

ORIGINAL ARTICLE

Peripheral neuropeptide Y Y1 receptors regulate lipid oxidation and fat accretion

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Objective: Neuropeptide Y and its Y receptors are important players in the regulation of energy homeostasis. However, while their functions in feeding regulation are well recognized, functions in other critical aspects of energy homeostasis are largely unknown. To investigate the function of Y1 receptors in the regulation of energy homeostasis, we examined energy expenditure, physical activity, body composition, oxidative fuel selection and mitochondrial oxidative capacity in germline Y1^{-/-} mice as well as in a conditional Y1-receptor-knockdown model in which Y1 receptors were knocked down in peripheral tissues of adult mice.

Results: Germline Y1^{-/-} mice of both genders not only exhibit a decreased respiratory exchange ratio, indicative of increased lipid oxidation, but interestingly also develop late-onset obesity. However, the increased lipid oxidation is a primary effect of Y1 deletion rather than secondary to increased adiposity, as young Y1^{-/-} mice are lean and show the same effect. The mechanism behind this is likely because of increased liver and muscle protein levels of carnitine palmitoyltransferase-1 (CPT-1) and maximal activity of key enzymes involved in β -oxidation; β -hydroxyacyl CoA dehydrogenase (β HAD) and medium-chain acyl-CoA dehydrogenase (MCAD), leading to increased mitochondrial capacity for fatty acid transport and oxidation. These effects are controlled by peripheral Y1-receptor signalling, as adult-onset conditional Y1 knockdown in peripheral tissues also leads to increased lipid oxidation, liver CPT-1 levels and β HAD activity. Importantly, these mice are resistant to diet-induced obesity.

Conclusions: This work shows the primary function of peripheral Y1 receptors in the regulation of oxidative fuel selection and adiposity, opening up new avenues for anti-obesity treatments by targeting energy utilization in peripheral tissues rather than suppressing appetite by central effects.

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Introduction

Obesity, an epidemic disorder and the leading risk factor for type 2 diabetes, develops through a prolonged imbalance between energy intake and energy expenditure. Neuropeptide Y (NPY), a 36-amino-acid peptide, is a key regulator of energy metabolism. Increased central NPY-ergic tone elicits robust

hyperphagia and induces a series of obesogenic changes that lead to gain of body weight and fat.^{1–6} These obesifying changes include decreases in body temperature and thermogenic capacity in brown adipose tissue^{5,7–10}—indicative of decreased energy expenditure, glucose partitioning towards storage rather than utilization,^{2,11} increased lipogenic activity in white adipose tissue and liver,^{7,8,12} and increased respiratory exchange ratio (RER)^{13–15}—indicative of a higher preference for carbohydrate versus lipid as a fuel source. Importantly, when NPY-induced hyperphagia is prevented by pair feeding, central administration of NPY for 5–7 days in rodents still leads to significant fat gain without changes in body weight,^{2,12} showing an important function of NPY in increasing adiposity independent of changes in food intake and body weight.

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NPY exerts its effects through activation of at least five G-protein-coupled receptors: Y1, Y2, Y4, Y5 and y6.¹⁶ The Y1 receptor has received particular attention because of its status as a potential 'feeding receptor'.^{15,17–21} Recent pharmacological studies suggest that Y1 receptors are not only involved in NPY-mediated feeding, but also in the control of energy metabolism.¹⁵ Thus, agonism of Y1 receptors increases RER, indicative of lower lipid oxidation, without significant effects on energy expenditure.¹⁵ However, as Y-receptor ligands are renowned for their lack of bioavailability and specificity, effects of antagonizing Y1 receptors on energy metabolism *in vivo* are difficult to examine pharmacologically. Therefore, we performed a systematic investigation of energy expenditure and substrate oxidation, combined with measurements of physical activity and body composition, in mouse models with Y1-receptor deficiency. As germline Y1-receptor-knockout mice develop late-onset obesity,^{22–25} and as weight gain in itself is known to have significant effects on energy metabolism,²⁶ we also studied groups of younger germline Y1-knockout mice to enable definitive investigation of the primary effects of Y1-receptor deficiency on energy metabolism.

It is important to note that both NPY and Y1 receptors are expressed in peripheral tissues as well as in the brain.^{27–30} Peripheral Y1-receptor signalling could, therefore, conceivably be involved in the regulation of energy balance, as is the case for peripheral Y2 receptors.³¹ Although pharmacological studies with central administration of NPY and/or Y1 ligands have established a function of central Y1 receptors in mediating NPY's effects on food intake,^{15,32,33} little is known about the function of peripherally expressed Y1 receptors in the regulation of energy homeostasis. Moreover, NPY and the Y-receptor system are known for their complexity and plasticity, as evidenced by compensatory changes in the expression of Y receptors when the ligand³⁴ or Y-receptor subtype(s)^{35,36} are completely ablated. Such compensatory changes in the NPY-ergic system in response to complete germline knockout of one of its components may at least contribute to some discrepant findings that have been reported between germline knockout studies and pharmacological studies.^{15,25,37} Therefore, to study the function of peripheral Y1 receptors in the regulation of energy homeostasis while reducing the impact of possible compensatory responses to gene deletion, we generated a conditional Y1-receptor-knockdown mouse model in which Y1 receptor was partially deleted specifically in peripheral tissues of adult mice. Understanding the functions of central versus peripheral Y1 receptors in the regulation of energy homeostasis is of critical importance for the development of novel anti-obesity pharmaceuticals based on Y-receptor antagonism. For instance, rather than interfering with Y1-receptor-mediated appetite control in the brain, with its potential side effects on behaviour and emotionality, targeting peripheral Y1-receptor-mediated control of energy utilization could be an attractive alternative.

Materials and methods

Animals

All research and animal care procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 °C) and illumination (12-h light cycle, lights on at 07:00). All mice were fed a normal chow diet *ad libitum* (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate and 2.6 kcal g⁻¹; Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia). Details of generation of the germline Y1-receptor-knockout mice were published earlier.³⁸ Y1-conditional-knockdown mice were generated by crossing mice harbouring the Y1-receptor gene flanked by recombinase recognition (LoxP) sites (Y1^{lox/lox}) onto transgenic mice expressing Cre-recombinase under the control of interferon-responsive Mx1 promoter. This Mx1-activated Cre-mediated gene deletion has been shown to be partial and to preferentially target tissues in the periphery, with almost no effect on gene expression in the brain.³⁹ In our model, deletion of the Y1-receptor gene was induced in mice at 13 weeks of age through i.p. injection of synthetic double-stranded RNA (polyinosinic-polycytidylic acid; Poly I:C 300 µg per injection; Sigma-Aldrich Inc., St Louis, MO, USA) three times at 2-day intervals as described earlier.³⁹ Commencement of induction was defined as the day of the first Poly I:C injection. At 5 weeks after commencement of induction, various tissues and organs (the hypothalamic region of the brain, liver, heart and skeletal muscle, pancreas, retroperitoneal white adipose tissue and brown adipose tissue) from control mice and conditional Y1-knockdown mice were collected and genomic DNA was isolated. Mx1-Cre-induced Y1-receptor gene deletion in these tissues and organs were assessed by PCR using primer sets A and B that give PCR products in the absence or presence of the Y1 gene, as described earlier.³⁸

Indirect calorimetry studies were carried out on male and female Y1^{-/-} and wild-type mice at 9 and 15 weeks of age. Female Y1-receptor conditional knockdown mice were studied by indirect calorimetry at 3 and 5 weeks after commencement of induction of gene deletion. Data obtained at these two time points were comparable, thus data at 5 weeks after the commencement of induction of gene deletion were presented. Food intake and rectal temperature were measured in conditional Y1-receptor-knockdown mice at 4 weeks after the commencement of induction of gene deletion. Rectal temperature was measured at ~09.00 h with a thermometer connected to a rectal probe (BAT-10 multi-purpose thermometer; Physitemp Instruments, Clifton, NJ, USA). Temperature readings were taken within 10 s of removing the mouse from its cage. At the completion of each study, mice were culled between 12.00 and 15.00 h by cervical dislocation followed by decapitation for collection of trunk blood. Serum was stored at -20 °C for subsequent

analysis as described below. White adipose tissue depots (inguinal, epididymal retroperitoneal and mesenteric), brown adipose tissue, brain, pancreas, liver, heart and skeletal muscle were removed, weighed and frozen until further analysis by PCR genotyping, radio-ligand binding assay, western blotting and/or enzyme activity assays as described below. A separate set of female conditional Y1-receptor-knockdown and control mice at 13 weeks of age were fed with a high fat diet (23% calories from fat, 19.4% calories from protein, 4.7% calories from crude fibre, 4.7% calories from acid detergent fibre and 20 MJ kg⁻¹; Specialty Feeds, Glen Forrest, WA, Australia) for 21 weeks. Body composition through dual-energy X-ray absorptiometry, energy metabolism, food intake and rectal temperature were measured after 15 weeks of high fat feeding. On completion of the study, mice were culled for collection of trunk blood for determination of serum insulin levels, and white adipose tissue depots (inguinal, epididymal retroperitoneal and mesenteric) were dissected and weighed as described above.

