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Y2 and Y4 receptor signaling synergistically act on energy expenditure and physical activity

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Abstract

Neuropeptide Y receptors are critical regulators of energy homeostasis and are well known for their powerful influence on feeding, but their roles in other important aspects of energy homeostasis, such as energy expenditure and their functional interactions in these processes, are largely unknown. Here we show that mice lacking both Y2 and Y4 receptors exhibited a reduction in adiposity, more prominent in intra-abdominal vs. subcutaneous fat, and an increase in lean mass as determined by dual-energy X-ray absorptiometry. These changes were more pronounced than those seen in mice with Y2 or Y4 receptor single deletion, demonstrating the important roles and synergy of Y2 and Y4 signaling in the regulation of body composition. These changes in body composition occurred without significant changes in food intake, but energy expenditure and physical activity were significantly increased in Y4−/− and particularly in Y2−/−Y4−/− but not in Y2−/− mice, suggesting a critical role of Y4 signaling and synergistic interactions with Y2 signaling in the regulation of energy expenditure and physical activity. Y2−/− and Y4−/− mice also exhibited a decrease in respiratory exchange ratio with no further synergistic decrease in Y2−/−Y4−/− mice, suggesting that Y2 and Y4 signaling each play important and independent roles in the regulation of substrate utilization. The synergy between Y2 and Y4 signaling in regulating fat mass may be related to differences in mitochondrial oxidative capacity, since Y2−/−Y4−/− but not Y2−/− or Y4−/− mice showed significant increases in muscle protein levels of peroxisome proliferator-activated receptor (PPAR)γ coactivator (PGC)-1α, and mitochondrial respiratory chain complexes I and III. Taken together, this work demonstrates the critical roles of Y2 and Y4 receptors in the regulation of body composition and energy metabolism, highlighting dual antagonism of Y2 and Y4 receptors as a potentially effective anti-obesity treatment.

Neuropeptide y (NPY), a 36-amino acid peptide expressed in the central and peripheral nervous system, plays a critical role in regulating energy homeostasis. An increase in hypothalamic NPY-ergic tone elicits robust hyperphagia, decreases energy expenditure, and induces many endocrine and metabolic changes that ultimately lead to excessive weight gain and fat gain (**22**, **23**, **27**, **36**, **42**, **50**). NPY exerts its effects through activation of the G protein-coupled Y receptors, notably Y1, Y2, Y4, Y5, and y6 (**8**). Interestingly, whereas much work has focused on mechanisms underlying NPY-induced hyperphagia, few studies have examined the relative contribution and functional interactions of Y receptors in regulating energy expenditure, the other side of the energy balance equation. Energy expenditure is an important component of energy balance since excessive fat gain in response to central elevation of NPY persists even when NPY-induced hyperphagia is prevented by pair feeding, demonstrating that NPY regulates adiposity independent of food intake, and that aspects of energy homeostasis other than feeding play a pivotal role in NPY's obesogenic effects (**42**, **55**). Moreover, low energy expenditure predicts subsequent weight gain in humans (**17**, **35**), demonstrating the relevance of mechanisms regulating energy expenditure to human health.

Y2 and Y4 receptors have been shown to play an important role in the regulation of

adiposity, with pronounced synergies between Y2 and Y4 signaling on fat mass as demonstrated using germline knockout mice (**26**, **40**, **41**, **43**, **44**). While germline knockout models can show developmental adaptations to gene deletion that may mask primary effects of the gene deletion (**31**), they are a useful experimental tool for identifying key players in physiological processes, such as energy homeostasis. Thus, mice that lack Y4 and/or Y2 receptors had reduced fat mass with a greater reduction in fat mass observed in mice with germline Y2 and Y4 double deletions (**40**). This marked reduction in fat mass in Y2 and Y4 double knockouts was associated with significant decreases in circulating insulin and leptin levels in the nonfasted state (**41**). Interestingly, massive obesity in leptin-deficient *ob/ob* mice is attenuated by Y2 (**44**) but not by Y4 (**45**) receptor deletion, suggesting different mechanisms and capacity of Y2 and Y4 signaling in the control of adiposity. Importantly, dual deletion of Y2 and Y4 receptors in *ob/ob* mice produced marked reductions in body weight and fat mass that were more pronounced than those observed in *ob/ob* mice with Y2 receptor single deletion (**26**). This finding demonstrates that the synergistic effects of Y2 and Y4 receptor ablation on adiposity observed on a lean background prevail in the massively obese *ob/ob* background.

Despite this compelling evidence that dual antagonism of Y2 and Y4 signaling may provide potent anti-obesity benefits, the mechanisms underlying the individual control and synergistic interactions between Y2 and Y4 signaling in the regulation of adiposity and energy homeostasis are unclear. Changes in the activity of several hypothalamopituitary axes may be involved in the regulation of body composition and energy metabolism by Y2 and Y4 signaling. For instance, germline Y2 or Y4 receptor knockout reduces expression of corticotropin-releasing hormone mRNA in the paraventricular nucleus of the hypothalamus in association with a tendency for decreased serum corticosterone concentrations on a lean background and normalization of hypercorticosteronemia of *ob/ob* mice (**26**, **40**, **44**, **45**). In addition to modulating output from the hypothalamo-pituitary-adrenal axis, Y2 signaling has been shown to mediate responses to glucocorticoids, since the obesity syndrome induced by exogenous corticosterone administration to wild-type mice is abolished in mice lacking Y2 signaling (**43**). Ablation of Y2 signaling abolishes fasting-induced reduction in the activity of somatotropic axis (**29**) and restores the low-serum IGF-1 levels in *ob/ob* mice (**44**), suggesting a role of Y2 signaling in regulating activity of the somatotropic axis, activation of which is known to promote the accretion of lean mass at the expense of fat mass (**19**). On the other hand, lack of Y4 signaling eliminates fasting-induced inhibition on the gonadotropic axis (**29**) and restores the low testosterone levels and fertility of *ob/ob* mice (**45**), indicating Y4 signaling may regulate energy metabolism via redistributing energy toward reproductive function. Surprisingly, changes in the activity of somatotropic and gonadotropic axes induced by Y2 and Y4 single deletion were not detected in nonfasted mice lacking both Y2 and Y4 receptors on a lean (**40**) or *ob/ob* background (**26**), suggesting that mechanisms other than changes in somatotropic and gonadotropic axis function may be involved in the synergistic control of energy balance by Y2 and Y4 signaling.

