original article

NPY modulates PYY function in the regulation of energy balance and glucose homeostasis

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Aims: Both the neuronal-derived neuropeptide Y (NPY) and the gut hormone peptide YY (PYY) have been implicated in the regulation of energy balance and glucose homeostasis. However, despite similar affinities for the same Y receptors, the co-ordinated actions of these two peptides in energy and glucose homeostasis remain largely unknown.

Methods: To investigate the mechanisms and possible interactions between PYY with NPY in the regulation of these processes, we utilized NPY/PYY single and double mutant mouse models and examined parameters of energy balance and glucose homeostasis.

Results: $PYY^{-/-}$ mice exhibited increased fasting-induced food intake, enhanced fasting and oral glucose-induced serum insulin levels, and an impaired insulin tolerance, − changes not observed in $NPY^{-/-}$ mice. Interestingly, whereas PYY deficiency-induced impairment in insulin tolerance remained in $NPY^{-/-}PYY^{-/-}$ mice, effects of PYY deficiency on fasting-induced food intake and serum insulin concentrations at baseline and after the oral glucose bolus were absent in $NPY^{-/-}PYY^{-/-}$ mice, suggesting that NPY signalling may be required for PYY's action on insulin secretion and fasting-induced hyperphagia. Moreover, $NPY^{-/-}PYY^{-/-}$, but not $NPY^{-/-}$ or $PYY^{-/-}$ mice had significantly decreased daily food intake, indicating interactive control by NPY and PYY on spontaneous food intake. Furthermore, both $NPY^{-/-}$ and $PYY^{-/-}$ mice showed significantly reduced respiratory exchange ratio during the light phase, with no additive effects observed in $NPY^{-/-}PYY^{-/-}$ mice, indicating that NPY and PYY may regulate oxidative fuel selection via partly shared mechanisms. Overall, physical activity and energy expenditure, however, are not significantly altered by NPY and PYY single or double deficiencies.

Conclusions: These findings show significant and diverse interactions between NPY and PYY signalling in the regulation of different aspects of energy balance and glucose homeostasis.

Keywords: energy regulation, glucose metabolism, insulin secretion

Introduction

A complex picture has emerged as to the regulation of energy balance and glucose homeostasis in mammals, which involve co-ordinated actions from neuronal factors and endocrine signals from peripheral tissues such as the gut [1]. Peptides from the neuropeptide Y (NPY) family are unique in this regard in that they comprise both a neuronal molecule, NPY, as well as the gut-derived hormones, peptide YY (PYY) and pancreatic polypeptide.

NPY is well known for its orexigenic effects. It has a wide distribution in the central and peripheral nervous system, with high expression levels in the hypothalamus, the key brain region involved in the control of energy and glucose

homeostasis [2,3]. PYY is primarily released from the endocrine L cells of the lower gastrointestinal tract in response to food ingestion. It exists in the circulation in two forms; the full-length PYY1-36, and the truncated form, PYY3-36, which is cleaved from PYY1-36 by the specific cell surface enzyme dipeptidyl peptidase-IV [4–8]. It is thought that the postprandial release of PYY from the gut induces satiety. Recently, it was shown that PYY is also synthesized by taste cells in the taste buds of the tongue, with PYY from this source also being implicated in the satiety effect [9]. In addition to their well-documented effects on feeding behaviour, both NPY and PYY play key roles in regulating a spectrum of parameters of energy and glucose metabolism, such as body weight, body composition, energy expenditure, oxidative fuel oxidation, physical activity and insulin sensitivity [1]. At least five G-protein-coupled Y receptors—Y1, Y2, Y4, Y5 and Y6—have been identified in central and peripheral tissues as mediating effects of NPY family peptides [10,11]. NPY

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and PYY, share a similar pharmacological profile for all Y receptors, with a rank order of potency of Y2 > Y1 > Y5 ≫ Y4 > Y6 [10,11]. On the other hand, the processed forms of NPY3-36 and PYY3-36 preferentially bind to Y2 receptors and to a lesser extent to Y5 receptors [10,11]. Interestingly, whereas all Y receptors have been shown to be involved in various aspects of energy balance and glucose homeostasis [12], different Y receptors are likely to elicit differential effects. For instance, conditional knockdown of peripheral Y2 receptors results in an increased respiratory exchange ratio (RER, an index of oxidative fuel selection), indicative of decreased lipid oxidation and/or increased lipogenesis [13], whereas diminished peripheral Y1 signalling leads to the opposite effect [14]. Moreover, Y receptors in the periphery and in the brain may also elicit differential effects. For example, activation of Y1 signalling in the ventromedial hypothalamus is thought to mediate hyperinsulinaemia [15], whereas Y1 receptors expressed in pancreatic tissue have been suggested to inhibit insulin secretion [16–18]. In light of these differential effects mediated by different Y receptors located in central versus peripheral sites, and considering the different expression patterns and pharmacological profiles of NPY, PYY and its truncated versions, it is possible that NPY and PYY co-ordinately interact to regulate components of energy balance and glucose homeostasis. Whereas NPY and PYY have each been studied intensively with NPY or PYY single mutant mouse models being generated to study various aspects of energy balance and glucose homeostasis [19–24], the complex interactions between NPY and PYY signalling has not been addressed. To investigate the mechanisms via which NPY and PYY regulate energy and glucose metabolism, as well as the possible interactions between PYY with NPY in the regulation of these processes, we generated NPY/PYY double mutant mice and examined parameters of energy balance and glucose homeostasis in these animals.