Food intake

Food intake was measured in wild-type and Y1^{-/-} mice at 9–10 weeks of age, chow-fed control and conditional Y1-receptor-knockdown mice at 4 weeks after the commencement of induction of gene deletion, as well as high fat diet-fed control and conditional Y1-receptor-knockdown mice after 15 weeks of high fat feeding. Mice were transferred from group housing on soft bedding to individual cages with paper towel bedding and allowed to acclimatize for 3 days. Food intake determined as the averages of triplicate readings were taken over 3 consecutive days. Actual food intake was calculated as the weight of pellets taken from the food hopper minus the weight of food spillage in the cage. Foetal weight was also determined in triplicate during these analyses.

Indirect calorimetry

Metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH, USA). Pre-weighed mice were housed individually in specially built Plexiglas cages (20.1 × 10.1 × 12.7 cm). Temperature was maintained at 22 °C with airflow of 0.6 l min⁻¹. Food and water were available *ad libitum*. Mice were singly housed 3 days before transferring into Plexiglas cages and were acclimatized to the cages for 24 h before recordings commenced. Mice were subsequently monitored in the system for 24 h. Oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured every 27 min. The RER was calculated as the quotient of VCO₂/VO₂, with the value of 1 representing 100% carbohydrate oxidation and the value of 0.7 representing 100% fat oxidation.^{40,41} Energy expenditure (kcal heat produced) was calculated as calorific value (CV) × VO₂, where CV is 3.815 + 1.232 × RER.⁴² Data for the 24-h

monitoring period was averaged for 1-h intervals for energy expenditure and RER.

Measurement of physical activity

Ambulatory activity of individually housed mice was evaluated within the metabolic chambers using an OPTO-M3 sensor system (Columbus Instruments), whereby ambulatory counts were a record of consecutive adjacent photo-beam breaks. Cumulative ambulatory counts of X and Y directions were recorded every minute and summed for 1-h intervals.

Analysis of body composition

On completion of indirect calorimetry and physical activity measurements, animals were anesthetized with isoflurane and then scanned using dual-energy X-ray absorptiometry (Lunar PIXImus2 mouse densitometer; GE Healthcare, Waukesha, WI, USA) to determine whole body lean and fat mass. The head and the tail were excluded from the analysis of body composition.

Serum insulin and free fatty acid analyses

Serum insulin and free fatty acid levels were determined with an Insulin ELISA from Linco Research (St Louis, MO, USA) and a kit from Wako (Osaka, Japan), respectively.

Western blotting

Western blotting was performed on liver and quadriceps muscle samples following procedures described earlier⁴³ to determine protein levels of key enzymes involved in mitochondrial oxidation. Briefly, liver and powdered muscle samples were resuspended in radio-immunoprecipitation assay buffer (PBS, pH 7.5; 1% non-ident NP-40; 0.5% sodium deoxy-cholate and 0.1% SDS), supplemented with protease and phosphatase inhibitors (10 µg ml⁻¹ phenylmethylsulfonyl fluoride, 10 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin, 1 mmol l⁻¹ Na₃VO₄ and 10 mmol l⁻¹ NaF) and solubilized for 2 h at 4 °C. Equal amounts of tissue lysate (12.5 µg protein) were resolved by SDS-PAGE and immunoblotted with appropriate antibodies against peroxisome proliferator-activated receptor γ coactivator (Calbiochem, Merck PTY, Kilsyth VIC, Australia), carnitine palmitoyltransferase-1 (CPT-1) (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) and an antibody cocktail that recognizes several subunits of the mitochondrial respiratory chain (MS601; Mitosciences, Eugene, OR, USA). Immunolabelled bands were quantitated by densitometry. Relative protein levels of the mutant mice as a percentage of that of control mice are presented.

Enzyme activity measurements

Liver and powdered muscle samples were homogenized 1:19 (wt/vol) in 50 mmol l⁻¹ Tris-HCl, 1 mmol l⁻¹ EDTA and

0.1% Triton X-100, pH 7.2, using a Polytron instrument (Kinematica, Littau-Lucerne, Switzerland) and were subjected to three freeze-thaw cycles. Activity of key enzymes involved in lipid oxidation including citrate synthase, β -hydroxyacyl CoA dehydrogenase (β HAD), medium-chain acyl-CoA dehydrogenase (MCAD) and cytochrome *c* oxidase were determined at 30 °C, as described earlier^{43–45} using a Spectra Max 250-microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Enzyme activities were expressed as units per gram wet weight of tissue, in which units are defined as micro-moles per minutes. Relative enzyme activities of the mutant mice as a percentage of that of control mice are presented.

RNA extraction and quantitative real-time PCR

To determine the Y1-receptor deletion efficiency in conditional Y1-receptor-knockdown mice, we measured Y1-receptor mRNA expression levels in liver and brain tissues from conditional Y1-receptor-knockdown and control mice. Total RNA from liver and the hypothalamic region of the brain was extracted using TRIzol reagent (Sigma, St Louis, MO, USA) following the manufacturer's protocol. The quality and concentration of RNA was determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (NanoDrop 1000, NanoDrop Technologies, LLC, Wilmington, DE, USA), and RNA integrity was confirmed by agarose gel electrophoresis. One microgram of total RNA was taken for DNase treatment (RQ1 RNase-Free DNase, Promega, Madison, WI, USA) to remove genomic DNA contamination. Subsequently, cDNA was synthesized with oligo(dT)₂₀ and random hexamers using Superscript III First-strand Synthesis System for reverse transcription-PCR (Invitrogen, Mount Waverley, VIC, Australia). Quantitative real-time PCR was then carried out on an ABI Prism 7900 HT instrument (Applied Biosystems Inc., Foster City, CA, USA) using the TaqMan Universal PCR master mix (Applied Biosystems Inc.) following the manufacturer's instructions. Probes and primers for the target gene, the Y1 receptor (Mm00650798_g1) and the reference gene, β -actin (Mm00697939_s1), were selected from TaqMan gene expression assay reagents (Applied Biosystems Inc.). Fluorescent signals generated during PCR amplifications were monitored and analysed with ABI Prism 7900 HT SDS software (Applied Biosystems Inc.). To determine the efficiency of each TaqMan gene expression assay, standard curves were generated by serial dilution of cDNA, and quantitative evaluations of target and reference gene levels were obtained by measuring threshold cycle numbers (Ct). The relative expression of target mRNA was computed from the Y1 Ct values and the β -actin Ct value using the standard curve method (Sequence Detection Systems Chemistry Guide, Applied Biosystems Inc.).

Radio-ligand binding assay

Receptor radio-ligand binding assays were conducted as described earlier.³⁶ Briefly, 20 μ m-coronal slides were cut

from frozen mouse brains and thaw mounted on charged slides. Matching brain sections from the same portion of the hippocampus (−1.94 mm from Bregma) were collected. Slides were thawed and pre-incubated at room temperature for 30 min in Krebs–Henseleit–Tris buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM MgSO₄, 1.2 mM CaCl₂, 50 mM glucose, 15 mM NaHCO₃, 1.2 mM KH₂PO₄, 10 mM Tris, pH 7.4). Slides were then incubated with Krebs–Henseleit–Tris buffer supplemented with 0.1% bovine serum albumin, 0.5% bacitracin and 25 pM ¹²⁵I-PYY (PerkinElmer Life Sciences, Inc., Boston, MA, USA) for 2 h at room temperature. To determine the expression level of Y1 receptors, the addition of 1229U91 (a ligand specific for Y1 receptors; NeomPS S.A., Strasbourg, France) at 1 μ M was added to compete with ¹²⁵I-PYY binding. Non-specific binding was determined in the presence of 1 μ M PYY. The sections were washed by dipping twice in ice-cold Krebs–Henseleit–Tris buffer and once in ice-cold deionized water followed by air drying. Finally, the slides were developed by exposure to β max films (Kodak, Rochester, NY, USA) for 4 days at −80 °C. The autoradiograms were developed and scanned. The relative optical density was determined in the dentate gyrus of the hippocampus⁴⁶ using software from ImageJ (ImageJ 1.38x, NIH, Bethesda, MD, USA). Specific binding was calculated by subtracting non-specific binding from total binding. Non-specific labelling was uniform and never exceeded 5% of total signal.