Whether altered food intake contributes to the lean phenotype of mice with single or double Y2 and Y4 receptor deletion is not clear. Earlier studies on food intake in these mice were conducted at various ages (8, 12, or 16 wk of age), with food spillage not being adjusted for in the majority of studies (**41**, **43**–**45**). Interestingly, however, several lines of evidence suggest an involvement of Y2 and Y4 signaling in the control of energy expenditure. Indeed, the expression of thyrotrophin-releasing hormone mRNA in the paraventricular nucleus of the hypothalamus is increased in mice lacking Y4 or Y2 and Y4 receptors (**40**), suggesting a role of Y4 signaling in the control of thyroid function, an important determinate of energy expenditure (**6**, **48**). Furthermore, the expression of uncoupling protein-1 in brown adipose tissue, an important regulator of thermogenesis and energy expenditure (**24**), is markedly decreased in *ob/ob* mice but is restored by Y2 receptor deletion (**44**). However, thyroid function and uncoupling protein-1 expression are indirect estimates of energy expenditure, and the control of energy expenditure by Y2 and Y4 signaling and the functional interaction of these two pathways in this process are thus unknown.

To allow direct and definitive investigation of the individual control and functional interaction between Y2 and Y4 signaling in the regulation of energy metabolism, we examined food intake in mice with single or double deletion of Y2 and Y4 receptors at a uniform age and accounted for spillage, which was recently shown to be significantly altered by Y receptor

ablation (**3**, **41**). We also employed indirect calorimetry to study the effects of single or double deletion of Y2 and Y4 receptors on energy expenditure as well as substrate oxidation, combined with measurement of physical activity and body composition. Furthermore, we examined the expression of key molecules involved in mitochondrial oxidation that may be mechanistically involved in the coordinated regulation of energy expenditure and substrate utilization by Y2 and Y4 signaling.

EXPERIMENTAL PROCEDURES

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; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia). Details of generation of the germline Y2 and Y4 receptor single and double-knockout mice have been previously published (**40**, **43**, **45**). Briefly, targeting constructs were designed that contain loxP flanking sequence upstream and downstream of the single coding exons of the Y2 and Y4 genes, respectively. After Cre-mediated recombination, this allows for the generation of null mice that do not contain any coding sequence including the translation start codon, thereby preventing any interference from minor remaining transcripts. Germline deletion of Y2 and Y4 receptor genes was achieved by crossing conditional-knockout floxed mice (Y2lox/lox or Y4lox/lox) with oocyte-specific Cre recombinase-expressing C57BL/6 mice. Y2–/–Y4–/– double-knockout mice were obtained by crossing Y2–/– and Y4–/– mice. All mice were on a mixed C57BL/6–129/SvJ background.

Food intake.
Food intake was measured in male Y2–/–, Y4–/–, and Y2–/–Y4–/– and wild-type mice at 11 wk of age. 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 was determined as the averages of triplicate readings taken over three 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. Fecal weight was also determined in triplicate during these analyses.

Indirect calorimetry.
Studies of indirect calorimetry were carried out on male Y2–/–, Y4–/–, Y2–/–Y4–/– and wild-type mice at 14–15 wk of age as described previously (**56**). Briefly, metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH). Preweighed 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. Food and water were available ad libitum. Mice were singly housed for 3 days prior to transferring into the calorimeter 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 ($\dot{V}o2$) and carbon dioxide production ($\dot{V}co2$) were measured every 27 min. The respiratory exchange ratio (RER) was calculated as the quotient of $\dot{V}co2/\dot{V}o2$, with 100% carbohydrate oxidation resulting in an RER of 1 and 100% fat oxidation resulting in an RER of 0.7 (**15**, **16**). Energy expenditure (kcal heat produced) was calculated as Calorific Value (CV) × $\dot{V}o2$, where CV is 3.815+1.232 × RER (**34**). Data for the 24-h monitoring period was averaged for 1-h intervals for energy expenditure (kcal/h) and RER.

Measurement of physical activity.
Physical activity was recorded continuously by infrared beam sensors using an OPTO-M3 sensor system with a 60-s data download interval (Columbus Instruments, Columbus, OH) at the same time as the indirect calorimetry measurements. This system provides both total counts (every time a beam is broken) and ambulatory counts (when a consecutive adjacent beam is broken) in the *x*- and *y*-axes directions. The recording of ambulatory counts does not

include the same beam being broken repeatedly and thus measures actual locomotion. Therefore, ambulatory counts in the *x*- and *y*-axes directions were used to measure physical activity, and continuous recording of individual mouse data were summed for 1-h intervals. To estimate resting metabolic rate and determine the possible contribution of changes in physical activity to changes in energy expenditure, a correlation analysis between physical activity and energy expenditure was performed as described previously (**7**). Briefly, each 1-h point of physical activity was plotted against the corresponding 1-h point of energy expenditure, and the correlation analysis was based on data collected over 24 h in individual mice. The function of the trend line was extrapolated to set the physical activity to zero and the *x*-axis intercept was used as an estimate of resting metabolic rate.

Analysis of body composition.
Upon completion of indirect calorimetry and physical activity measurements, animals were anesthetized with isoflurane and then subjected to dual-energy X-ray absorptiometry (DXA; Lunar PIXImus2 mouse densitometer; GE Healthcare, Waukesha, WI) to determine whole body fat mass and nonfat, nonbone mass. Nonfat, nonbone mass is referred to as lean mass in the manuscript for easier presentation. The head and the tail were excluded from the analysis of body composition.

Tissue collection.
Upon completion of the study, mice at 15 wk of age were culled between 12:00 and 15:00 h by cervical dislocation followed by decapitation. White adipose tissue depots (inguinal, epididymal retroperitoneal, and mesenteric) were removed and weighed. The quadriceps skeletal muscles were frozen until further analysis by Western blotting as described below.

Western blot analysis.
Western blot analysis was performed on quadriceps muscle samples following procedures described previously (**56**) to determine protein levels of key enzymes involved in mitochondrial oxidation. Briefly, powdered muscle samples were resuspended in radioimmunoprecipitation assay buffer (PBS, pH 7.5; 1% nonident NP-40; 0.5% sodium deoxy-cholate; and 0.1% SDS) supplemented with protease and phosphatase inhibitors (10 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na3VO4, and 10 mmol/l NaF) and solubilized for 2 h at 4°C. Equal amounts of tissue lysate (20 μg protein) were resolved by SDS-PAGE and immunoblotted with anti-bodies against peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α (Calbiochem, Merck, Kilsyth VIC, Australia), carnitine palmitoyltransferase-1 (CPT-1; Alpha Diagonistic San Antonio, TX), or an anti-body cocktail that recognizes several subunits of the mitochondrial respiratory chain (cat. no. MS601; Mitosciences, Eugene, OR). Immunolabeled bands were quantified by densitometry. Relative protein levels of the mutant mice as a percentage of that of control mice are presented.

Statistical analyses.
All data are expressed as means ± SE. 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) were carried out by ANCOVA with lean body mass as covariate, and the slopes of the regression lines were compared by a test of homogeneity of slopes by ANCOVA. Subsequent multiple post hoc comparisons were performed via the method of Bonferroni and the adjusted means of energy expenditure at a common lean mass were generated by ANCOVA. Equality of variance between groups was tested with the Levene's test. Statistical analyses were performed with SPSS for Mac OS X, version 16.0.1 (SPSS, Chicago, IL). Statistical significance was defined as $P < 0.05$.

