (**Figure 2c**), daily fecal output and daily water intake (data not shown) were also not significantly different in Poly I:C-injected mice compared to saline-injected mice. Both groups of mice recovered lost body weight after 24-h fasting in an equivalent manner (data not shown). Nasal-anal length was also not significantly different between Poly I:C- and saline-injected control mice (data not shown).

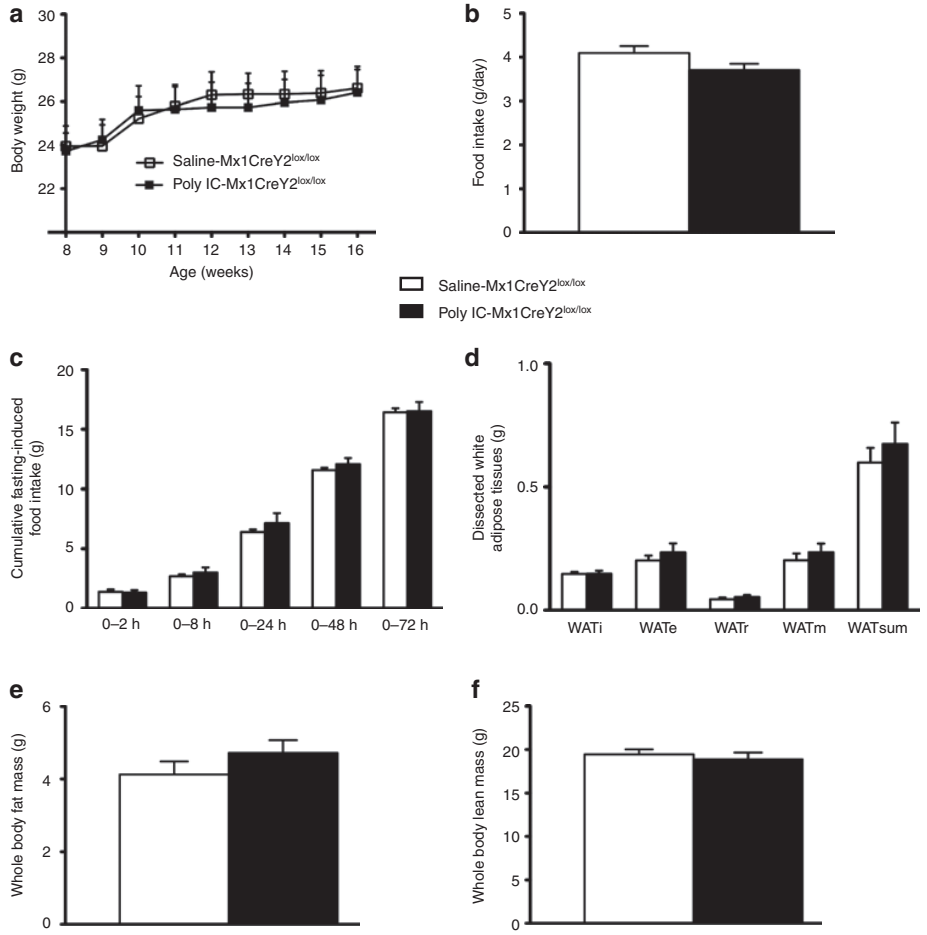

Figure 2 Effects of conditional peripheral Y2 knockdown on metabolic parameters on a normal chow diet. (**a**) Body weight, (**b**) spontaneous daily food intake, (**c**) cumulative fasting-induced food intake, (**d**) the dissected weights of each white adipose tissue (WAT) depot and the summed dissected weights of WATs, (**e**) whole body fat mass and (**f**) whole body lean mass, determined by dual-energy X-ray absorptiometry, are not altered by adult-onset peripheral Y2 receptor knockdown. Data are mean ± SEM of 8–12 male mice per group. WATe, epididymal WAT; WATi, inguinal WAT; WATm, mesenteric WAT; WATr, retroperitoneal WAT; WATsum, summed fat weights of four depots.

Effect of conditional peripheral Y2 receptor knockdown on body composition

To investigate the possibility that peripheral Y2 receptors influence body composition, Poly I:C-injected Mx1Cre;Y2lox/lox mice were compared with saline-injected Mx1Cre;Y2lox/lox control mice on a normal chow diet. The weights of dissected WAT depots and the summed weights of all depots in Poly I:C-injected mice did not differ from that of saline-injected mice (**Figure 2d**). Whole body fat mass, measured by DXA, also showed no significant difference between the two groups of mice (**Figure 2e**). Knockdown of Y2 receptors predominantly in peripheral tissues also does not affect whole body lean mass as determined by DXA and as shown in **Figure 2f**. Furthermore, the weights of other organs and tissues were unaltered in conditional Y2 receptor knockdown mice compared to wild types (BAT: 0.064 ± 0.004 g vs. 0.056 ± 0.004 g; pancreas: 0.253 ± 0.012 g vs. 0.254 ± 0.010 g; liver: 1.276 ± 0.055 g vs. 1.304 ± 0.061 g; spleen: 0.071 ± 0.006 g vs. 0.074 ± 0.008 g; kidney: 0.217 ± 0.008 g vs. 0.217 ± 0.005 g; heart: 0.120 ± 0.002 g vs. 0.127 ± 0.004 g; testis: 0.129 ± 0.003 g vs. 0.127 ± 0.004 g in knockdown vs. wild-type mice.)