Statistical analyses

All data are expressed as means \pm s.e.m. RER and physical activity over the continuous 24-h period were averaged for the whole 24-h period, as well as for the light and dark periods. Differences between knockout and wild-type mice were assessed by ANOVA or repeated-measures ANOVA. Comparisons of energy expenditure (kcal h^{−1}) were carried out by analysis of covariance with lean body mass and fat mass as the covariates. The adjusted means of energy expenditure at a common lean mass and fat mass for the comparison were generated by analysis of covariance. Statistical analyses were performed with SPSS for Mac OS X, version 16.0.1 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

Results

Germline lack of Y1 signalling significantly increases lipid oxidation and physical activity independently of obesity

Body weight (Table 1) and 24-h food intake (data not shown) were not significantly different between Y1^{−/−} and wild-type mice in both genders at 15 weeks of age (Table 1). Interestingly, while male Y1^{−/−} mice at this age showed a slightly, but significantly reduced adiposity, female Y1^{−/−} mice had a whole body adiposity double that of wild-type mice (Table 1). Furthermore, serum insulin and free fatty acid levels were significantly increased in female Y1^{−/−} mice, but male Y1^{−/−}

Table 1 Effects of germline Y1 deletion on body composition, serum parameters and energy metabolism in mice at 15 weeks of age

Parameter	Male		Female	
	Wild type	Y1 ^{-/-}	Wild type	Y1 ^{-/-}
Body weight (g)	30.5 ± 1.0	27.5 ± 1.0	22.6 ± 0.5	22.7 ± 0.7
Whole body fat mass (% BW)	22.79 ± 0.81	17.52 ± 0.8*	18.19 ± 0.9	34.23 ± 1.18*
Serum insulin (pM)	148.9 ± 30.1	219.6 ± 50.9	99.4 ± 11.3	185.3 ± 31.1*
Serum FFA (mEq l ⁻¹)	1.55 ± 0.13	2.18 ± 0.27	0.86 ± 0.09	1.66 ± 0.19*
<i>Physical activity (ambulatory counts per hour)</i>				
24 h	311 ± 53	600 ± 83*	534 ± 77	1064 ± 208*
Dark phase	481 ± 85	917 ± 142*	808 ± 153	1554 ± 327*
Light phase	141 ± 24	282 ± 43*	260 ± 34	575 ± 131*
<i>Energy expenditure (kcal per hour)</i>				
24 h	0.458 ± 0.01	0.440 ± 0.02	0.408 ± 0.02	0.377 ± 0.04
Dark phase	0.483 ± 0.01	0.481 ± 0.02	0.435 ± 0.02	0.403 ± 0.05
Light phase	0.433 ± 0.01	0.398 ± 0.02	0.382 ± 0.02	0.350 ± 0.04
<i>Respiratory exchange ratio</i>				
24 h	1.01 ± 0.02	0.957 ± 0.02 (P=0.06)	1.01 ± 0.02	0.936 ± 0.01**
Dark phase	1.03 ± 0.01	1.01 ± 0.02	1.03 ± 0.01	0.962 ± 0.01*
Light phase	0.983 ± 0.02	0.901 ± 0.01*	1.00 ± 0.02	0.909 ± 0.03**

Abbreviations: BW, body weight; FFA, free fatty acid. Whole body fat mass was determined by dual-energy X-ray absorptiometry. Physical activity, energy expenditure and respiratory exchange ratio were determined in metabolic chamber and expressed as averages for 24-h, dark (1900–0700 h) and light (0700–1900 h) periods. For comparison of energy expenditure by analysis of covariance, common lean and fat mass are 21.58 and 5.74 g, respectively, for male mice and 16.63 and 5.08 g, respectively, for female mice. Data are means ± s.e.m. of 6–10 mice per group. *P<0.05 and **P<0.001 versus gender-matched wild-type mice.

mice only showed a trend to increased serum insulin and free fatty acids (Table 1). Despite the striking sex difference in body composition, both male and female Y1^{-/-} mice showed significant increases in physical activity during the dark and light periods relative to controls (Table 1) without significant changes in energy expenditure (Table 1), suggesting that basal metabolic rate is decreased in the knockout mice. Importantly, compared with wild-type controls, both male and female Y1^{-/-} mice had significantly decreased RER (Table 1), suggesting greater use of lipid as an oxidative fuel to provide energy. As obesity is often associated with increased fatty acid influx to metabolically active tissues and subsequently increases lipid oxidation,⁴³ the reduced RER observed in obese 15-week-old female Y1^{-/-} mice with significantly elevated serum free fatty acids levels (Table 1) raises the possibility that their increased lipid oxidation may be secondary to the increased adiposity rather than a primary effect of Y1 deletion.

Thus, to investigate the primary effects of Y1-receptor deletion on energy metabolism in the absence of obesity, particularly in female mice, we examined Y1^{-/-} mice at a younger age, before the onset of obesity. Both male and female Y1^{-/-} mice at 9 weeks of age weighed significantly less than wild-type controls despite no significant change in daily food intake or daily faecal output (Table 2). Furthermore, while 9-week-old male Y1^{-/-} mice exhibited a lean phenotype compared with matched wild types, as was also seen in 15-week-old male Y1^{-/-} mice, 9-week-old female Y1^{-/-} had a similar level of adiposity to that of age-matched

female wild-type mice, as evidenced by a similar whole body fat mass as determined by dual-energy X-ray absorptiometry and the weight of dissected adipose tissue depots (Table 2). Moreover, both male and female Y1^{-/-} mice at 9 weeks of age were not different from their gender- and age-matched wild-type controls in regards to circulating insulin or free fatty acid levels (Table 2). Importantly, 9-week-old male and female Y1^{-/-} mice showed a pattern of changes in physical activity, energy expenditure and RER that was broadly similar to that seen in 15-week-old Y1^{-/-} mice, in that physical activity was significantly increased without changes in energy expenditure, and RER was significantly decreased (Figure 1), indicative of increased lipid oxidation. It is interesting that the increases in physical activity and lipid oxidation seen in female Y1^{-/-} versus wild-type mice seems more pronounced at 15 than at 9 weeks of age, as differences between genotypes in these parameters were significant in both the dark and light phases in the older female Y1^{-/-} mice (Table 1), but were only significant in one phase in the younger female Y1^{-/-} mice (Figures 1b and f). Therefore, the more significant increases in physical activity and lipid oxidation seen in the older obese female Y1^{-/-} mice may be due to compensatory responses to increase adiposity, in addition to primary effects of Y1-receptor deletion on these parameters as seen in younger non-obese male and female Y1^{-/-} mice.

Taken together, these data show that germline Y1-receptor deletion in both male and female mice leads to increased lipid oxidation and physical activity without significant

Table 2 Effects of germline Y1 deletion on body composition, serum and feeding parameters and energy metabolism in mice at 9 weeks of age

Parameter	Male		Female	
	Wild type	Y1 ^{-/-}	Wild type	Y1 ^{-/-}
Body weight (g)	24.3 ± 0.4	22.1 ± 0.5*	19.6 ± 1.2	15.2 ± 0.6*
Daily food intake (g)	3.58 ± 0.19	3.69 ± 0.13	2.92 ± 0.11	2.83 ± 0.19
Daily faecal output (g)	0.98 ± 0.11	0.98 ± 0.10	0.74 ± 0.07	0.65 ± 0.08
Whole body fat mass (% BW)	15.25 ± 1.38	11.68 ± 0.30*	14.57 ± 0.86	15.77 ± 0.77
Inguinal WAT (% BW)	1.436 ± 0.122	1.035 ± 0.082*	1.813 ± 0.215	2.06 ± 0.068
Epididymal WAT (% BW)	1.419 ± 0.112	0.960 ± 0.115*	1.488 ± 0.274	1.440 ± 0.094
Mesenteric WAT (% BW)	0.741 ± 0.066	0.415 ± 0.059*	0.953 ± 0.147	0.586 ± 0.064*
Retroperitoneal WAT (% BW)	0.375 ± 0.034	0.245 ± 0.037*	0.217 ± 0.039	0.457 ± 0.025*
Serum insulin (pM)	108.1 ± 10.5	110.1 ± 20.3	96.4 ± 2.3	77.7 ± 12.0
Serum FFA (mEq l ⁻¹)	1.31 ± 0.32	0.92 ± 0.15	1.07 ± 0.01	0.75 ± 0.35
<i>Physical activity (ambulatory counts per hour)</i>				
24-h	777 ± 114	957 ± 134	846 ± 116	1124 ± 285
Dark phase	1235 ± 202	1220 ± 212	1408 ± 219	1709 ± 499
Light phase	319 ± 49	697 ± 110*	285 ± 52	539 ± 107*
<i>Energy expenditure (kcal per hour)</i>				
24-h	0.418 ± 0.01	0.405 ± 0.011	0.374 ± 0.009	0.396 ± 0.014
Dark phase	0.459 ± 0.011	0.424 ± 0.012	0.408 ± 0.011	0.432 ± 0.017
Light phase	0.377 ± 0.01	0.385 ± 0.011	0.339 ± 0.011	0.360 ± 0.016
<i>Respiratory exchange ratio</i>				
24-h	0.994 ± 0.016	0.938 ± 0.014*	0.998 ± 0.011	0.939 ± 0.025*
Dark phase	1.03 ± 0.013	0.987 ± 0.022	1.04 ± 0.009	0.988 ± 0.033
Light phase	0.957 ± 0.021	0.889 ± 0.016*	0.955 ± 0.017	0.889 ± 0.018*

Abbreviations: BW, body weight; FFA, free fatty acid; WAT, white adipose tissue. Whole body fat mass determined by dual-energy X-ray absorptiometry and weight of individual WAT depots were expressed as percentage of BW. For comparison of energy expenditure by analysis of covariates, common lean mass and fat mass were 17.7 and 2.8 g for male and 13.9 and 2.7 g for female mice, respectively. Data are means ± s.e.m. of 5–9 mice per group. **P* < 0.05 versus gender-matched wild-type mice.

changes in energy expenditure. Although female Y1^{-/-} mice developed obesity at 15 weeks of age, these changes in energy metabolism are the primary effects of germline Y1-receptor deletion as non-obese male Y1^{-/-} and younger female Y1^{-/-} mice showed the same effects.