RESULTS

Synergistic reduction in adiposity and increase in lean (nonfat, nonbone) tissue mass in response to double deletion of Y2 and Y4 receptors with no decrease in food intake.
Body weight of Y2−/−Y4−/− mice was no different from that of wild-type mice, while Y2−/−

and Y4–/– mice weighed significantly less than wild types (**Table 1**). Importantly, however, mice with single or double deletion of Y2 and Y4 receptors exhibited a lean phenotype, with Y2–/–Y4–/– mice being the leanest. Thus, whereas the epididymal white adipose tissue mass relative to body weight was significantly reduced in all knockouts compared with wild types, the reduction in weight of this tissue was significantly greater in Y2–/–Y4–/– than that in Y2–/– or Y4–/– mice (**Fig. 1*A***). This pattern of changes in adiposity among wild-type, Y2, and Y4 single or double knockouts exists in other dissected white adipose depots, namely the mesenteric, retroperitoneal, and inguinal white adipose tissue depots (**Fig. 1*A***), and is also apparent when adiposity was expressed as the summed weight of these dissected white adipose depots (**Fig. 1*B***) as well as whole body fat mass as determined by DXA scan (**Fig. 1*C***). The absolute weights of individual and summed dissected white adipose tissues as well as whole body fat mass as determined by DXA in wild-type, Y2–/–, Y4–/–, and Y2–/–Y4–/– mice (**Table 1**) showed similar differences to those seen when fat masses were expressed as relative weights (**Fig. 1**, *A–C*). These results suggest a synergy between Y2 and Y4 receptor signaling in the regulation of adiposity. Importantly, the ratio of the summed weight of the intraperitoneal white adipose tissue depots measured (epididymal, mesenteric, and retroperitoneal) vs. that of the subcutaneous depot measured (inguinal) was significantly reduced in Y2–/–Y4–/– but not in Y2–/– or Y4–/– mice compared with wild-type mice (**Table 1**), suggesting more prominent effects of the Y2 and Y4 synergistic interaction on intra-abdominal vs. subcutaneous fat depots. Furthermore, the whole body nonfat, nonbone mass (hereafter referred to as lean tissue mass) as determined by DXA was significantly increased in Y2–/–Y4–/– and to a lesser extent in Y4–/– mice (**Fig. 1*D***), suggesting also a synergy between Y2 and Y4 signaling in the regulation of lean tissue mass. In conjunction with the comparable body weight between wild-type and Y2–/–Y4–/– mice (**Table 1**), the synergistic reduction in fat mass (**Fig. 1**, *A–C*) and the concomitant increase in lean tissue mass (**Fig. 1*D***) in Y2–/–Y4–/– double-knockout mice suggests formation of lean tissue mass at the expense of fat mass in these animals. Interestingly, daily food intake was not significantly different among different genotypes, albeit there was a trend toward decreased food intake in Y2–/– and Y4–/– mice and a more apparent trend toward increased food intake in Y2–/–Y4–/– mice relative to wild types (3.47 ± 0.24, 3.19 ± 0.11, 3.21 ± 0.18, and 4.06 ± 0.16 g/day for wild-type, Y2–/–, Y4–/–, and Y2–/–Y4–/– mice, respectively, data are means ± SE of 6–10 mice per group at 14–15 wk of age measured over 3 consecutive days, not significant). Furthermore, daily fecal output was similar among groups, with a trend toward increased output in Y2–/–Y4–/– mice, in keeping with the food intake in these mice [0.926 ± 0.14, 0.856 ± 0.06, 0.862 ± 0.09 and 1.08 ± 0.10 g (wet wt)/day for wild-type, Y2–/–, Y4–/–, and Y2–/–Y4–/– mice, respectively, data are means ± SE of 6–10 mice per group at 14–15 wk of age, not significant].

Table 1. *Effects of single or double deletion of Y2 and Y4 receptors on body weight and adiposity*

	Wild Type	$Y2^{-/-}$	$Y4^{-/-}$	$Y2^{-/-}Y4^{-/-}$
Body weight	25.2 ± 0.7	22.9 ± 0.7*	21.3 ± 0.4*	25.5 ± 0.6
Epididymal white adipose tissue	0.544 ± 0.03	0.370 ± 0.03†	0.255 ± 0.01†‡	0.160 ± 0.004†‡
Mesenteric white adipose tissue	0.271 ± 0.03	0.216 ± 0.01	0.1542 ± 0.01*	0.131 ± 0.01*
Retroperitoneal white adipose tissue	0.151 ± 0.01	0.083 ± 0.01†	0.047 ± 0.003†	0.037 ± 0.002†‡
Inguinal white adipose tissue	0.545 ± 0.04	0.360 ± 0.02†	0.232 ± 0.01†‡	0.251 ± 0.02†
Summed weight of measured white adipose tissue depots	1.51 ± 0.1	1.03 ± 0.06†	0.688 ± 0.03†‡	0.579 ± 0.03†‡
Intra-abdominal-to-subcutaneous fat ratio	1.81 ± 0.08	1.88 ± 0.10	2.00 ± 0.11	1.33 ± 0.07*‡§
Whole body fat mass	3.60 ± 0.23	3.00 ± 0.16	2.21 ± 0.10†	2.45 ± 0.08†

Data are means ± SE of 6–10 mice per group in grams. Data were collected from mice at 14–15 wk of age. Whole body fat mass was determined by X-ray absorptiometry. The ratio of the summed weight of the intraperitoneal depots measured (epididymal, mesenteric, and retroperitoneal) vs. that of the subcutaneous depot measured (inguinal) is used as an indicator of intra-abdominal-to-subcutaneous fat ratio. *$P < 0.05$ and †$P < 0.001$ vs. wild type mice; ‡$P < 0.05$ vs. $Y2^{-/-}$ mice; §$P < 0.05$ vs. $Y4^{-/-}$ mice.