Importantly, despite no observed change in body weight, food intake or body composition, oxygen consumption (**Figure 3a**), carbon dioxide production (**Figure 3b**) and energy expenditure (**Figure 3c**) in chow-fed Poly I:C-injected Mx1Cre;Y2lox/lox mice were significantly lower than those of saline-injected Mx1Cre;Y2lox/lox control mice, particularly in the light phase. Moreover, RER, an index of metabolic fuel selection, in Poly I:C-injected mice was also higher than that in saline-injected control mice, notably in the light phase (**Figure 3d**). Interestingly, peripheral Y2 receptor knockdown mice showed a marked trend towards increased physical activity relative to saline-injected control mice ($P = 0.07$, **Figure 3e**). However, rectal temperature was not different between the two groups (**Figure 3f**).

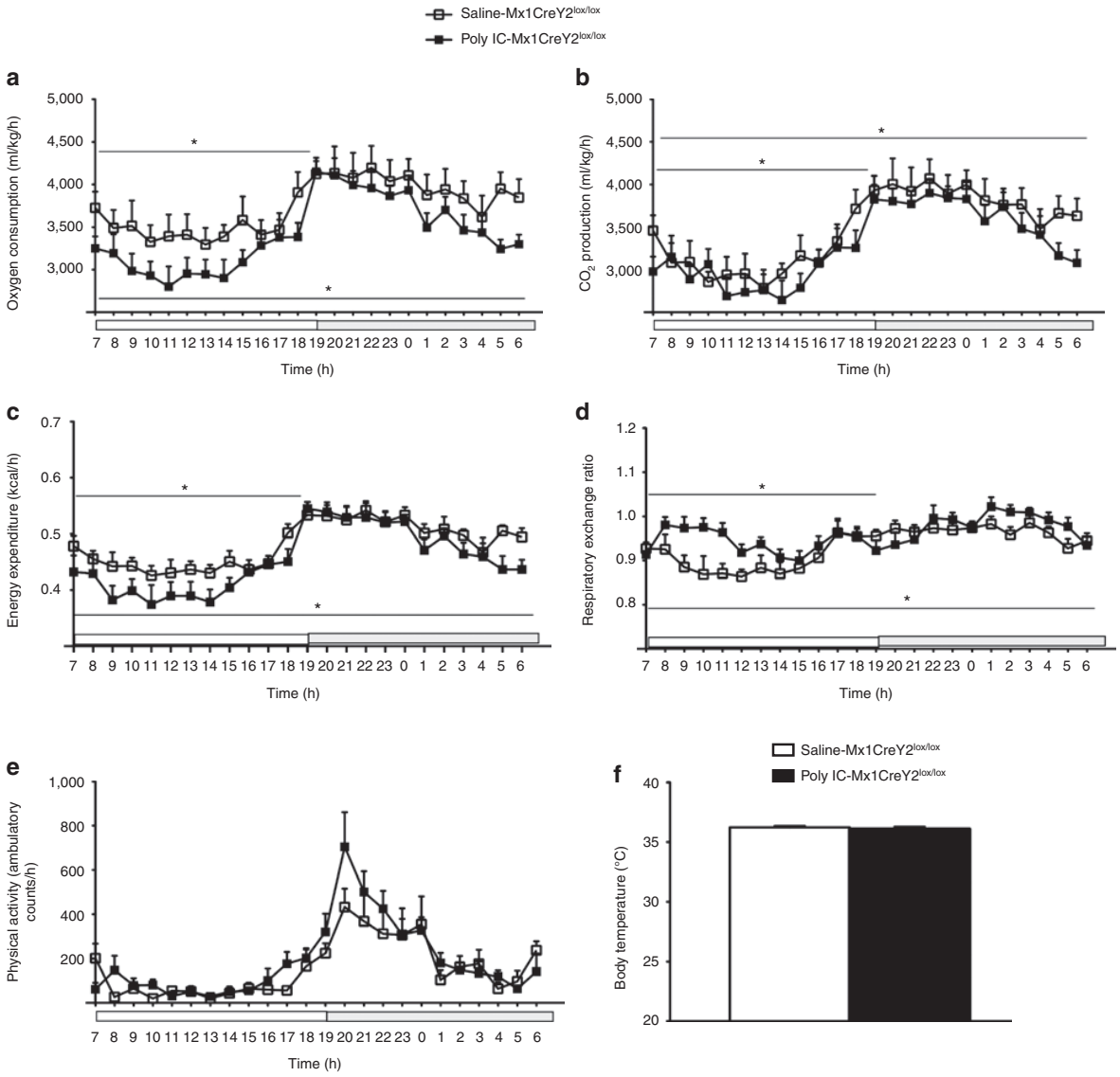

Figure 3 Effects of peripheral Y2 knockdown on energy metabolism in mice at 15 weeks of age. (**a**) O_2 consumption, (**b**) CO_2 production, (**c**) energy expenditure, (**d**) respiratory exchange ratio, and (**e**) physical activity were determined in metabolic chambers during indirect calorimetry studies. (**f**) Rectal temperature was also measured. For comparison of energy expenditure by analysis of covariates, common lean mass and fat mass were 18.87 and 4.1, respectively. Open and gray horizontal bars indicate light and dark phases, respectively. Data are mean ± SEM of 8–12 male mice per group. $^*P < 0.05$ vs. saline-injected control mice.