Altered muscle and liver mitochondria function in Y1^{-/-} mice

To determine the mechanisms by which Y1 deletion might increase fat oxidation, we investigated key mitochondrial functions that control lipid oxidation. By western blotting of muscle and liver extracts, we determined the protein levels of CPT-1, the mitochondrial transmembrane enzyme controlling entry of fatty acid into mitochondria and the rate-limiting enzyme for fatty acid oxidation.^{47,48} There was a 1.3- to 1.6-fold increase in the protein levels of CPT-1 in the muscle and liver of 9-week-old male and female Y1^{-/-} mice compared with wild-type mice (Figures 2a and b), showing increased capacity for transporting fatty acids into mitochondria for oxidation. To examine mitochondrial fatty acid utilization at a functional level, we also determined the maximal activity of key enzymes of the β -oxidation pathway, namely, β HAD and MCAD in muscle tissue. The maximal activities of β HAD and MCAD in muscle from Y1^{-/-} mice of both genders were increased relative to wild-type controls

(Figures 2c and d), although the increase in muscle β HAD activity from male Y1^{-/-} mice did not reach statistical significance. This shows enhanced capacity for β -oxidation of fatty acids in Y1^{-/-} mice. Taken together, these data show that lack of Y1 signalling is associated with increased capacity for mitochondrial fatty acid transport and β -oxidation, which is likely the major contributor to the increased lipid oxidation indicated by the reduced RER seen in Y1^{-/-} mice.

Male Y1^{-/-} mice exhibit greater muscle mitochondrial oxidative capability

Having seen an increased capacity for fatty acid transport and oxidation in Y1 deficiency, we subsequently investigated whether the overall mitochondrial oxidative capability is also increased by Y1 deletion. Thus, we examined the muscle protein levels of several subunits of the mitochondrial respiratory chain complexes (I, II, III and V) by western blotting, as well as peroxisome proliferator-activated receptor γ coactivator 1 α , (PGC-1 α) an important regulator of mitochondrial biogenesis.⁴⁹ The protein levels of subunits of the respiratory chain complex I, II, III and V were all significantly increased in the muscle of 9-week-old male, but not female Y1^{-/-} mice compared with wild-type controls (Figures 2e and f). In addition to the increased level of

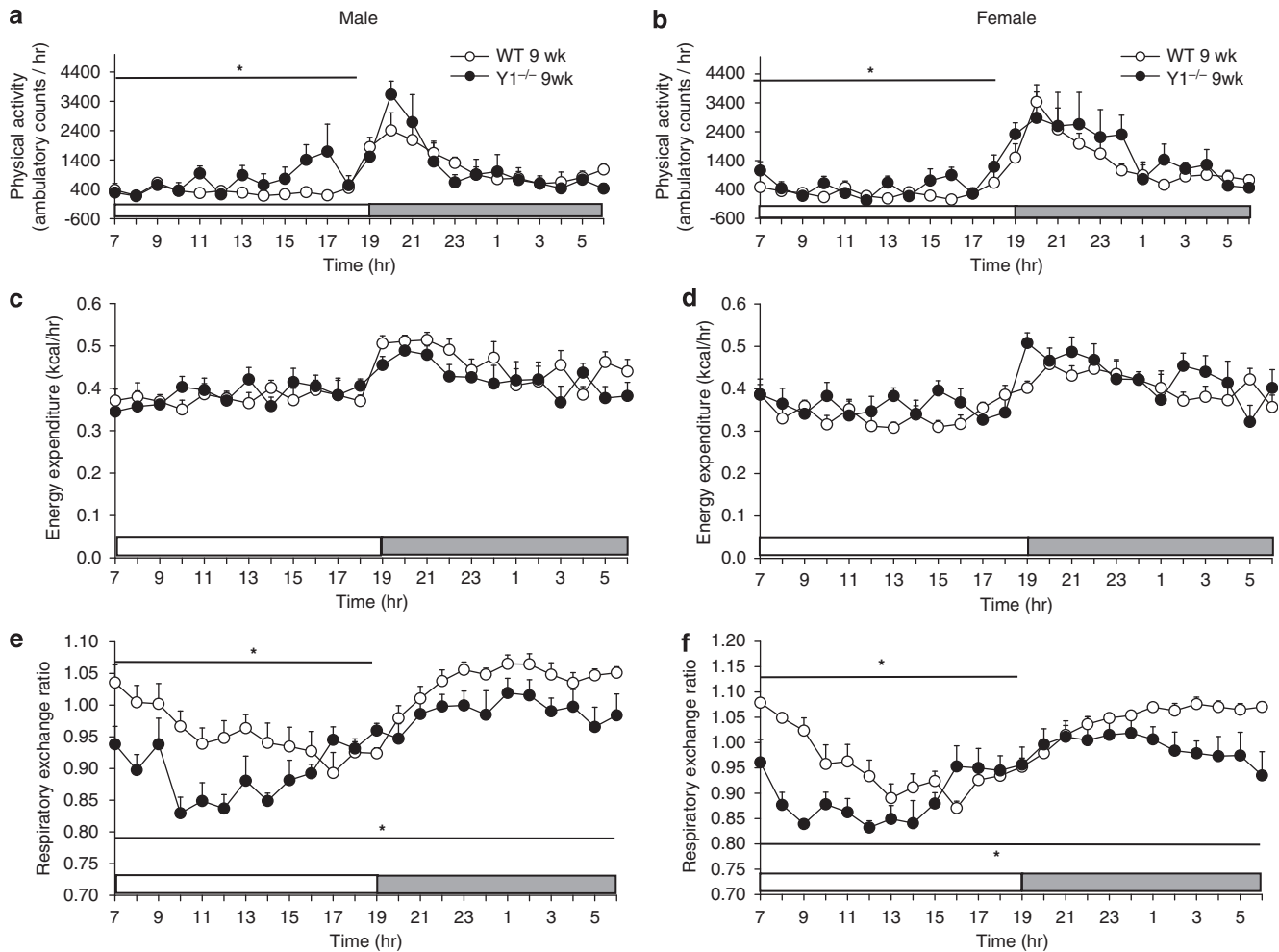


Figure 1 Effects of germline Y1-receptor deletion on energy metabolism in mice at 9 weeks of age. Physical activity (a, b), energy expenditure (c, d) and respiratory exchange ratio (e, f) were determined in metabolic chamber. For comparison of energy expenditure by analysis of covariates, common lean mass and fat mass were 17.7 and 2.8 g for male and 13.9 and 2.7 g for female mice, respectively. Open and grey bars indicate light and dark phases, respectively. Data are means \pm s.e.m. of 6–10 mice per group. * $P < 0.05$ versus gender-matched wild type (WT).

mitochondrial respiratory chain complex proteins, male $Y1^{-/-}$ mice had a 1.7-fold increase in muscle protein levels of PGC-1 α compared with male wild-type mice, whereas no such increase was seen in female $Y1^{-/-}$ mice (Figure 2g). These data suggest an increased mitochondrial oxidative capacity in male $Y1^{-/-}$ mice. To examine the mitochondrial oxidative capacity at a functional level, we also determined in muscle tissue the maximal activity of citrate synthase, a key enzyme of the Krebs cycle, and cytochrome *c* oxidase, representing complex IV of the respiratory chain. Although both male and female $Y1^{-/-}$ mice showed a significant increase in muscle citrate synthase activity (Figure 2h), only male $Y1^{-/-}$ mice had significantly increased cytochrome *c* oxidase activity compared with corresponding wild-type controls (Figure 2i). Collectively, these data suggest that Y1 deficiency in male mice leads to increased mitochondrial oxidative capacity, whereas this effect in female mice is absent or less pronounced. This gender difference in changes

in mitochondrial oxidative capacity because of Y1 deletion may contribute to the difference in adiposity observed between male and female $Y1^{-/-}$ mice.

Increased lipid oxidation in Y1 deficiency involves peripheral Y1 receptors

To study the possible function of peripherally expressed Y1 receptors in the control of energy metabolism, we generated a conditional Y1-receptor-knockdown mouse model in which partial deletion of Y1 receptors was induced specifically in peripheral tissues of adult mice. This was achieved by crossing mice harbouring the Y1-receptor gene flanked by recombinase recognition (LoxP) sites ($Y1^{lox/lox}$) onto transgenic mice expressing Cre-recombinase under the control of interferon-responsive Mx1 promoter. PCR on genomic DNA shows that the Mx1-activated Cre-mediated Y1-gene partial deletion was achieved in the hypothalamus as well as in

various peripheral tissues and organs (Figure 3a), consistent with an earlier report.³⁹ Importantly, no detectable change in Y1-mRNA expression was seen in the hypothalamic region of the brain of conditional Y1-receptor-knockdown mice compared with that of control mice, as quantitated by quantitative real-time PCR (Figure 3b), in keeping with an earlier study showing poor Mx1-activated Cre-mediated gene deletion in the brain.³⁹ In contrast, a significant reduction of Y1-mRNA expression was seen in the liver of conditional Y1-receptor-knockdown mice (Figure 3b), in which it is known that Mx1-activated Cre-mediated gene deletion has the highest efficiency.³⁹ To confirm the unchanged expression levels of central Y1 receptors in our conditional knockdown

mice at a functional protein level, we performed competitive autoradiography on brain sections using a Y1-selective ligand, 1229U91,⁵⁰ to compete with ¹²⁵I-PYY binding. The ¹²⁵I-PYY-binding intensity, with or without 1229U91 as a competitor, was analysed in the dentate gyrus of the hippocampus in which Y1 receptors are highly expressed.³⁶ The total specific ¹²⁵I-PYY-binding intensity in the dentate gyrus of conditional Y1-receptor-knockdown mice was similar to that of control mice (Figure 3c), suggesting that the functional protein levels of total Y receptors are not changed in the brain of these animals. Furthermore, the percentage of ¹²⁵I-PYY binding displaced by 1229U91 in the dentate gyrus area was comparable between conditional