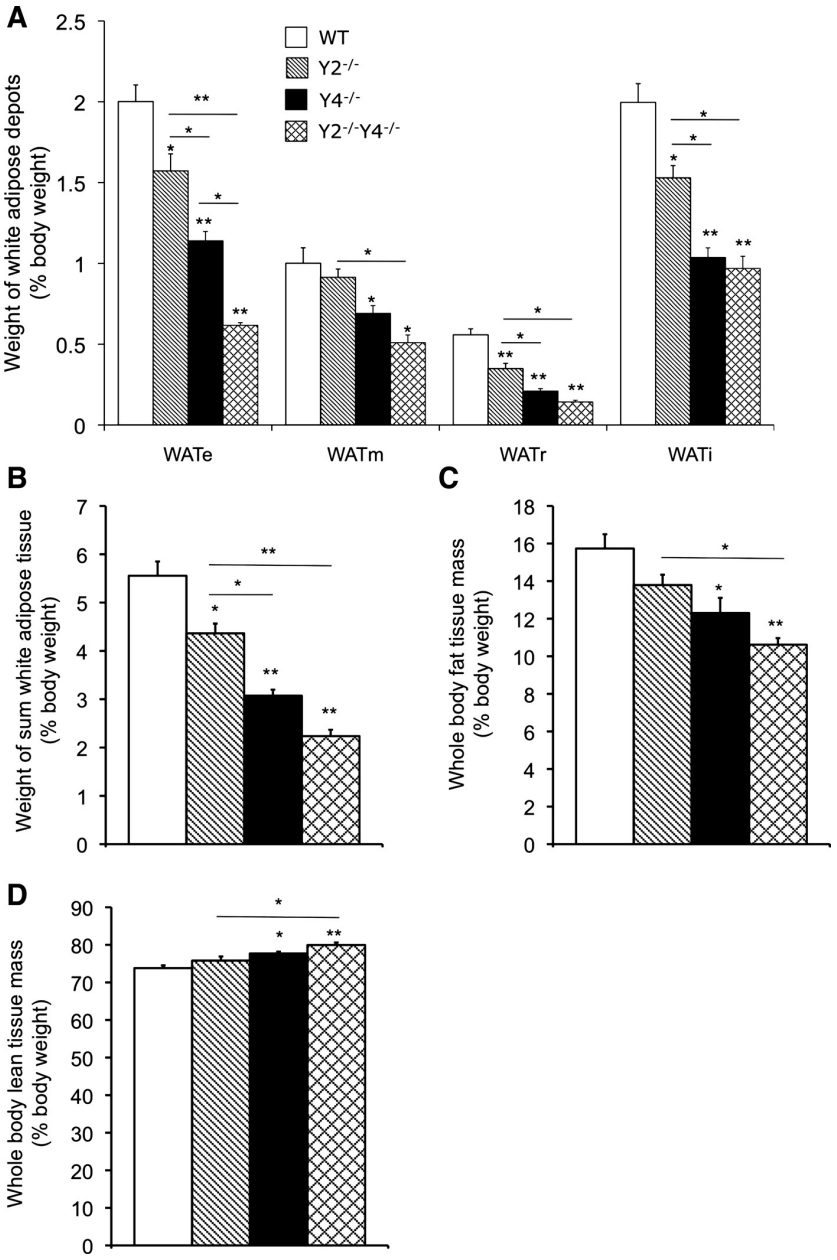

Fig. 1. Synergy between Y2 and Y4 receptors in the regulation of body composition. Mass of individual white adipose depots (*A*), namely epididymal (WATe), mesenteric (WATm), retroperitoneal (WATr), inguinal (WATi), and combined weight of these depots (*B*) as % body weight in wild-type (WT), $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice. Whole body fat mass (*C*) and nonfat, nonbone mass defined as lean mass (*D*) relative to body weight determined by dual-energy X-ray absorptiometry (DXA) in WT, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice. Data are means ± SE from 6–10 mice/group. $*P < 0.05$, $**P < 0.001$ vs. WT mice or comparison indicated by horizontal bars.

Synergistic increase in energy expenditure by double deletion of Y2 and Y4 receptors. To investigate mechanisms underlying the synergistic control of adiposity and lean mass by Y2 and Y4 receptors, we examined energy expenditure, the other side of the energy balance equation, and RER, an index of oxidative fuel source, by indirect calorimetry with concurrent measurement of physical activity in wild-type, Y2–/–, Y4–/–, and Y2–/–Y4–/– mice. Since energy expenditure is, in part, determined by body size, we normalized energy expenditure via ANCOVA (Fig. 2) to account for differences in lean tissue mass, the best determinant of energy expenditure (37) (see Table 2 for nonnormalized energy expenditure data). Energy expenditure followed a circadian rhythm in all groups, with higher energy expenditure in the dark period (Fig. 2*A*). Y2–/– mice had similar daily energy expenditure to wild types, with a slight decrease in energy expenditure during the light period (Fig. 2, *A* and *B*). In contrast, Y4–/– mice showed a significant increase in energy expenditure compared with wild-type mice during the dark period (Fig. 2, *A* and *B*). Interestingly, mice with double deletion of Y2 and Y4 receptors showed a marked increase in overall energy expenditure, which is significant during the dark period (Fig. 2, *A* and *B*). Importantly, the energy expenditure of Y2–/–Y4–/– mice in the dark phase was not only significantly higher than that

of wild types, also it was significantly higher than that of Y2–/– and Y4–/– mice (Fig. 2, *A* and *B*), suggesting a synergy between Y2 and Y4 signaling in the regulation of energy expenditure, which may contribute to their synergistic effects on adiposity.

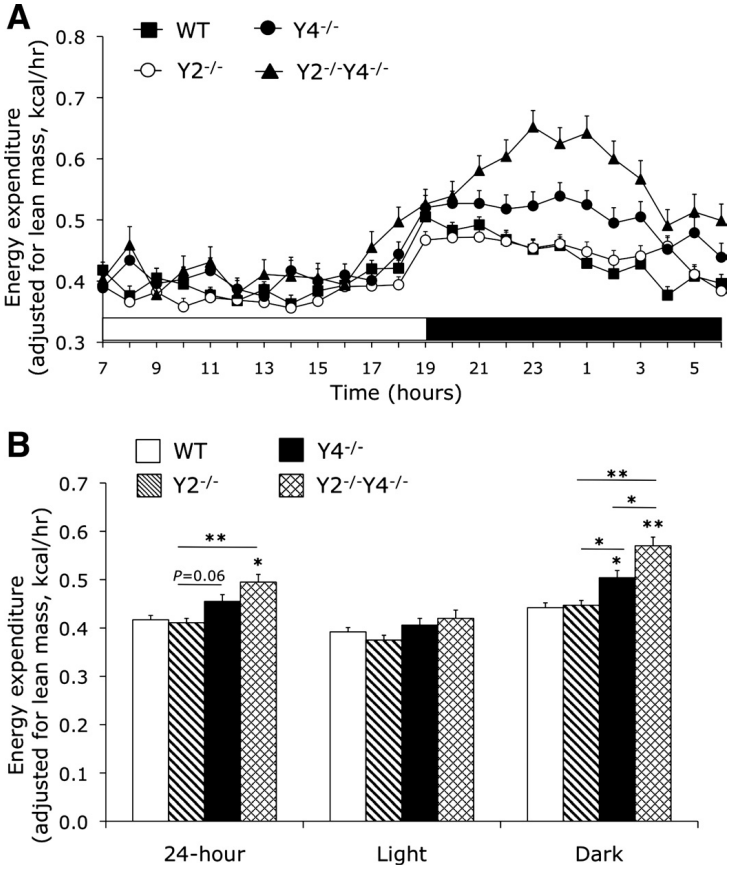

Fig. 2. Synergy between Y2 and Y4 receptors in the regulation of energy expenditure. Time course of energy expenditure (*A*) and averages for 24-h, light and dark phases (*B*) in WT, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice. Energy expenditure was adjusted for lean mass via ANCOVA: adjusted energy expenditure (common lean mass = 19.33 g) was presented. White and black horizontal bars on *A* indicate light and dark phase, respectively. Data are means ± SE of 6–10 mice per group. $*P < 0.05$, $**P < 0.001$ vs. WT mice or comparison indicated by horizontal bars.