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 hours). All mice were fed a normal chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia) *ad libitum* unless otherwise stated. Water was available *ad libitum* at all times. Germline $PYY^{-/-}$ and $NPY^{-/-}$ mice were generated by homologous recombination in embryonic stem cells as described previously [23,25]. $NPY^{-/-}$ and $PYY^{-/-}$ mice were crossed to generate double heterozygotes, and subsequent double knockout $NPY^{-/-}PYY^{-/-}$ mice. Double knockout was confirmed by Southern blot analysis and immunohisto-chemistry [26]. All mice were on a mixed C57Bl/6-129/SvJ background.

Measurements of Food Intake and Body Weight

Body weight was monitored weekly from 5 to 16 weeks of age in wild type (WT), $NPY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice. Subsets of 12-week-old WT, $PYY^{-/-}$, $NPY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice were examined for daily food intake and 24-h fasting-induced food intake. Mice were transferred from group housing on soft bedding to individual cages with paper towel bedding and allowed to acclimatize for 2–4 nights. Food intake was determined as the average 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 spilled in the cage. During the following week, the effect of 24 h fasting on feeding and body weight was examined. Food intake was determined as described above after 1 day of re-feeding. Body weight was determined before and after the 24-h fast, as well as after 1 day of re-feeding.

Oral Glucose Tolerance Test

Mice at 14 weeks of age were used for an oral glucose tolerance test. As NPY plays an important role in regulating stress responses, and since stress induced by oral glucose administration by gavage may hinder the investigation in mice lacking NPY, we trained mice to voluntarily eat a vehicle jelly that would contain glucose on the day of experimentation. Training and vehicle jelly preparation were as described previously [27,28]. Mice were fasted for 24 h and were then given an oral glucose bolus (3 g/kg) delivered as a glucose jelly that was consumed within 1 min. Glucose (0.52 g/ml) was incorporated into a jelly containing 4.9% wt/vol gelatin and 7.5% imitation strawberry flavouring essence. Tail vein blood was collected at 0, 5, 15, 30, 60 and 120 min after the mouse had finished eating the glucose jelly, and blood was collected for the determination of serum glucose and insulin levels as described below. Glucose tolerance curves for serum glucose and insulin are presented as absolute values. Area under the serum glucose or insulin concentration curves between 0 and 60 min after glucose ingestion were calculated after subtracting glucose or insulin concentrations before glucose ingestion.

Insulin Tolerance Test

For a subset of 14-week old mice, food was removed from cage hoppers at 8.30 h, and 5–6 h later a dose of insulin (0.5 IU/kg, Actrapid, Novo Nordisk, Baulkham Hills, NSW, Australia) was injected into the peritoneal cavity. Approximate 5 μl blood was taken from the tip of the tail at 0, 15, 30, 45, 60 and 75 min after insulin injection for blood glucose measurement using a Glucometer (Accu-Check Go, Roche, Basel, Switzerland).

Determination of Metabolic Rate, RER and Physical Activity

Metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH, USA) as described previously [14]. Briefly, pre-weighed mice were housed individually in specially built Plexiglass cages (20.1 × 10.1 ×

12.7 cm). Temperature was maintained at 22 °C with airflow of 0.6 l/min. Mice were singly housed for at least 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 (VO_2) and carbon dioxide production (VCO_2) were measured every 27 min. The RER was calculated as the quotient of VCO_2/VO_2, with 100% carbohydrate oxidation giving rise to a value of 1, and 100% fat oxidation giving rise to a value of 0.7 [29,30]. Energy expenditure (kcal heat produced) was calculated as calorific value (CV) $\times VO_2$, where CV is $3.815 + 1.232 \times$ RER [31]. Data for the 24-h monitoring period was averaged for 1-h intervals for energy expenditure and RER. Ambulatory activity of individually housed mice was evaluated within the metabolic chambers using an OPTO-M3 sensor system (Columbus Instruments, Columbus, OH, USA), 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

Upon completion of indirect calorimetry and physical activity measurements, animals were anaesthetized with isoflurane and then scanned for whole-body lean and fat mass using Dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus2 mouse densitometer; GE Healthcare, Waukesha, WI, USA). The head and tail were excluded in DEXA scans.