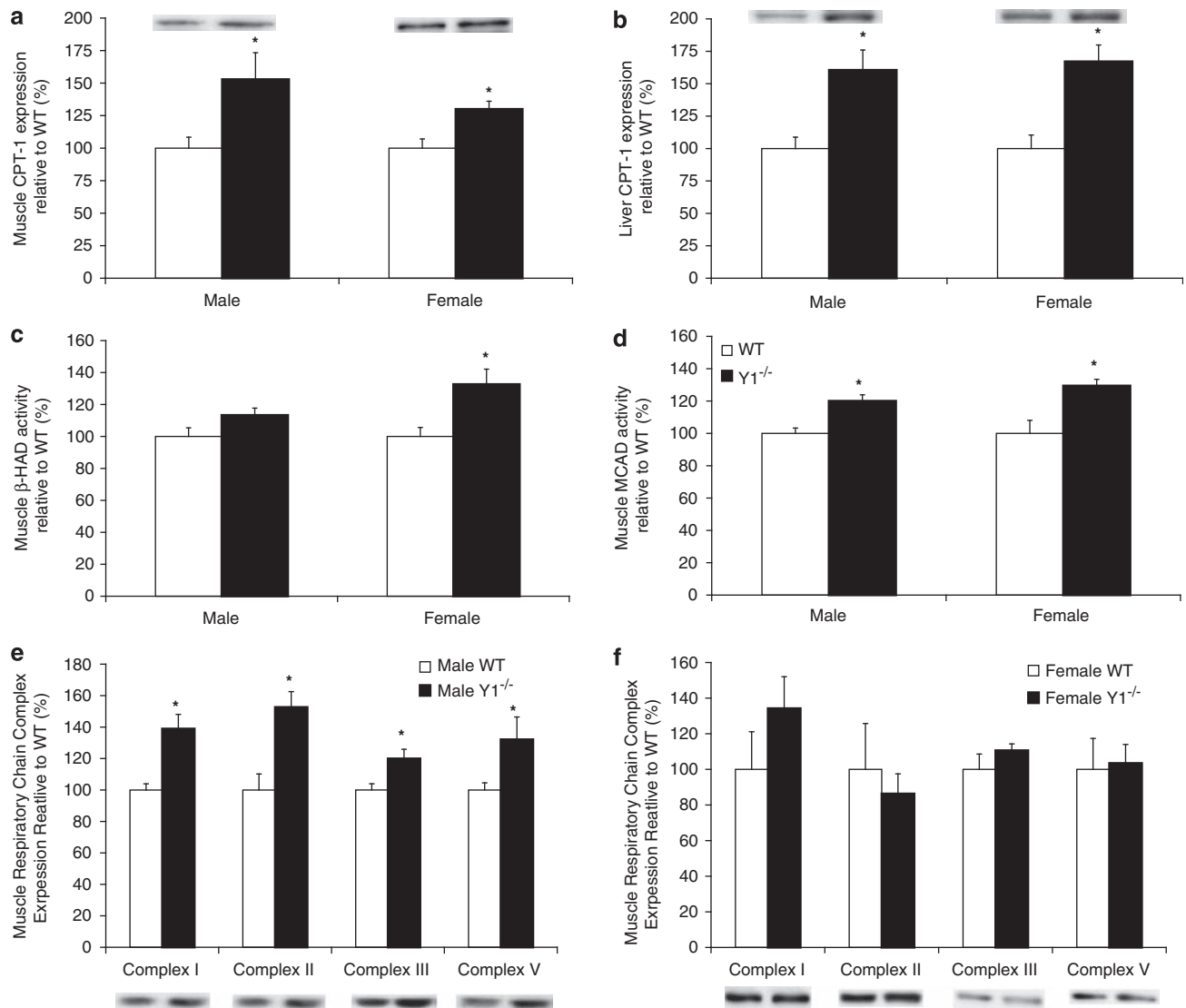


Figure 2 Mitochondrial oxidation in germline Y1^{-/-} mice. Protein levels of CPT-1 in muscle (a) and liver (b), and maximal activity of muscle βHAD (c) and MCAD (d), muscle protein levels of mitochondrial respiratory chain complexes I, II, III and V (e, f) and peroxisome proliferator-activated receptor (PPAR) γ coactivator -1α (PGC-1α, g), maximal activity of citrate synthase (CS, h) and cytochrome c oxidase (COX, i) in wild type (WT) and Y1^{-/-} at 9 weeks of age. Data are means ± s.e.m. of 5–8 mice per group and are expressed as a percentage of WT values. **P* < 0.05 versus gender-matched WT.

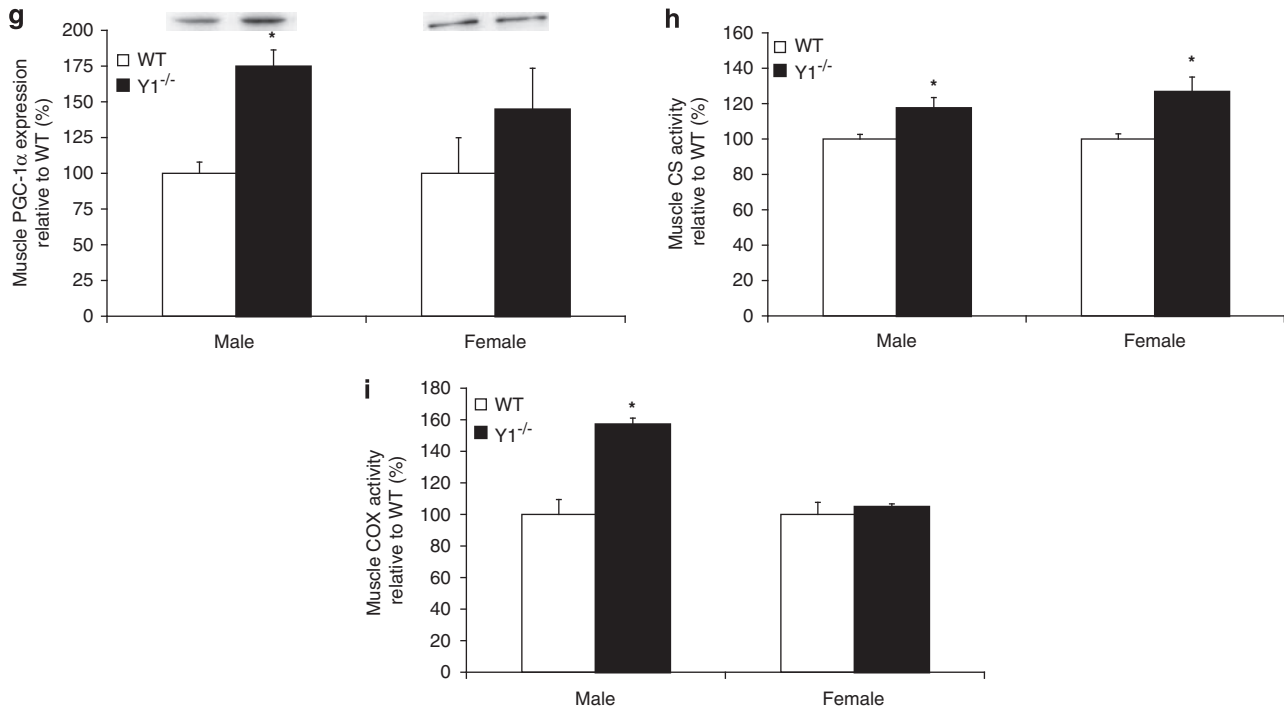


Figure 2 Continued.

knockdown and control mice (Figure 3d), indicating a similar proportion of Y1 receptors expressed in the dentate gyrus of both groups. These radio-ligand binding data suggest unaltered central Y1-receptor expression in conditional Y1-knockdown mice, consistent with the real-time PCR results presented in Figure 3b.

Having established a conditional Y1-knockdown model in which Y1 receptors are only deleted in peripheral tissues, we investigated energy metabolism in these mice 5 weeks after induction of Y1-gene deletion. The conditional Y1-knockdown mice exhibited similar body weight, daily food intake and faecal output, body composition, and circulating levels of insulin and free fatty acids as well as rectal temperature compared with corresponding measures in control mice (Table 3). Furthermore, the conditional Y1 knockdown in peripheral tissues of adult mice had no effect on physical activity (Figure 3e; Table 3) or energy expenditure (Figure 3f; Table 3), suggesting that the increased physical activity seen in germline Y1^{-/-} mice (Table 1; Figures 1a and b) is due to a lack of central Y1 signalling. Importantly, however, the conditional Y1 knockdown in mice induced a significantly reduced RER during the light period (Figure 3g; Table 3), showing an important function of peripheral Y1 receptors in the regulation of oxidative fuel selection. Furthermore, consistent with an increased lipid oxidation, conditional Y1 knockdown in mice induced a 1.4-fold increase in hepatic CPT-1 protein levels (protein levels of CPT-1 as percentage of control mice: 100 ± 7 and $140.7 \pm 13\%$ for control and conditional Y1-knockdown mice, respectively, data are

mean \pm s.e.m. of 5–7 mice for each group; $P < 0.05$) and a moderate, but significant increase in maximal hepatic activity of β HAD (activity as percentage of control mice: 100 ± 5.7 and $118 \pm 2.5\%$ for control and conditional Y1-knockdown mice, respectively, data are mean \pm s.e.m. of 4–6 mice per group, $P < 0.05$). These data show that similar to germline Y1-receptor deletion, peripheral Y1-receptor knockdown leads to increased lipid oxidation without changes in energy expenditure, showing the important function of peripheral Y1 receptors in the regulation of oxidative fuel selection. Furthermore, in contrast to the significant increase in physical activity seen in germline Y1-receptor-knockout mice, peripheral Y1-receptor knockdown did not alter physical activity. This suggests an important function of central Y1 receptors in the regulation of physical activity.