Effect of peripheral Y2 receptor knockdown on high-fat diet-induced obesity

To examine the effect of peripheral Y2 receptor knockdown on diet-induced obesity, we challenged male saline- and Poly I:C-injected Mx1Cre;Y2lox/lox mice with *ad libitum* access to a high-fat diet for 10 weeks. Initial body weight at 8 weeks of age was not different between experimental groups (20.89 ± 0.69 g vs. 20.67 ± 0.71 g in saline- and Poly I:C-injected Mx1Cre;Y2lox/lox mice, respectively). One week after Poly I:C injection, high-fat diet was commenced. Interestingly, mice with peripheral Y2 receptor knockdown gained significantly less weight on the high-fat diet compared to saline-injected control mice (**Figure 4a**). Intriguingly, this reduced weight gain occurred in spite of increased 24-h spontaneous food intake (**Figure 4b**). In keeping with increased food intake, daily faecal output in conditional Y2 receptor knockdown mice was significantly increased relative to saline-injected control mice (**Figure 4c**). Food intake in response to 24-h fasting in Poly I:C-injected mice was also increased compared to control mice, and the difference reached statistical significance at 72 h after refeeding (**Figure 4d**). No changes in fecal consistency, suggestive of diarrhea or other gastric perturbations, were observed. Moreover, consistent with the reduced body weight, peripheral Y2 receptor

knockdown mice exhibit reduced weight of dissected WAT depots after 10 weeks on the high-fat diet relative to saline-injected control mice, significantly so in the inguinal and retroperitoneal depots, as well as a significant decrease in the summed weight of these depots, whether expressed in absolute weight (**Figure 4e**) or as a percent of body weight (data not shown). Whole body fat mass, determined by DXA, also confirmed decreased fat level in Y2 receptor knockdown mice (**Figure 4f**) with no change in lean mass (**Figure 4g**). These data thus show that peripheral Y2 receptor knockdown reduces body weight gain and fat accretion under a high-fat diet without influencing whole body lean mass.