Table 2. *Nonnormalized energy expenditure (EE, kcal/h) in wild-type, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice*

	Wild Type	$Y2^{-/-}$	$Y4^{-/-}$	$Y2^{-/-}Y4^{-/-}$
24-hour EE	0.432 ± 0.012	0.405 ± 0.011	0.428 ± 0.013	0.501 ± 0.019*‡§
Light phase EE	0.406 ± 0.012	0.369 ± 0.010	0.381 ± 0.014	0.425 ± 0.021
Dark phase EE	0.459 ± 0.012	0.440 ± 0.013	0.475 ± 0.014	0.577 ± 0.021†‡§
Resting EE	0.340 ± 0.012	0.301 ± 0.014	0.344 ± 0.011	0.387 ± 0.021‡
Resting EE, adjusted for lean mass	0.334 ± 0.012	0.312 ± 0.013	0.355 ± 0.019	0.384 ± 0.022‡

Data are means ± SE of 6–10 mice/group. Data is averaged for 24 h as well as in the light and dark phases, and resting EE (nonnormalized and adjusted for lean mass), as determined by correlation analysis between energy expenditure and physical activity. ANCOVA was performed on resting metabolic rate using lean tissue mass as covariate, and the adjusted value was generated on the common lean mass of 19.36 g. $*P < 0.05$ and $†P < 0.001$ vs. wild-type mice; $‡P < 0.05$ vs. $Y2^{-/-}$ mice; $§P < 0.05$ vs. $Y4^{-/-}$ mice.

Synergistic increase in physical activity by double deletion of Y2 and Y4 receptors. All groups showed a clear circadian rhythm in physical activity, with markedly higher activity levels during the dark period when rodents are most active (**Fig. 3*A***). Importantly, physical activity, an important factor influencing energy expenditure, was significantly increased in Y4–/– but not Y2–/– mice in the dark phase, resulting in an overall significantly increased daily activity levels in Y4–/– mice (**Fig. 3**, *A* and *B*). These data demonstrate an important role of Y4 signaling in the control of physical activity and suggest that an increase in physical activity may contribute to the increase in energy expenditure observed in Y4–/– mice. Interestingly, whereas Y2 receptor single deletion had no significant effect on physical activity (**Fig. 3**, *A* and *B*), double deletion of Y2 and Y4 receptors lead to a marked and significant increase in physical activity relative to wild-type levels that was also significantly greater than that induced by Y4 receptor deletion per se (**Fig. 3**, *A* and *B*). These data reveal an indirect control of physical activity by Y2 signaling via a synergistic interaction with Y4, likely contributing to the increases in energy expenditure and whole body lean mass observed in Y2–/–Y4–/– mice.

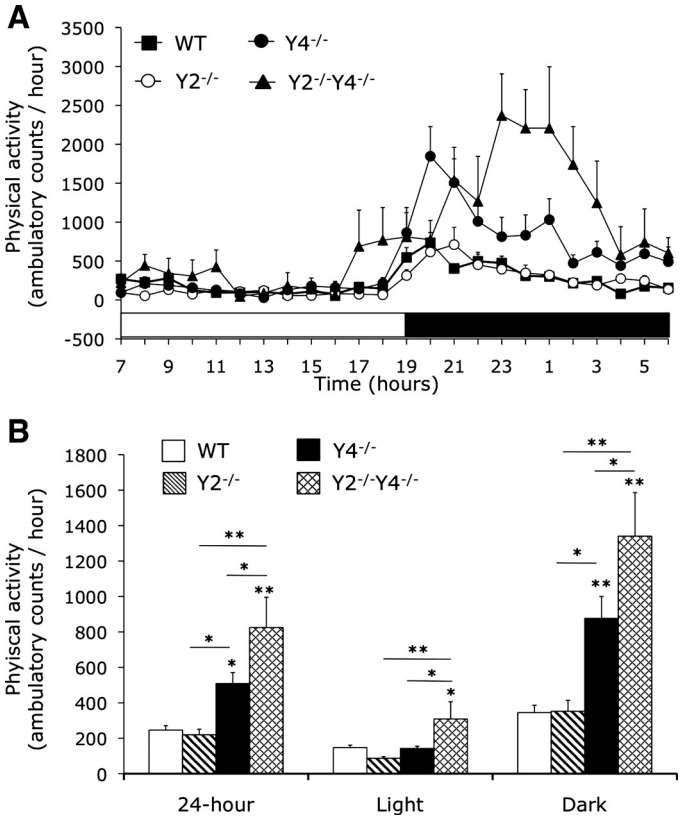

Fig. 3. Synergy between Y2 and Y4 receptors in the regulation of physical activity. Time course of physical activity (*A*) and averages for 24-h, light and dark phases (*B*) in WT, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice. White and black horizontal bars on *A* indicate light and dark phase, respectively. Data are means ± SE of 6–10 mice per group. $*P < 0.05$, $**P < 0.001$ vs. WT mice or comparison indicated by horizontal bars.

To determine whether an increase in basal metabolic rate, in addition to the measured increase in physical activity, may contribute to the increases in total energy expenditure observed in Y4–/– and Y2–/–Y4–/– mice, we performed a correlation analysis between physical activity and energy expenditure. When hourly data from individual mice were analyzed for correlations between energy expenditure and physical activity, there was a positive correlation between the two parameters ($P < 0.05$ by Pearson correlation for all animals studied). Using the function from the trend line and extrapolating to set the physical activity to zero, we found that the *x*-axis intercept, an index of resting metabolic rate (**7**), was higher in Y4–/– and particularly in Y2–/–Y4–/– compared with wild-type control mice, although this increase was not statistically significant (**Table 2**). These data suggest that an

increase in basal metabolism may be an important contributor to the increased total energy expenditure observed in Y4–/– and Y2–/–Y4–/– mice.

Altered substrate utilization in response to either Y2 or Y4 receptor deletion.