Peripheral Y1-receptor deletion protects against diet-induced obesity

To examine whether peripheral Y1-receptor knockdown and the resultant increase in lipid oxidation had functional effects to protect against diet-induced obesity, we challenged conditional Y1-knockdown mice with an *ad libitum* high fat diet for 21 weeks. Basal body weight determined before the commencement of high fat feeding was comparable between conditional Y1 knockdown and controls (20.7 ± 0.5 and 20.0 ± 0.8 g for control and conditional Y1-knockdown mice, respectively; means \pm s.e.m. of 5 mice per group; NS). Interestingly, conditional Y1-knockdown mice on a high

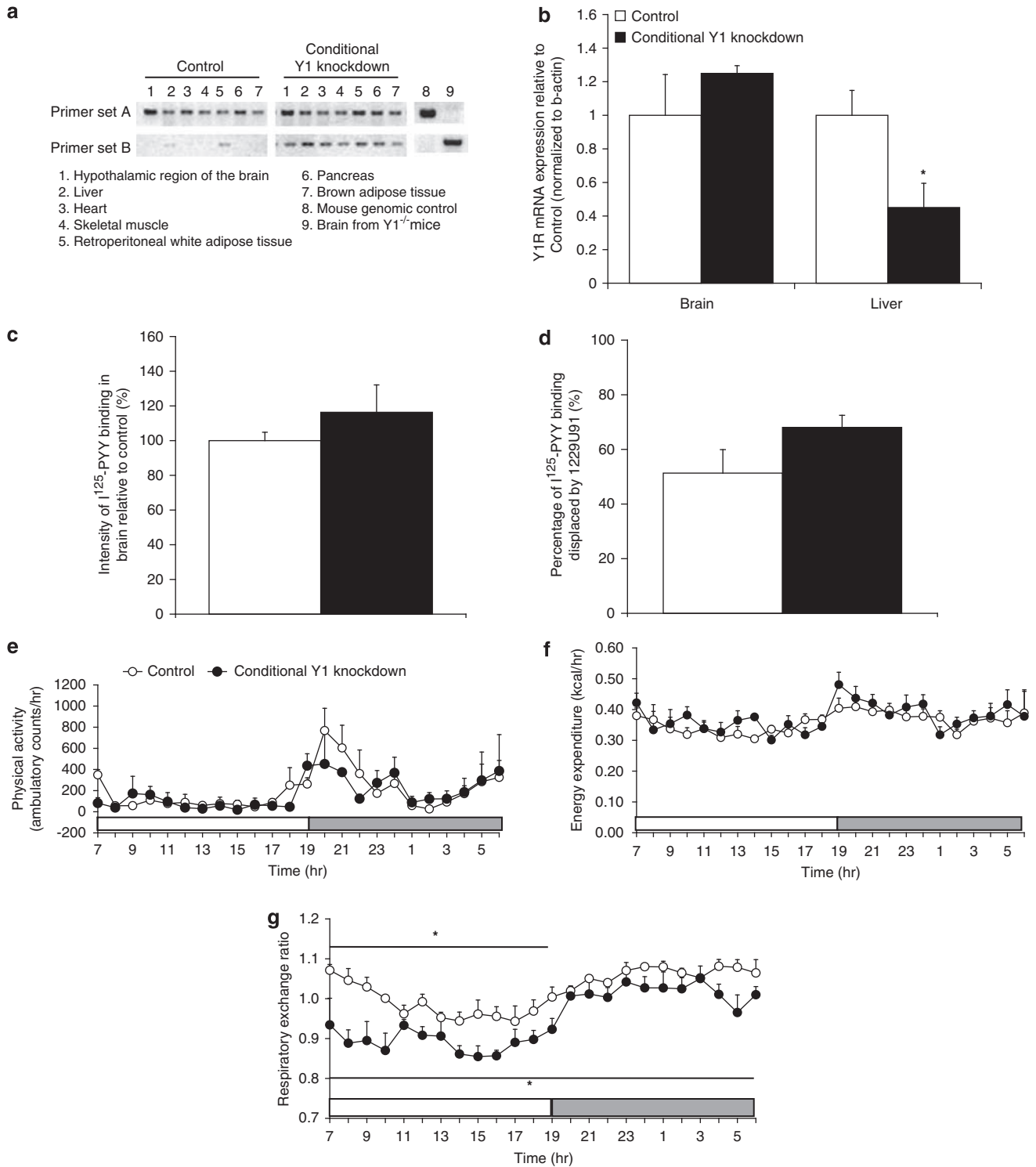


Figure 3 Unaltered brain Y1-receptor expression in peripheral conditional Y1-knockdown mice and subsequent effects on energy metabolism. (a) Mx1-activated Cre-mediated Y1-gene deletion was assessed by PCR on genomic DNA isolated from conditional Y1-knockdown and control mice; primer sets A and B give PCR product in the absence and presence of Y1-gene deletion, respectively. (b) Y1 mRNA expression levels in the hypothalamic region of the brain and liver of conditional Y1-knockdown versus control mice. Intensity of specific [¹²⁵I]-PYY binding (c) and the percentage of [¹²⁵I]-PYY binding displaced by the Y1-selective ligand (1229U91) (d) were examined in the dentate gyrus region of the brain of conditional Y1-knockdown and control mice. Physical activity (e), energy expenditure (f) and respiratory exchange ratio (g) were determined in metabolic chamber. For comparison of energy expenditure by analysis of covariance, common lean mass and fat mass were 14.3 and 5.1 g, respectively. Open and grey bars indicate light and dark phases, respectively. Data are means \pm s.e.m. of 4–6 mice per group. **P* < 0.05 versus control mice.

Table 3 Effects of peripheral Y1-receptor knockdown on body composition, serum and feeding parameters and energy metabolism

	Control	Conditional Y1 knockdown
Body weight (g)	21.89 ± 0.89	19.66 ± 0.30
Daily food intake (g)	2.86 ± 0.10	2.88 ± 0.10
Daily faecal output (g)	0.68 ± 0.05	0.62 ± 0.08
Whole body lean mass (g)	14.53 ± 0.39	13.87 ± 0.12
Whole body fat mass (g)	5.57 ± 0.58	4.67 ± 0.15
Whole body fat mass (% BW)	27.63 ± 2.04	25.27 ± 0.74
Inguinal WAT (% BW)	2.88 ± 0.37	2.84 ± 0.16
Epididymal WAT (% BW)	2.81 ± 0.28	3.01 ± 0.16
Retroperitoneal WAT (% BW)	0.77 ± 0.20	0.61 ± 0.03
Mesenteric WAT (% BW)	1.26 ± 0.05	1.15 ± 0.07
Total WAT (% BW)	7.61 ± 0.32	7.61 ± 0.12
Serum insulin (pM)	101.15 ± 18.89	85.22 ± 8.19
Serum free fatty acid (mEq l ⁻¹)	1.121 ± 0.078	1.214 ± 0.184
Rectal temperature (°C)	36.3 ± 0.2	37.0 ± 0.2
<i>Energy expenditure (kcal per hour)</i>		
24-h	0.358 ± 0.014	0.374 ± 0.017
Dark phase	0.378 ± 0.02	0.397 ± 0.024
Light phase	0.339 ± 0.009	0.351 ± 0.012
<i>Physical activity (ambulatory counts per hour)</i>		
24-h	197 ± 13	171 ± 68
Dark phase	283 ± 14	269 ± 107
Light phase	111 ± 29	72 ± 29
<i>Respiratory exchange ratio</i>		
24-h	1.02 ± 0.013	0.95 ± 0.017*
Dark phase	1.06 ± 0.011	1.01 ± 0.027
Light phase	0.986 ± 0.015	0.891 ± 0.016*

Abbreviations: BW, body weight; WAT, white adipose tissue. Whole body lean and fat masses were determined by dual energy X-ray absorptiometry. Weight of individual WAT depots was expressed as percentage of BW. For comparison of energy expenditure by analysis of covariance, common lean mass and fat mass were 14.3 and 5.1 g, respectively. Data were obtained at 5 weeks after the commencement of induction of gene deletion and are means ± s.e.m. of 4–6 mice per group. * $P < 0.05$ versus control mice.