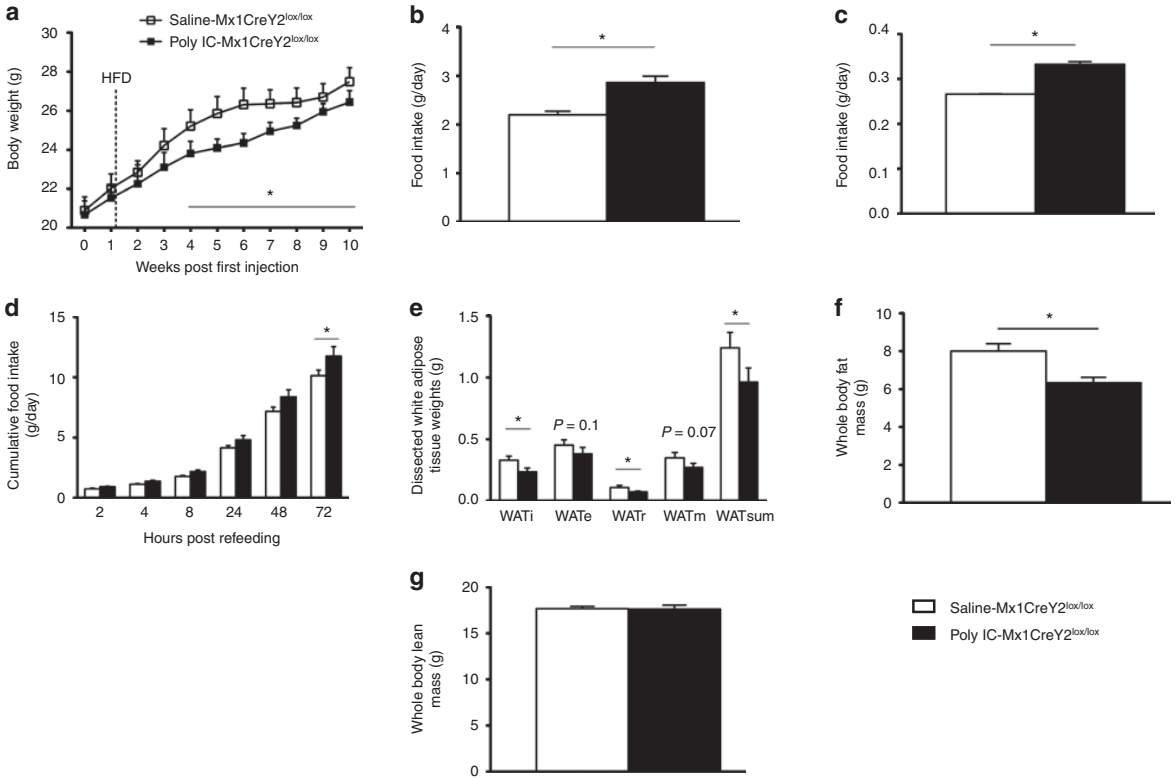

Figure 4 Reduced body weight gain and adiposity in conditional peripheral Y2 receptor knockdown mice on a high-fat diet. (**a**) Body weight, (**b**) daily food intake, (**c**) daily fecal output, (**d**) cumulative fasting-induced food intake, (**e**) dissected weights of each white adipose tissue depot and summed weights of dissected white adipose tissue depots, (**f**) whole body fat mass and (**g**) whole body lean mass, determined by dual-energy X-ray absorptiometry. Data are mean ± SEM of 8–12 male mice per group. *$P < 0.05$ vs. saline-injected control mice.

The reduced body weight and fat mass observed in peripheral Y2 receptor knockdown mice on a high-fat diet was associated with a significant increase in both oxygen consumption (**Figure 5a**) and carbon dioxide production (**Figure 5b**), notably in the dark phase, which resulted in a marked trend towards increased energy expenditure in the dark phase (**Figure 5c**). As is the case under chow-fed conditions, mice with peripheral Y2 receptor knockdown on a high-fat diet also exhibit a higher RER compared to saline-injected control mice (**Figure 5d**), indicating that peripheral Y2 signalling regulates oxidative fuel selection. Physical activity was also significantly increased during the dark phase in knockdown compared to wild-type mice (**Figure 5e**), similar to the trend observed in chow-fed animals. There is a trend toward an increase in rectal temperature in peripheral Y2 receptor knockdown mice compared to controls (**Figure 5f**), indicating increased thermogenesis.

Effect of peripheral Y2 receptor knockdown on glucose metabolism

Some factors that regulate energy homeostasis do so in association with changes in glucose metabolism, even in the absence of effects on body weight or food intake (**10**). We therefore investigated the effect of peripheral Y2 receptor knockdown on whole body glucose metabolism by examining glucose clearance during an intraperitoneal glucose tolerance test. When fed a chow diet, mice with conditional Y2 receptor knockdown did not exhibit any difference in glucose clearance after intraperitoneal glucose injection compared to control mice (**Figure 6a**),

however, when fed a high-fat diet the Y2 receptor knockdown mice displayed significantly improved glucose tolerance relative to controls (**Figure 6b**). This is consistent with the reduced adiposity seen in these animals, which has been shown to improve insulin action (**27**). In order to test whether peripheral Y2 receptor knockdown may have influenced the response to insulin, we performed insulin tolerance tests. We did not detect any difference between Poly I:C- or saline-injected Mx1Cre;Y2lox/lox mice with respect to the hypoglycemic response to intraperitoneal insulin injection when fed on normal chow diet or a high-fat diet. However, when fed on a high-fat diet, Y2 receptor knockdown mice show a more rapid restoration in serum glucose levels ($P <$ 0.05 at time = 45 min) (**Figure 6d**). Taken together, these findings suggest that peripheral Y2 receptor knockdown improves glucose tolerance and also leads to a more rapid glucose restoration in response to intraperitoneal insulin.