To identify whether alterations in substrate utilization may contribute to the lean phenotypes of the single and double Y2 and Y4 knockout models, we measured the RER of these mice. Y2–/– and Y4–/– mice exhibited significant reductions in overall RER compared with wild-type mice, suggesting a greater use of lipid as an oxidative fuel source and/or reduced lipogenesis (**Fig. 4**, *A* and *B*). Furthermore, Y4 signaling appears to have a greater control on fuel selection than Y2 signaling, as the decrease in RER during the light period was more pronounced in Y4–/– than in Y2–/– mice (**Fig. 4**, *A* and *B*). Interestingly, in contrast to the synergy between Y2 and Y4 receptors in the control of energy expenditure and physical activity, the Y2 and Y4 receptor seem not to regulate substrate partitioning pathways synergistically, because, whereas Y2–/–Y4–/– mice had reduced RER compared with wild types, the pattern and magnitude of this reduction was similar to that in Y4–/– mice (**Fig. 4**, *A* and *B*). Taken together, these data demonstrate the critical role Y2 and Y4 receptor signaling in the control of oxidative fuel metabolism and also suggest an important contribution to increased lipid oxidation and/or decreased lipogenesis by Y2 and Y4 receptors. However, other mechanisms, such as increased energy expenditure and physical activity, may play the key role in the synergistic reduction in adiposity in Y2–/–Y4–/– mice.

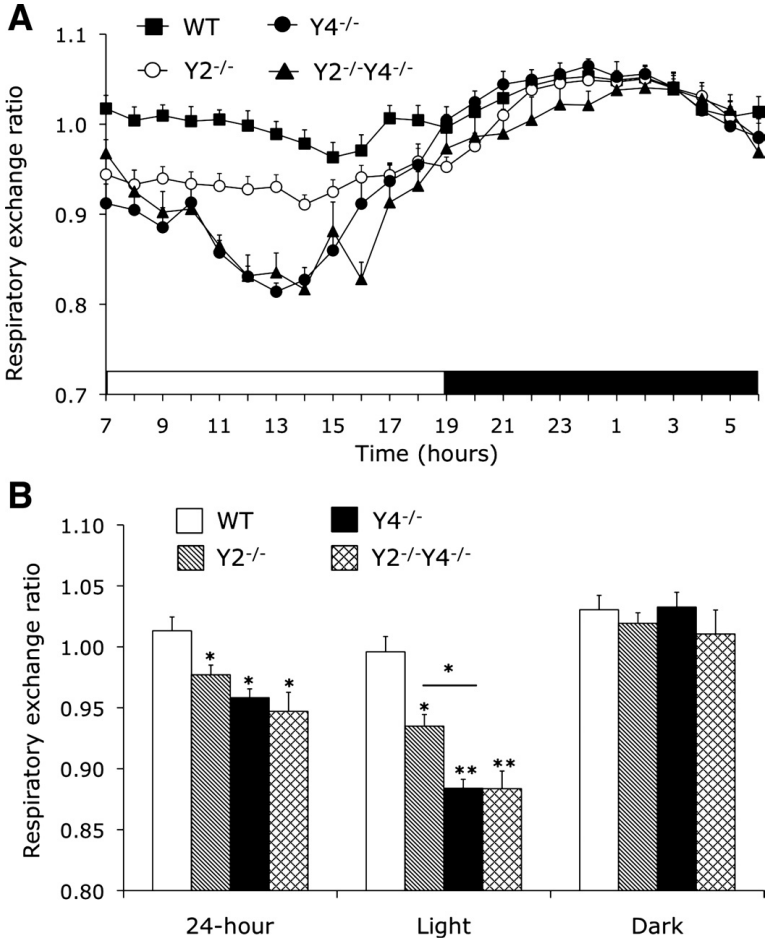

Fig. 4. Altered substrate utilization by single or double deletion of Y2 and Y4 receptors. Time course of respiratory exchange ratio (an index of oxidative fuel) (*A*) and averages for 24 h, light, and dark phases (*B*) in WT, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice. White and black horizontal bars on *A* indicate light and dark phase, respectively. Data are means ± SE of 6–10 mice per group. $*P < 0.05$, $**P < 0.001$ vs. WT mice or comparison indicated by horizontal bars.

Enhanced muscle mitochondrial oxidative capacity in mice with double deletion of Y2 and Y4 receptors.

To investigate mechanisms by which Y2 and Y4 receptors regulate energy expenditure, we examined by Western blot analysis the muscle protein levels of several key molecules involved in mitochondrial oxidation, the key cellular processes that convert biochemical energy to a form that can be used for biological work. Y2–/– and Y4–/– (**Fig. 5**, *A* and *B*) mice were comparable to wild types with regard to the muscle protein levels of subunits of the respiratory chain complexes I, II, III, and V, as well as that of the PGC-1α, an important regulator of mitochondrial biogenesis (**28**). In contrast, muscle protein levels of subunits I and III of the respiratory chain complex, as well as that of PGC-1α, were significantly increased in Y2–/–Y4–/– mice compared with wild types (**Fig. 5*C***), suggesting an increased mitochondrial oxidative capacity caused by dual deletion of Y2 and Y4 receptors. These data suggest a synergy between Y2 and Y4 signaling in the control of mitochondrial oxidative capacity and are consistent with the synergistic increase in energy expenditure measured in Y2–/–Y4–/– mice (**Fig. 2**). Interestingly, all knockout mice exhibited a significant increase relative to wild types in muscle 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 (**33**, **39**), with no synergistic effects observed in Y2–/–Y4–/– mice (**Fig. 5**). These data suggest an increased capacity to transport fatty acids into mitochondria for oxidation in mice lacking either one or both of the Y2 and Y4 receptor, likely contributing to the increased lipid oxidation indicated by the reduced RER seen in Y2–/–, Y4–/–, and Y2–/–Y4–/– mice.