fat diet showed significantly reduced weight gain compared with controls (Figure 4a). Importantly, this reduced weight gain in the conditional knockdown mice was associated with no difference in daily food intake or faecal output and a significant reduction in whole body fat mass without any changes in lean mass as determined by dual-energy X-ray absorptiometry scan after 15 weeks of high fat diet (Table 4). Moreover, when individual white adipose tissue depots were dissected out and weighed after 21 weeks on the high fat diet, the conditional Y1-knockdown mice showed significant reductions in the absolute weight of all dissected white adipose tissue depots investigated (namely, the inguinal, epididymal, retroperitoneal and mesenteric white adipose tissue depots) (Figure 4b) as well as the summed weight of these tissues (Figure 4c). In addition, the summed or individual weights of white adipose tissue depots as a percentage of body weight was decreased in conditional Y1-knockdown mice, albeit this decrease did not reach statistical significance for the inguinal and retroperitoneal

depots (Figures 4d and e). Interestingly, the significantly reduced adiposity in conditional Y1-knockdown mice on the high fat diet occurred in the absence of significant effects on serum insulin, overall physical activity or rectal temperature compared with corresponding measures in control mice (Table 4). Although there was no significant difference in energy expenditure between the knockdown and control mice on the high fat diet (Table 4), the conditional Y1-knockdown animals had generally higher energy expenditure than controls, with total 24-h energy expenditure being ~6% higher in knockout mice (Table 4). Notably, conditional Y1-knockdown mice on the high fat diet showed a decrease in RER relative to control mice during the light period ($P = 0.07$) (Figure 4f), suggesting that higher lipid oxidation could contribute to the reduced fat gain in conditional Y1-knockdown mice during high fat feeding. It is important to note that high fat feeding itself increases lipid oxidation,^{26,51–53} and this is evident in this study in which the average 24-h RER of chow-fed control mice is 1.02 ± 0.02 compared with 0.89 ± 0.02 in control mice fed on a high fat diet (means ± s.e.m. of 3–5 mice per group, $P < 0.05$). A similar difference in RER is seen between chow-versus fat-fed conditional Y1-knockdown mice; 0.95 ± 0.03 versus 0.85 ± 0.05 , respectively (means ± s.e.m. of 3–5 mice per group, $P < 0.05$). Therefore, the significant decrease in RER seen in chow-fed conditional Y1-knockdown versus control mice (Figure 3g) may have been masked by the effects of the high fat diet *per se* during the high fat diet. In summary, these data show that mice with conditional Y1-receptor knockdown are resistant to diet-induced obesity, and this effect may be attributable to increased lipid oxidation.

Discussion

This study shows that the regulation of oxidative fuel selection and physical activity is critically dependent on Y1 signalling, as germline lack of Y1 receptors resulted in a reduction in RER, consistent with a preference for lipid as an oxidative fuel source and a significantly increased physical activity with no change in energy expenditure. Importantly, these effects of germline Y1-receptor deletion on energy metabolism occurred in both male and female mice, but show differences in the age of onset and the degree of obesity induced by germline Y1-receptor deletion.^{23,24} Moreover, the mechanism behind the greater utilization of lipid as an oxidative fuel source in mice lacking Y1 receptors most likely involves increases in hepatic and muscle protein levels of CPT-1 as well as increases in hepatic and muscle activity of enzymes involved in β -oxidation, suggesting that Y1-receptor-signalling controls mitochondrial capacity for fatty acid transport and oxidation. Peripherally expressed Y1 receptors have a function in mediating these effects, as mice with conditional knockdown of Y1 receptors, in which Y1-gene

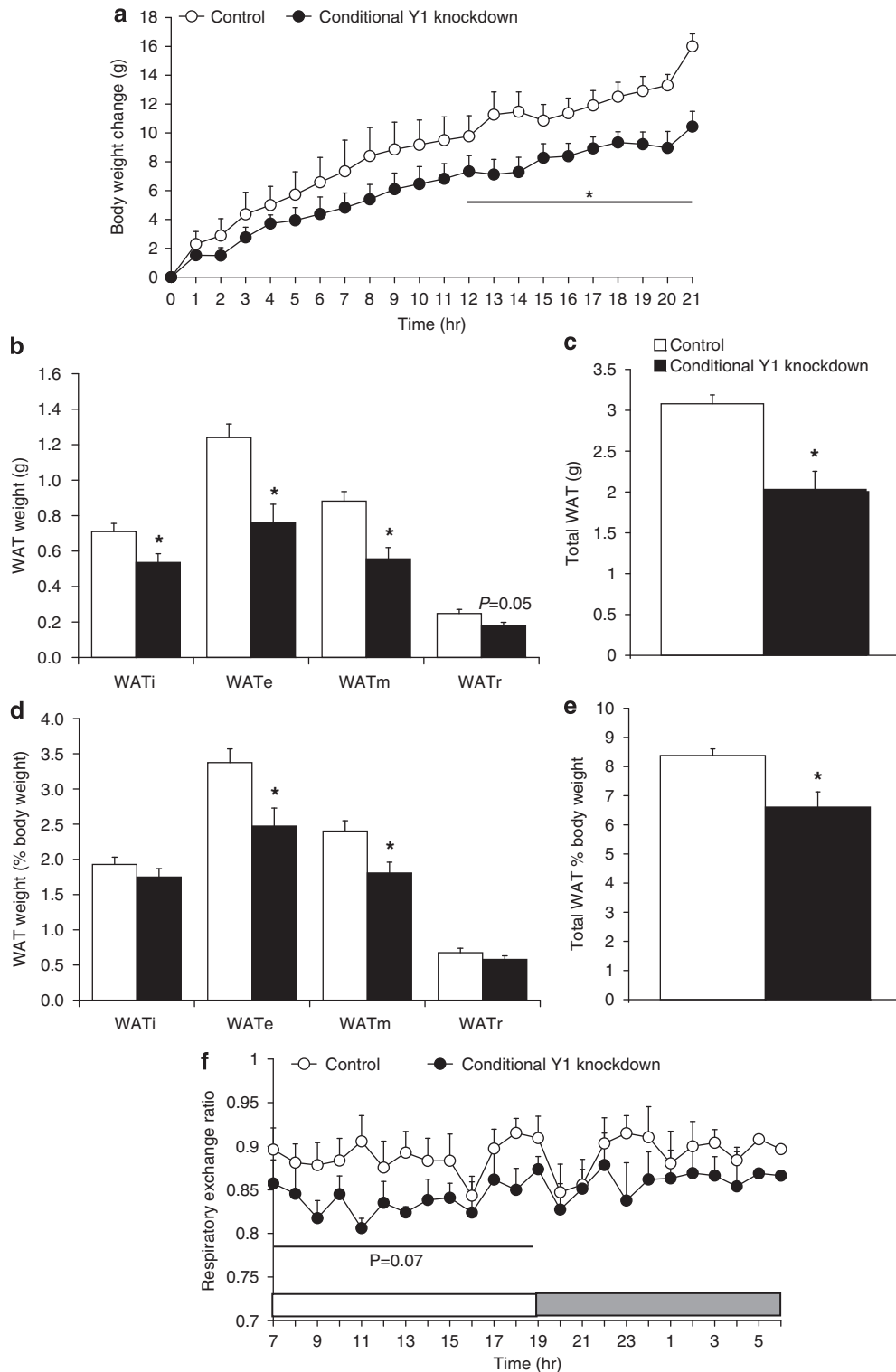


Figure 4 Reduced weight gain and adiposity with increased lipid oxidation in conditional Y1-receptor-knockdown mice on a high fat diet. **(a)** Body weight change over 21 weeks of high fat feeding. White adipose tissue (WAT) including inguinal (WATi), epididymal (WATe), retroperitoneal (WATr) and mesenteric (WATm) depots were dissected and weighed after 21 weeks of high fat feeding. Individual and summed weights of WAT depots are presented in absolute values **(b and c)** and as percentage of body weight **(d and e)**. Respiratory exchange ratio **(f)** was determined in metabolic chamber. Open and grey horizontal bars in **(f)** represent light and dark phases, respectively. Data are means \pm s.e.m. of five mice per group. * $P < 0.05$ versus control mice.

Table 4 Effects of peripheral Y1 receptor knockdown in mice on a high fat diet

	Control	Conditional Y1 knockdown
Body weight (g)	32.1 ± 0.7	28.4 ± 0.8*
Daily food intake (g)	2.38 ± 0.22	2.40 ± 0.29
Daily faecal output (g)	0.46 ± 0.06	0.45 ± 0.12
Whole body lean mass (g)	15.98 ± 0.28	15.56 ± 0.25
Whole body fat mass (g)	13.76 ± 0.81	10.60 ± 0.93*
Whole body fat mass (% BW)	46.16 ± 1.9	40.22 ± 2.4 (<i>P</i> = 0.08)
Serum insulin (pM)	253.28 ± 40.13	202.95 ± 19.03
Rectal temperature (°C)	36.88 ± 0.24	37.02 ± 0.15
<i>Physical activity (ambulatory counts per hour)</i>		
24-h	220 ± 48	142 ± 43
Dark phase	331 ± 87	234 ± 69
Light phase	110 ± 14	51 ± 18*
<i>Energy expenditure (kcal per hour)</i>		
24-h	0.462 ± 0.019	0.489 ± 0.019
Dark phase	0.483 ± 0.023	0.526 ± 0.023
Light phase	0.442 ± 0.017	0.453 ± 0.017
<i>Respiratory exchange ratio</i>		
24-h	0.890 ± 0.017	0.848 ± 0.021
Dark phase	0.893 ± 0.018	0.860 ± 0.031
Light phase	0.886 ± 0.017	0.837 ± 0.017 (<i>P</i> = 0.075)

Abbreviation: BW, body weight. Whole body lean and fat masses were determined by dual energy X-ray absorptiometry. Physical activity and energy expenditure were determined in metabolic chamber and expressed as averages for 24-h, dark (1900–0700 h) and light (0700–1900 h) periods. For comparison of energy expenditure by analysis of covariance, common lean and fat mass are 15.77 and 12.18 g, respectively. Data were obtained between 15 and 21 weeks of high fat feeding and are means ± s.e.m. of five mice per group. **P* < 0.05 versus control mice.

deletion occurs in a large variety of tissues with the highest efficiency in liver, but with no effect on Y1-receptor expression in the brain, led to similar changes in RER and hepatic CPT-1 and β -oxidative enzyme activity compared with germline Y1^{-/-} mice. Most importantly from a clinical perspective, peripheral Y1-receptor knockdown protected against high fat diet-induced increases in body weight and total adiposity in the absence of effects on lean mass or inguinal fat mass. As preservation of lean mass and inguinal fat during loss of excess adiposity is desirable because of known beneficial effects of these tissues on metabolic rate and disease risk,^{54–58} and as these benefits could be achieved without the need to deliver Y1 antagonists to the brain, this finding has significant implications for the pharmacological management of obesity.