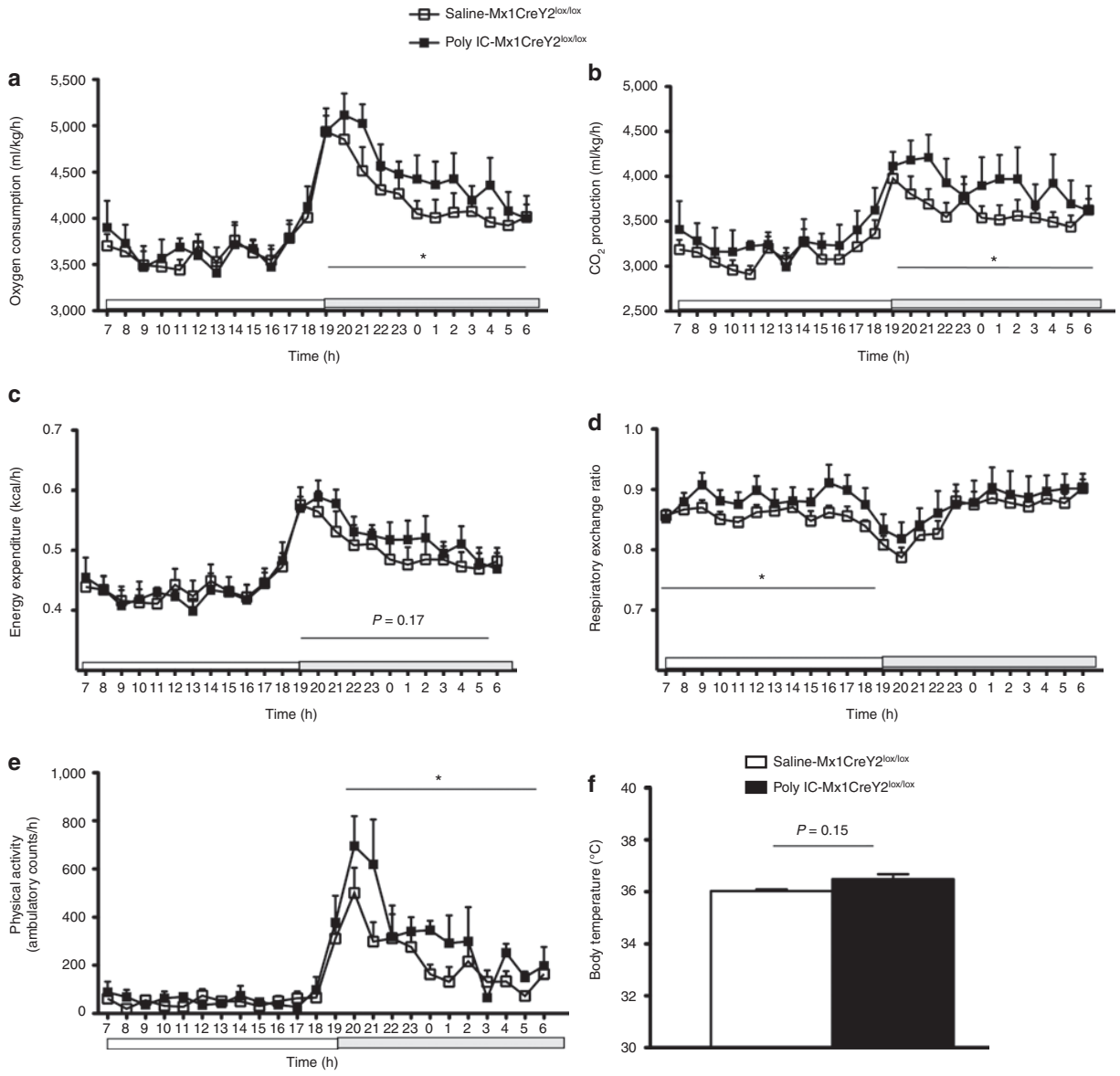

Figure 5 Effects of peripheral Y2 knockdown on energy metabolism in mice on a high-fat diet. (**a**) O_2 consumption, (**b**) CO_2 production, (**c**) energy expenditure, (**d**) respiratory exchange ratio, (**e**) physical activity were determined in metabolic chambers during indirect calorimetry studies. (**f**) Rectal temperature was also measured. For comparison of energy expenditure by analysis of covariates, common lean mass and fat mass were 17.11 and 6.34, respectively. Open and gray horizontal bars indicate light and dark phases, respectively. Data are mean ± SEM of 8–12 male mice per group. *$P < 0.05$ vs. saline-injected control mice.

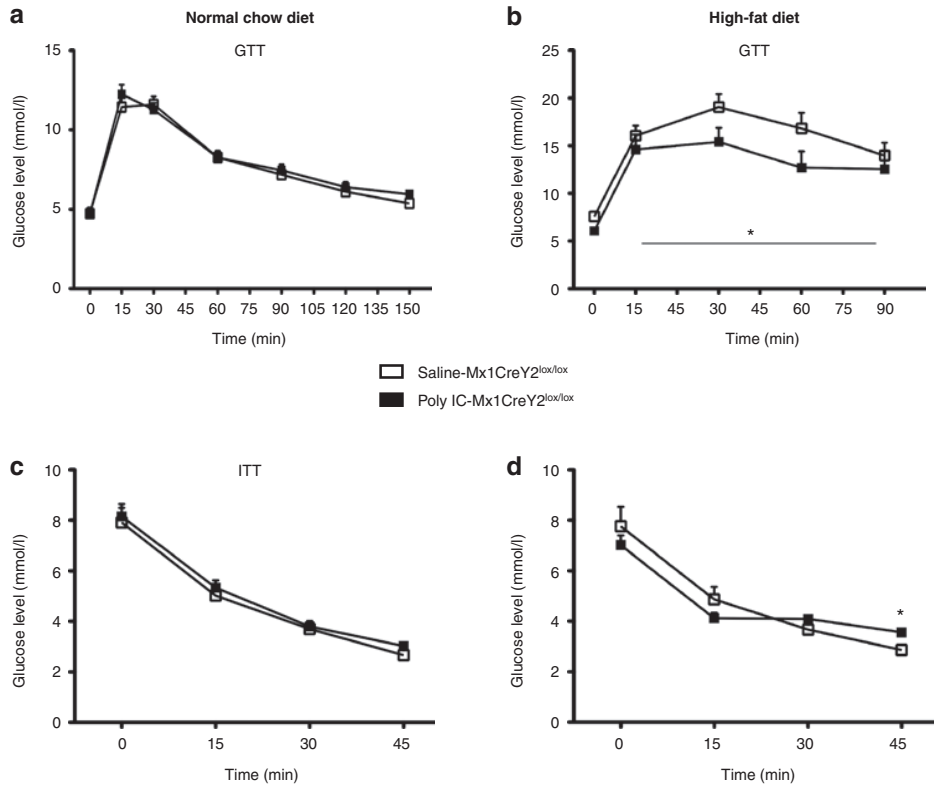

Figure 6 Effects of peripheral Y2 receptor knockdown on glucose metabolism in mice on a chow or a high-fat diet. (**a**,**b**) Glucose (1 g/kg) tolerance tests (GTTs) and (**c**,**d**) insulin (1 IU/kg) tolerance tests (ITTs) on a chow or a high-fat diet were carried out in mice at 17–18 weeks of age. Data are mean ± SEM of 5–6 male mice per group. **P* < 0.05 vs. saline-injected mice.