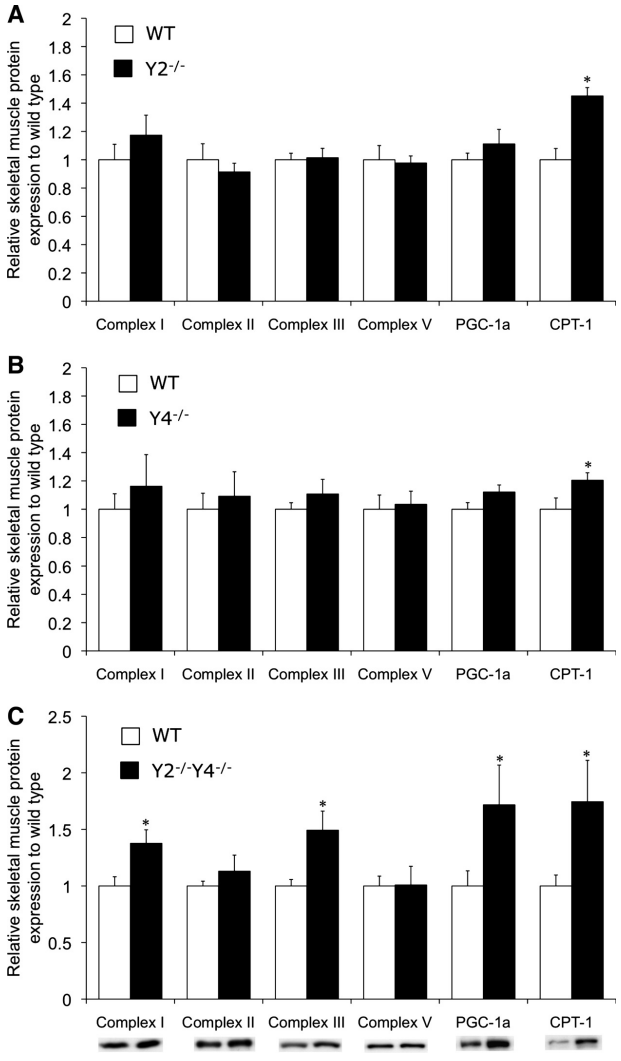

Fig. 5. Mitochondrial oxidation in $Y2^{-/-}$ (*A*), $Y4^{-/-}$ (*B*), and $Y2^{-/-}Y4^{-/-}$ (*C*) mice. Protein levels of subunits I, II, III, and V of the mitochondrial respiratory chain complex, peroxisome proliferator-activated receptor (PPAR)γ coactivator −1α (PGC-1α), and carnitine palmitoyltransferase-1 (CPT-1) in the skeletal muscle. Data are means ± SE of 5–8 mice per group and are expressed as % wild-type values. $*P < 0.05$ vs. WT.

DISCUSSION

This study shows that signaling through Y2 and Y4 receptors is critical for the regulation of body composition, energy expenditure, and physical activity, since lack of Y2 and Y4 receptors in mice results in a striking and synergistic reduction in adiposity (particularly intra-abdominal adiposity) and a marked increase in lean body mass (nonfat nonbone mass as determined by DXA) with no significant change in food intake, concomitant with marked and significant synergistic increases in energy expenditure and physical activity. Moreover, these findings reveal a direct role of Y4 signaling and a synergistic interaction with Y2 signaling in the regulation of energy expenditure and physical activity, whereas Y2 and Y4 signaling each play important roles in the regulation of substrate utilization without synergistic action. Double deletion of Y2 and Y4 receptors leads to significant increases in muscle protein levels of subunits I and III of the respiratory chain complex as well as that of PGC-1α that are not seen in mice with single knockouts, suggesting that Y2 and Y4 signaling may interact to regulate mitochondrial oxidative capacity and thereby contributing to the synergistic increase in energy expenditure and decrease in fat mass seen in Y2–/–Y4–/– mice.

Lack of Y2 signaling has been shown to provide protection against obesity induced by corticosterone infusion (**43**), a high-fat diet (**41**), Y1 receptor deficiency (**41**), or leptin deficiency (**44**). Our new data now show that the protection conferred by Y2 receptor deletion against obesity is not likely to come from changes in energy expenditure or physical activity, as Y2–/– mice had similar overall energy expenditure and physical activity to that of wild-type controls. In keeping with the lack of increase in overall energy expenditure in Y2–/– mice, chronic central activation of Y2 receptors by the Y2 receptor agonist Ac-NPY(24–36)(Leu28, Leu30) did not change oxygen consumption (**18**). Interestingly, our study shows that Y2–/– mice had a slight but significant decrease in RER during the light phase, indicating an increased lipid oxidation and/or decreased lipogenesis. It should be noted that energy balance is a factor influencing RER (**32**), and our Y2–/– mice showed a trend toward decreased daily food intake. Although energy expenditure during the light period is also decreased in Y2–/– mice, it is possible that Y2–/– mice are in energy deficiency at least during some of the 24-h day, and this may contribute to the lower body weight, the lean phenotype, and also the reduced RER seen in these mice. However, the increased muscle protein levels of CPT-1, an increase that enhances lipid oxidation (**11**), in the muscle of Y2–/– mice support a primary role of Y2 signaling on substrate utilization. Importantly, this change in substrate utilization in conjunction with other actions induced by Y2 receptor deletion such as stimulation of the somatotropic axis (**29**, **44**) may contribute to the anti-obesity effects of Y2 receptor ablation. Indeed, it has recently been shown that activation of Y2 receptors by NPY stimulates fat angiogenesis and adipogenesis in preadipocytes primed for differentiation and promotes adipoctye proliferation (**25**). Thus it appears likely that Y2 receptor signaling controls nutrient uptake in adipose tissue and promotes lipogenesis. Lack of Y2 receptor signaling therefore results in reduced white adipose tissue mass with coinciding increases in nonfat tissues such as bone, the latter effect being due to increased osteoblast activity and an increased rate of bone mineralization and formation (**4**). Taken together, our current data suggest that Y2 signaling plays a role in the regulation of fuel selection but not in the control of energy expenditure or physical activity, and that the protection against obesity upon Y2 receptor deletion is more likely the result of altered substrate utilization and energy partitioning between fat and lean mass.

In contrast to the lack of effect of Y2 receptor ablation on energy expenditure and physical activity, our study demonstrates a prominent role of Y4 signaling in the regulation of these processes. Interestingly, Y2 signaling appears to interact and enhance the effects mediated by Y4 signaling, since energy expenditure and physical activity is significantly more increased in Y2–/–Y4–/– than in Y4–/– mice. The synergistic effects on energy expenditure induced by Y2 and Y4 receptors may be mediated at least in part by their concomitant synergistic actions on physical activity. Furthermore, we showed that increased basal metabolic rate may also contribute to the increased total energy expenditure in mice lacking Y4 receptors. This is consistent with the increase in activity of the hypothalamo-pituitary-thyroid axis that has been reported in Y4–/– and Y2–/–Y4–/– mice (**40**), thyroid function being an important regulator of metabolic rate (**6**, **48**). Furthermore, the daily food intake in Y2–/–Y4–/– mice tended to be higher than that of wild types, in contrast to a trend toward a lower food intake in Y2–/– or Y4–/– mice relative to wild types. It is important to note that 5–

10% differences in daily food intake can have a significant impact on body weight and body composition over the long term, and such small differences in daily food intake may not be detectable or reach statistical significance when measurement is performed over a short period of time such as the 3 days in the current study. Thus, whereas it is possible that a decrease in daily food intake in Y2–/– and Y4–/– mice may contribute to the decreased body weight and lean phenotype in these mice, the synergistic increase in energy expenditure induced by Y2 and Y4 receptor double deletion likely plays a critical role in the synergistic reduction in adiposity seen in Y2–/–Y4–/– mice. Moreover, it is interesting to note that a high energy turnover balance (i.e., higher energy intake and expenditure with maintained energy balance) has recently been shown to influence metabolism differently from low energy turnover balance, at least in the short term (**12**), which could conceivably have implications for the long-term regulation of body weight and composition. Although the energy balance status wasn't directly assessed in the present study, the trend to a higher daily food intake and significantly greater energy expenditure in Y2–/–Y4–/– mice is consistent with a higher energy flux, and this could mediate some of the metabolic effects seen in these mice. Interestingly, whereas increased lipid oxidation may contribute to the lean phenotype of Y2–/–Y4–/– mice, it is unlikely to play a key role in mediating the synergy between Y2 and Y4 signaling in the regulation of fat mass, as the reduction in RER, indicating increased lipid oxidation and/or decreased lipogenesis, as well as the increase in muscle protein levels of CPT-1, a key regulator of mitochondrial lipid oxidation, was comparable between Y2–/–Y4–/– and Y2–/– or Y4–/– mice. In conclusion, our data show the critical roles of Y4 signaling in the regulation of energy metabolism, physical activity, and substrate oxidation, with synergistic interactions with Y2 signaling in the control of energy expenditure and physical activity but not substrate oxidation, likely contributing to the synergistic reduction in adiposity observed in Y2–/–Y4–/– mice.