The increased fat oxidation seen in Y1-deficient mice is likely mediated by increased capacity for fatty acid transport into mitochondria and increased β -oxidation. Germline and conditional Y1-receptor-deficient mice exhibit increased protein levels of CPT-1 in skeletal muscle and/or liver, the two primary tissues contributing to basal metabolic rate and major sites of fatty acid oxidation.^{59–61} CPT-1 is a mitochon-

drial transmembrane enzyme and considered to be rate limiting for long-chain fatty acid entry into the mitochondria for β -oxidation.⁶² An increased CPT-1 expression in Y1-deficient mice suggests an increased capacity for fatty acid influx into mitochondria, which in itself is sufficient to increase fat oxidation.⁶³ This is confirmed by the significantly increased maximal activities of MCAD and/or β -HAD in germline and conditional Y1-receptor-knockout mice, which are key enzymes involved in β -oxidation.⁶⁴

In addition to increased lipid oxidation, small imbalances in energy intake versus expenditure may have contributed to the reduced diet-induced obesity seen conditional Y1-receptor-knockdown mice. Although food intake and energy expenditure were not significantly different between the high fat-fed conditional Y1-receptor-knockdown and control mice, daily energy expenditure was 5.8% higher in the conditional Y1-knockdown mice. Interestingly, this increase in energy expenditure and protection against diet-induced obesity²² was not observed in germline Y1^{-/-} mice despite significantly increased physical activity, possibly because of compensatory effects of germline gene deficiency. As a minor imbalance between energy intake and expenditure can lead to severe obesity over a long period of time,⁶⁵ this small difference in energy expenditure may have contributed to the reduced obesity seen in conditional Y1-receptor-knockdown mice on the high fat diet.

Consistent with earlier studies, our data show a gender difference in the effects of Y1-receptor deletion on adiposity, with late-onset obesity that is more pronounced and of earlier onset in female compared with male mice.^{23,25,66} Our data suggest that differential mitochondrial capacity for substrate oxidation between male and female mice may contribute to this gender difference in effects of Y1 signalling on adiposity. The increased overall mitochondrial capacity for substrate oxidation seen in male versus female mice may enhance the ability of male mice to cope with nutrient excess. Interestingly, studies have shown that hyperinsulinaemia is likely to be a primary aetiological factor in the late-onset obesity of germline Y1-deficient mice,^{23,25,66} being apparent in female, but not significantly so in male Y1^{-/-} mice before the onset of obesity. In addition, although enhanced mitochondrial capacity for substrate oxidation or reduced hyperinsulinaemia in male Y1^{-/-} mice may contribute to a delay in the onset of obesity because of global Y1-receptor blockade, male and female Y1^{-/-} mice *both* develop obesity at an older age.^{23,24} Taken together, this work highlights the importance of avoiding hyperinsulinaemic effects of novel anti-obesity drugs based on Y1-receptor antagonism, and the likelihood that any such drugs may best be used in the short term or intermittently.⁶⁷

As germline Y1-receptor deficiency has been associated with enhanced function of the gonadotropic axis in both male and female mice under conditions of energy deficit,^{68,69} and as sex hormones have pronounced effects on energy homeostatic mechanisms, it is questioned whether Y1-receptor deletion may mediate some of its effects on

energy balance through changes in sex hormone activity. For instance, oestrogen treatment in mice increases lipolysis in abdominal fat cells,⁷⁰ and female rodents show a dramatic increase in physical activity on the night of oestrous, which is abolished by removal of oestrogens (for example ovariectomy).⁷¹ Therefore, it is possible that the increased lipid oxidation and physical activity evident in germline Y1^{-/-} mice may be related to stimulation of the sex hormone axis, at least in females. In addition, as a decline in circulating oestrogen or androgen levels is associated with increased total and abdominal fat levels, which is reversed by oestrogen or androgen replacement,⁷²⁻⁷⁵ possible increases in androgen action could conceivably contribute to the reduced adiposity seen in young male Y1^{-/-} mice. However, as Y1-receptor ablation probably influences sex hormone activity through hypothalamic pathways, as evidenced by up-regulation of several key factors in the hypothalamo-pituitary-gonadal axis in Y1^{-/-} mice,^{68,69} it is unlikely that changes in sex hormone actions contribute to the metabolic phenotype seen in mice with peripheral knockdown of Y1-receptor signalling.

In addition to a function of peripheral Y1 receptors in the regulation of lipid oxidation and accretion, this work reveals an important function of central Y1 receptors in the regulation of physical activity. Germline Y1^{-/-} mice have significantly increased physical activity compared with wild-type controls. This is consistent with behavioural studies showing a hyperactive phenotype in Y1^{-/-} mice.⁷⁶ Although germline Y1-receptor deficiency increases adiposity, particularly in female mice, and although positive energy balance itself triggers compensatory increases in physical activity in human beings^{26,53} and rodents,⁵¹ the increased physical activity of Y1^{-/-} mice is a primary effect of Y1 deletion and not a secondary effect of increased adiposity, because younger female and male Y1^{-/-} mice showed the same increase in physical activity in the absence of increases in fat mass. Importantly, changes in physical activity were not seen in our peripheral-specific Y1-receptor-knockdown mice, showing an important primary function of Y1 receptors in the brain in the control of physical activity.

Our conditional Y1-knockdown model showed partial deletion of the Y1 gene in various peripheral tissues. As the NPY-ergic system is known for its complexity and plasticity and compensatory changes in expression in response to germline total ablation of one of the system components,³⁴⁻³⁶ the adult-onset general peripheral knockdown strategy used in this study may reduce compensatory effects of gene deletion, thus revealing the true function of peripheral Y1 receptors in the control of energy homeostasis. Our finding that peripheral Y1 receptors regulate lipid oxidation extends an earlier report that intracerebroventricular administration of a Y1-receptor agonist to mice increases RER, indicative of reduced lipid oxidation,¹⁵ suggesting involvement of central Y1 receptors in the control of oxidative fuel selection. Although the mechanism by which this may occur is unclear, central Y1-mediated inhibition of sympathetic

terminals innervating white adipose tissue could inhibit sympathetically mediated lipolysis,^{77,78} and the resultant reduction in fatty acid supply could also contribute to reduced lipid oxidation.⁴³ Indeed, elevated central NPY-ergic tone reduces sympathetic outflow,^{79,80} and central Y1 receptors have been implicated in these effects.^{81,82} In the periphery, one of the targets on which Y1 receptors may exert effects on lipid metabolism are adipocytes, which express Y1 receptors^{29,30} and which are innervated with sympathetic neurons containing NPY.^{31,83} Blocking Y1-receptor signalling on isolated adipocytes from rodents and human beings *in vitro* antagonizes the anti-lipolytic effects of NPY and enhances lipolysis,^{84-87,30} and this mechanism may contribute to the enhanced lipid oxidation seen in peripheral Y1-receptor deficiency. In addition, our findings suggest that Y1 receptors in the liver may have an important function in the regulation of lipid oxidation. Y1 receptors are expressed abundantly on hepatic blood vessels,⁸⁸ whereas their expression on hepatocytes remains to be determined. NPY co-localized with norepinephrine in hepatic nerves⁸⁹ and released with norepinephrine on sympathetic nerve stimulation⁹⁰ may act on Y1 receptors expressed on hepatic blood vessels or on hepatocytes to modulate blood flow, and thereby indirectly or directly influence liver functions such as substrate oxidation. Importantly, insights from this study form the basis for future studies looking into more specific effects of Y1 receptors in individual tissues, such as conditional deletion of Y1 receptors in the liver and white adipose tissue, to shed even more light onto the tissue-specific function of Y1 receptors in the regulation of energy metabolism.

In summary, this work has revealed the critical function that peripheral Y1 receptors have in the regulation of energy homeostasis, in particular by controlling fatty acid oxidation. This is of particular importance as it opens up novel therapeutic options for the treatment of obesity by directly targeting peripheral Y1 signalling rather than by interfering with central Y1-receptor-mediated control of appetite, which has proven difficult and ineffective. Indeed, targeting appetite-controlling systems in the brain generally only leads to 3-5% reductions in body weight.⁹¹ Targeting peripheral as opposed to central Y1 receptors would have the additional benefit that other important central Y1-mediated functions, such as control of anxiety and emotionality, would not be at risk of being affected. Thus, it will be of considerable interest to develop strategies to antagonize Y1 signalling in tissues that increases lipid oxidation without inducing hyperinsulinaemia or late-onset obesity.

Conflict of interest

The authors declare no conflict of interest.

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