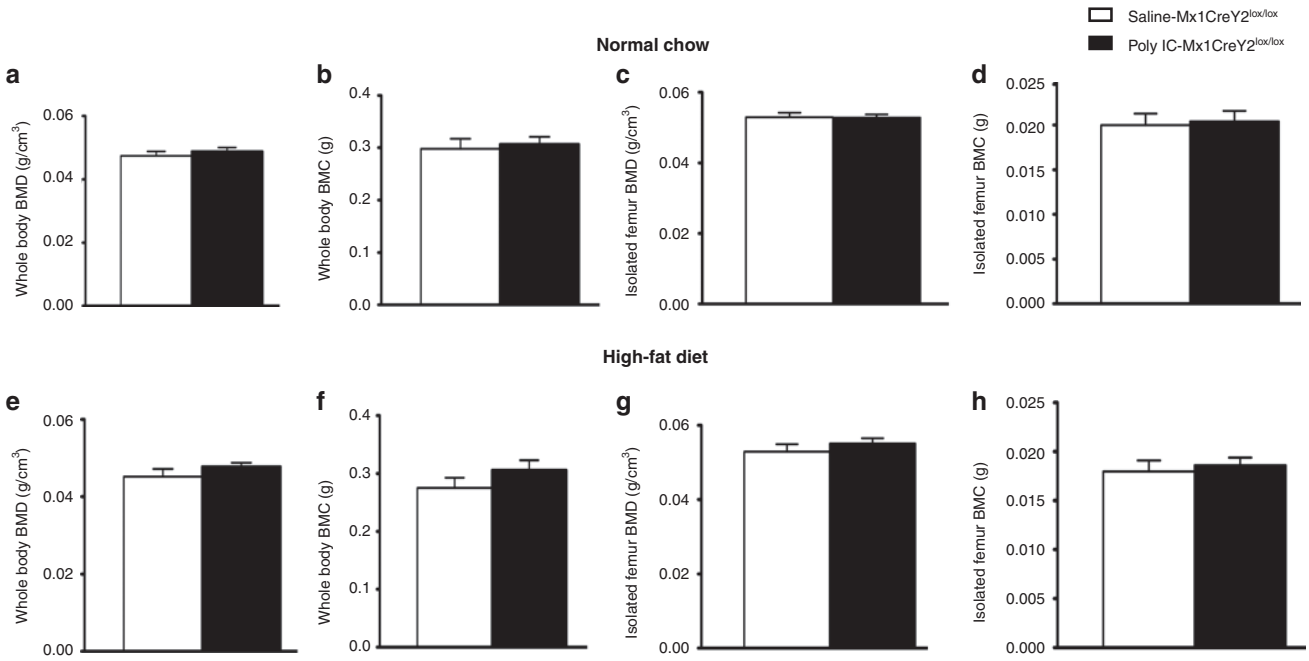

Figure 7 Unaltered bone parameters in peripheral Y2 receptor knockdown mice on a chow diet or a high-fat diet. Whole body bone mineral density (BMD), bone mineral content (BMC), isolated femur BMD and BMC were determined by dual-energy X-ray absorptiometry on (**a**–**d**) a chow diet or (**e**–**h**) a high-fat diet. Data are mean ± SEM of 8–12 male mice per group.

Effect of peripheral Y2 receptor knockdown on bone homeostasis

We previously showed that germline Y2 receptor knockout leads to pronounced anabolic effects on cortical (**28**) as well as trabecular bone mass (**18**), and that adult-onset hypothalamus-specific Y2 receptor deletion increases cortical and trabecular bone mass (**18**,**28**). Although there are no Y2 receptors being expressed on bone tissue *per se* (**29**), we wanted to investigate whether peripheral Y2 receptor knockdown in other tissues might be involved in any aspects of the anabolic bone phenotype seen in germline Y2 knockout mice. Interestingly, there was no significant effect of Y2 receptor knockdown, either on a chow (**Figure 7a-d**) or a high-fat diet (**Figure 7e-h**), with regards to whole body bone mineral density, whole body bone mineral content, isolated femur bone mineral density and isolated femur bone mineral content, determined by DXA.

Discussion

The present study demonstrates that peripheral Y2 receptors play an obesogenic role in the regulation of energy homeostasis and adiposity. Although mice with peripheral-specific Y2 receptor knockdown and fed a chow diet did not exhibit significant changes in body weight, food intake or body composition relative to controls, energy expenditure was decreased, notably in the light phase, in association with increased RER and physical activity. This suggests that peripheral Y2 receptor signalling may be directly involved in the regulation of energy expenditure, selection of oxidative fuel source and physical activity. Importantly, when fed a high-fat diet, mice with peripheral Y2 receptor knockdown gain significantly less weight and exhibit significantly reduced adiposity and improved glucose tolerance despite increased food intake relative to control mice. These results indicate that energy homeostatic regulatory mechanisms involving peripheral Y2 receptors are different under a normal chow diet and a high-fat diet, with peripherally-expressed Y2 receptor knockdown having protective effect against high-fat diet-induced obesity.