An increased capacity for mitochondrial oxidation, indicated by the significant and synergistic increase in the muscle protein levels of PGC-1α and subunits I and III of the respiratory chain complex in Y2–/–Y4–/– mice, may be mechanistically involved in the synergistic increase in total energy expenditure, and may particularly contribute to the increased basal metabolic rate seen in these animals. Furthermore, the altered substrate utilization favoring fat burning induced by single or double deletion of Y2 and Y4 receptors may be mediated by an increased capacity for fatty acid transport into mitochondria, as indicated by the significantly increased muscle protein levels of CPT-1, since overexpression of CPT-1 in skeletal muscle enhances fatty acids influx into mitochondria and increases lipid oxidation (**10**). It is unclear, however, whether Y2 and Y4 signaling regulates these mitochondrial functions via direct or indirect, peripheral or central mechanisms. The significant increases in muscle protein levels of PGC-1α in Y2–/–Y4–/– mice is in line with an indirect mechanism via Y2 and Y4 signaling-induced effects on physical activity, since PGC-1α is a downstream target of exercise (**1**, **51**) and an important regulator of mitochondrial biogenesis and oxidative capacity as well as substrate oxidation (**9**, **28**, **54**). It is interesting to note, however, that, whereas Y4–/– mice also showed increased physical activity, muscle capacity for mitochondrial oxidation was not increased, at least when assessed by the protein expression levels of molecules involved in mitochondrial oxidation. Thus it appears likely that the further increase in physical activity induced by the Y2 deletion in addition to Y4 is required to increase mitochondrial oxidative capacity. A central mechanism of effect on physical activity, as similarly suggested for Y1 receptors (**56**), may mediate the primary control by Y4 signaling and synergistic interaction with Y2 signaling on physical activity. On the other hand, regulation by Y2 receptors of the capacity for fatty acid transport into mitochondria likely involved a peripheral mechanism, since deletion of Y2 receptor in hypothalamic NPY-ergic neurons results in decreased rather than increased muscle protein level of CPT-1 (**47**).
The profound reduction in fat mass seen in our in Y2–/–Y4–/– mice is associated with a marked increase in lean mass, as determined by DXA, in the absence of any change in body weight. In conjunction with our previous report showing a synergy between Y2 and Y4 signaling to increase bone mass (**40**), these findings suggest the formation of lean and bone mass at the expense of fat mass in the absence of Y2 and Y4 signaling. Energy redistribution from fat to lean tissues via Y2 receptors as discussed above may contribute significantly to the observed synergistic effects on body composition in Y2–/–Y4–/– mice. Furthermore, although Y4 receptor deletion alone did not alter bone metabolism (**40**), Y4 deletion may

contribute to the effect of Y2 receptor deletion on lean mass and bone mass by increasing physical activity, which is known to modulate muscle mass and stimulate bone growth (**13**, **14**).

Whereas our findings suggest that dual antagonism of Y2 and Y4 receptors may provide potent anti-obesity effects while also promoting lean body mass and bone mass, it is noteworthy that PYY3–36 and PP, the endogenous agonists for Y2 and Y4 receptors, respectively, have been proposed as anti-obesity agents due to their inhibitory effects on food intake and gastric emptying (**5**, **38**, **46**). Additionally, there are reports of increased energy expenditure in response to acute PYY3–36 or PP administration (**2**, **49**). However, this effect of Y2 or Y4 receptor agonism appears to be transient, as no increase in energy expenditure was observed after long-term elevation of PYY3–36 or PP levels by chronic administration or overexpression (**52**, **53**). Our study on knockout mouse model suggests that long-term dual Y2 and Y4 receptor antagonism may increase energy expenditure. More importantly, dual Y2 and Y4 receptor antagonism reduces fat mass with concomitant increases in lean tissue mass, in contrast to the decrease in lean tissue mass often observed during weight/fat loss (**21**). Moreover, the more prominent reduction in intra-abdominal vs. subcutaneous fat induced by dual Y2 and Y4 receptor antagonism as seen in this study may confer a greater metabolic benefit than that of overall fat loss, since subcutaneous fat has been suggested to have protective effects on insulin sensitivity and glucose tolerance (**20**) and since waist-to-hip ratio, influenced by the ratio of intra-abdominal to subcutaneous fat, is emerging as a better predictor of health than central adiposity alone (**30**). Finally, as discussed previously, a peripheral-acting Y2 antagonist and a central-acting Y4 antagonist may be an effective combination as anti-obesity therapeutics.

Perspectives and Significance

This is the first work investigating the individual control and coordinated interactions between Y2 and Y4 signaling in the regulation of energy metabolism using indirect calorimetry. This study shows that Y4 signaling is critically involved and synergistically interacts with Y2 signaling in the regulation of energy expenditure and physical activity, whereas Y2 and Y4 signaling may each exert important roles in the control of oxidative fuel selection without significant interaction. These coordinated actions lead to greater reductions in adiposity with concomitant greater increases in lean tissue mass in Y2–/–Y4–/– mice than the changes seen in response to deletion of either receptor alone. One limitation of this study is that, whereas daily food intake was determined using a refined protocol that accounted for food spillage and was measured in mice of the same age, it was only measured over three consecutive days. As such, small difference in daily food intake that could have significant effects on body weight over a longer period of time may not have been detectable. However, the trend to an increase in food intake in Y2–/–Y4–/– mice suggests that the significant increase in energy expenditure and/or a high energy turnover seen in these mice may play a critical role in mediating the lean phenotype induced by ablation of both Y2 and Y4 receptors. We further show that an increased capacity for mitochondrial oxidation and fatty acid transport may be mechanistically involved to increase energy expenditure and alter substrate utilization favoring fat burning in mice with Y2 and Y4 receptor double deletion. However, it remains to be determined whether Y2 and Y4 signaling regulates these mitochondrial functions via direct or indirect, peripheral or central mechanisms. Conditional and tissue-specific Y2 and/or Y4 receptor knockout models will be needed to further investigate this effect. Importantly however, our findings highlight the possibility that dual antagonism of Y2 and Y4 receptors may offer a potent and effective adjunct in the treatment of obesity.

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