Decreased physical activity and increased RER have been shown to predict subsequent weight gain (**30**,**31**), but this is not the case in peripheral Y2 knockdown mice. This apparent discrepancy might be explained partly by increased physical activity, since it has been shown that increased physical activity *per se* can reduce adiposity in association with an actual decrease in energy expenditure (**32**). Intriguingly, we observed that chow-fed conditional knockdown mice have decreased energy expenditure whereas high fat-fed knockdown mice have increased energy expenditure relative to saline-injected wild types. The precise mechanism for this remains to be determined, but it may be due to a mosaic deletion pattern that allows for an increase in energy expenditure under conditions of a high-fat diet. Protection against obesity in the face of reduced energy expenditure in chow-fed peripheral Y2 receptor knockdown mice could be due to compensatory mechanisms by other important pathways that regulate energy balance, as well as by alteration of other Y receptors in response to down-regulation of peripheral Y2 receptors (**11**). It has been shown that germline Y2 receptor knockout mice gained significantly less weight when fed a high-fat diet (**14**). More interestingly, crossing germline Y2 receptor knockout mice with genetically obese leptin-deficient *ob/ob* mice attenuates the increased adiposity, hyperinsulinemia, hyperglycemia, and increased hypothalamo-pituitary-adrenal axis activity of *ob/ob* mice, indicating that Y2 receptors mediate obese and type 2 diabetic-like phenotypes of dietary or genetic origin in mice (**12**). It seems that peripheral Y2 receptor knockdown contributes significantly to the resistance to these obese phenotypes. Importantly from a clinical perspective, this protective effect of peripheral Y2 receptor knockdown occurred without any apparent adverse effect on lean or bone mass. Furthermore, the beneficial antiobesity effect of peripheral knockdown of Y2 receptors opens the possibility that novel Y2 antagonists that do not cross the blood brain barrier could be used as antiobesity agents. This is of particular importance given that specific deletion of hypothalamic Y2 receptors or hypothalamic NPY neuron-specific Y2 receptors has been shown to increase adiposity (**19**), possibly by preventing the satiety hormone PYY and its variant PYY3–36 from acting on Y2 receptors in hypothalamic regions such as the arcuate nucleus.

Conditional Y2 receptor knockdown in peripheral tissues had a considerable impact on glucose metabolism when mice were fed a high-fat diet. In the present study, with Y2 receptor expression having been markedly knocked down in many peripheral tissues, it is possible that the improved glucose tolerance seen in knockdown mice on a high-fat diet is the net outcome of Y2 receptor knockdown in multiple peripheral tissues, particularly skeletal muscle, liver and

WATs which are major peripheral tissues or organs involved in glucose metabolism. Y2 knockdown mice on a high-fat diet exhibited a more rapid restoration of serum glucose levels after intraperitoneal injection of insulin. It is thus possible that conditional Y2 receptor knockdown improved the counter-regulatory responses to hypoglycemia. Further examination of glucose counter-regulatory hormones—notably catecholamines, corticosterone, growth hormone and glucagon in Y2 receptor knockdowns, could shed light on this matter.

Interestingly, Y2 receptor deficiency in the periphery leads to an increase in physical activity. The exact mechanism for this is not clear, as physical activity was not altered in germline Y2 receptor deletion mice (**33**). It is possible that peripheral Y2 receptor deletion leads to an alteration of NPY levels in peripheral tissues such as muscle, causing increased activity, or that other Y receptors (such as Y1) become more responsive to NPY in the absence of Y2 receptors, leading to a less anxious phenotype with increased activity (**34**).

This study also suggests that peripheral Y2 receptor signalling plays a role in selection of fuel for oxidation, particularly during the light phase when lipid is generally a primary source of fuel for oxidation in rodents. In mice with peripheral knockdown of Y2 receptors, light-phase RER was increased, one explanation for which could be a shifting from lipid oxidation toward greater carbohydrate oxidation relative to control mice, and this effect is independent of food type as it was observed under both a chow and a high-fat diet. Importantly, this effect is likely to be specifically Y2 receptor-mediated, because it is not observed in mice with conditional peripheral Y1 receptor knockdown (**16**). As hypothalamic NPY administration to rodents has been shown to stimulate the consumption of carbohydrate-rich foods in preference to fat-rich foods (**35**), it is likely that the action of peripheral Y2 receptors are not involved in driving this process. Nevertheless, data from the present study suggest the regulatory role of peripheral Y2 receptors in energy expenditure, physical activity and the selection of fuel for oxidation, with subsequent effects on energy balance.

The major tissue contributors to the decreased adiposity in Y2 receptor knockdown mice are most likely skeletal muscle, liver, and WAT itself. Intraperitoneal Poly I:C injection induced a significant reduction of Y2 receptor mRNA expression in each of these three tissues, in association with a marked decrease in WAT depot weights, consistent with a previous report which showed that local knockdown of Y2 receptors in subcutaneous abdominal WAT reduced stress-induced visceral fat by 50% in 2 weeks (**17**). It is interesting to note that Y2 receptor expression was not decreased in BAT after poly I:C treatment. This may be due to low efficacy of activation of Mx1 promoter in BAT. In addition, Y2 mRNA expression level in BAT is lower than that in epididymal WAT, so it could be difficult to be reduced further by Poly I:C treatment in a chow diet. However, complete abolishment of Y2 mRNA in BAT from Poly I:C-injected mice fed a high-fat diet suggests that BAT may play a role in the reduced adiposity of knockout mice on a high-fat diet. Activation of fat-burning β-adrenergic activity is a possible pathway for the reduction in adiposity seen in Y2 receptor deficiency on adipose tissue (**17**). If a similar activation of β-adrenergic activity is occurring in the muscle of Y2 receptor knockdown mice, then this may lead to increased locomotion (**36**), thus contributing to reduced adiposity (**32**). Furthermore, as partial deletion of peripheral Y1 receptors exerts a similar antilipogenic effect (**16**), more efficacious fat reduction might be achieved by targeting different aspects of energy balance using dual Y1 and Y2 peripheral blocking strategies.

It is interesting to note that peripheral Y2 receptor knockdown led to increased food intake on a high-fat diet. One possible mechanism for that could be that increased food intake results from a compensatory response of the brain to the peripheral Y2 receptor knockdown-induced decrease in adiposity. It is also possible that partial deletion of Y2 receptors in peripheral tissues could trigger other signalling pathways that favor food intake. A number of studies have shown that gene alteration in peripheral tissues can influence food intake. For instance, deletion of uncoupling protein 1 or insulin receptor substrate-2 specifically in BAT increases food intake (**37**). Potentially, it is also possible that gut-derived satiety factors may be reduced due to the lack of Y2 signalling in these tissues, but further analysis will be required to prove this hypothesis.

In addition to effects on energy homeostasis, Y2 receptor signalling, notably in the hypothalamus, plays a critical role in the regulation of bone physiology, as evidenced by the fact that germline and adult-onset hypothalamus-specific Y2 receptor deletion increases cortical and trabecular bone mass (**18**), whereas ablation of Y2 receptors specifically from hypothalamic NPY-ergic neurons only partially increases trabecular bone mass and has no effect on cortical bone mass (**19**), suggesting the involvement of other neuronal populations in the regulation of

bone mass. The current study extends these findings to demonstrate that peripheral Y2 receptors do not seem to be involved in the regulation of bone mass, consistent with a previous report that detected Y1 but not Y2 receptor mRNA expression in mesenchymal progenitor cells or bone tissues (**29**). Taken together, these findings further highlight the key role of hypothalamic Y2 receptors on non-NPY-ergic neurons (**13**) as well as NPY-ergic neurons (**19**) in the control of bone formation.

In conclusion, these data suggest that under chow-fed conditions peripheral Y2 receptors may not be actively involved in the regulation of body weight or feeding behavior, but they are important regulators of aspects of energy homeostasis such as energy expenditure, fuel handling, and physical activity. In contrast, under conditions of diet-induced obesity, peripheral Y2 receptors attenuate fat accretion and improve glucose tolerance in association with increased physical activity and energy expenditure. These changes occur despite increased food intake and with no adverse effects on lean body mass or bone mass. Thus, a previous study (**17**) and our new results demonstrate that peripheral Y2 receptors exert pro-obesity effects by local effects in the WAT (stimulation of fat growth through adipogenesis and angiogenesis (**17**)), and by exerting systemic effects on glucose tolerance, fat accretion, and energy balance (decreased physical activity and energy expenditure), with Y2 receptors in skeletal muscle and BAT possibly also contributing to this phenotype. Since current antiobesogenic drugs that target central appetite-controlling systems in the brain generally only lead to 3–5% reductions in body weight over and above the effects of diet and exercise *per se* and have been proven to have several undesired side effects, mostly affecting mood and inducing nausea (**38**), data from the present study suggest that targeting peripheral as opposed to central Y2 receptors could have beneficial effects for the treatment of obesity and glucose intolerance without adverse effects on anxiety, emotionality (**39**) or weight gain (**19**) that have been reported with central Y2 receptor blockade.

Axknowedgment

We are grateful to Dr Turner and Prof Greg Cooney of the Garvan Institute of Medical Research for help with set-up and use of the Columbus Instruments Laboratory Animal Monitoring System. We thank the staff of the Garvan Institute Biological Testing Facility. This research was supported the National Health and Medical Research Council (NHMRC) of Australia grants #427661 and #427606, and A.S. and H.H. are supported by NHMRC Fellowships, M.S.B. is supported by Netherlands Organization for Scientific Research (2008/03609/ALW) and a Dutch Cancer Society Fellowship (BUIT-2009-4449) and the Cancer Council NSW (grant RG08-07).

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