Tissue Collection

At 15 weeks of age, animals were culled between 12:00 and 15:00 hours by cervical dislocation followed by decapitation. White adipose tissue depots (inguinal, epididymal, mesenteric and retroperitoneal) and interscapular brown adipose tissue depots were removed and weighed.

Serum Analyses

Serum insulin levels were measured using an ELISA Kit from Mercodia (Uppsala, Sweden). Serum glucose levels in oral glucose tolerance test samples were determined with a glucose oxidase kit (Trace Scientific, Melbourne, Australia).

Statistical Analyses

All data are expressed as means ± 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 WT mice were assessed by analysis of variance (ANOVA) or repeated measures ANOVA. Comparisons of energy expenditure (kcal/h) were carried out by analysis of covariance (ANCOVA) with lean body mass as the covariate. The adjusted means of energy expenditure at a common lean mass for the comparison were generated by ANCOVA. 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

Effects of NPY and PYY Deletion on Body Weight, Adiposity and Food Intake

While NPY$^{-/-}$ mice had a higher body weight than WT control mice—significantly so from 10 to 14 weeks of age (figure 1A), mice lacking PYY (i.e. PYY$^{-/-}$ [23] and NPY$^{-/-}$PYY$^{-/-}$ double knockout mice) (figure 1A), had a similar growth curve to that of WT mice. Interestingly, adiposity at 15 weeks of age, as assessed by the weight of dissected white adipose tissue depots (inguinal, epididymal, mesenteric and retroperitoneal) or their summed total mass, was unaltered relative to WT in PYY$^{-/-}$ mice but was markedly increased over WT values in mice lacking NPY (i.e. NPY$^{-/-}$ or NPY$^{-/-}$PYY$^{-/-}$ mice) (figure 1B). Mass of white adipose tissue depots expressed as a percentage of body weight showed a similar pattern (Table 1). The effects of NPY and/or PYY deletion on adiposity appear to be specific to white adipose tissue, as brown adipose tissue weight was comparable among different genotype groups (Table 1). Moreover, daily food intake was unaltered in mice with PYY or NPY single deletion (figure 1C). Mice lacking both NPY and PYY, however, showed significantly reduced daily food intake compared to WT mice (figure 1C), suggesting interactive control of NPY and PYY in the regulation of daily food intake. Interestingly, relative to WT mice, 24-h fasting-induced food intake was significantly increased in PYY$^{-/-}$ but not in mice lacking NPY (i.e. NPY$^{-/-}$ or NPY$^{-/-}$PYY$^{-/-}$ mice, figure 1D), indicating that NPY may be required in order for the lack of PYY to elicit effects on fasting-induced food intake. Furthermore, all knockout groups had greater weight loss after a 24-h fast than WT mice, with no additive effect observed in double knockouts (Table 1), suggesting that NPY and PYY play redundant roles to preserve energy stores during fasting. After 1 day of re-feeding, PYY$^{-/-}$ mice showed a greater body weight recovery than other groups, significantly so when compared to NPY$^{-/-}$ and NPY$^{-/-}$PYY$^{-/-}$ mice (Table 1), consistent with the increased food intake during re-feeding observed in PYY$^{-/-}$ mice (figure 1D, Table 1).

Effects of NPY and PYY Deletion on Energy Metabolism

To investigate the effects of NPY and PYY deficiency on the other side of the energy balance equation—energy expenditure—we conducted indirect calorimetry on WT, NPY$^{-/-}$, PYY$^{-/-}$ and NPY$^{-/-}$PYY$^{-/-}$ mice at 15 weeks of age with concomitant measurement of physical activity. All groups showed a clear circadian rhythm in energy expenditure (figure 2A), physical activity (figure 2C) and RER (an indicator of oxidative fuel source) (figure 2E), with a higher level for all parameters during the dark period (figure 2), consistent with rodents being more active, consuming more energy and using carbohydrate as a main fuel source during the dark phase. Energy expenditure was not significantly different between knockout groups and WT mice (figure 2A, B), and this lack of difference among genotypes was associated with comparable overall physical activity levels among all groups (figure 2C, D), although mice lacking NPY tended to be less active, significantly so in NPY$^{-/-}$PYY$^{-/-}$ mice during the light phase (figure 2D). Interestingly, mice with single
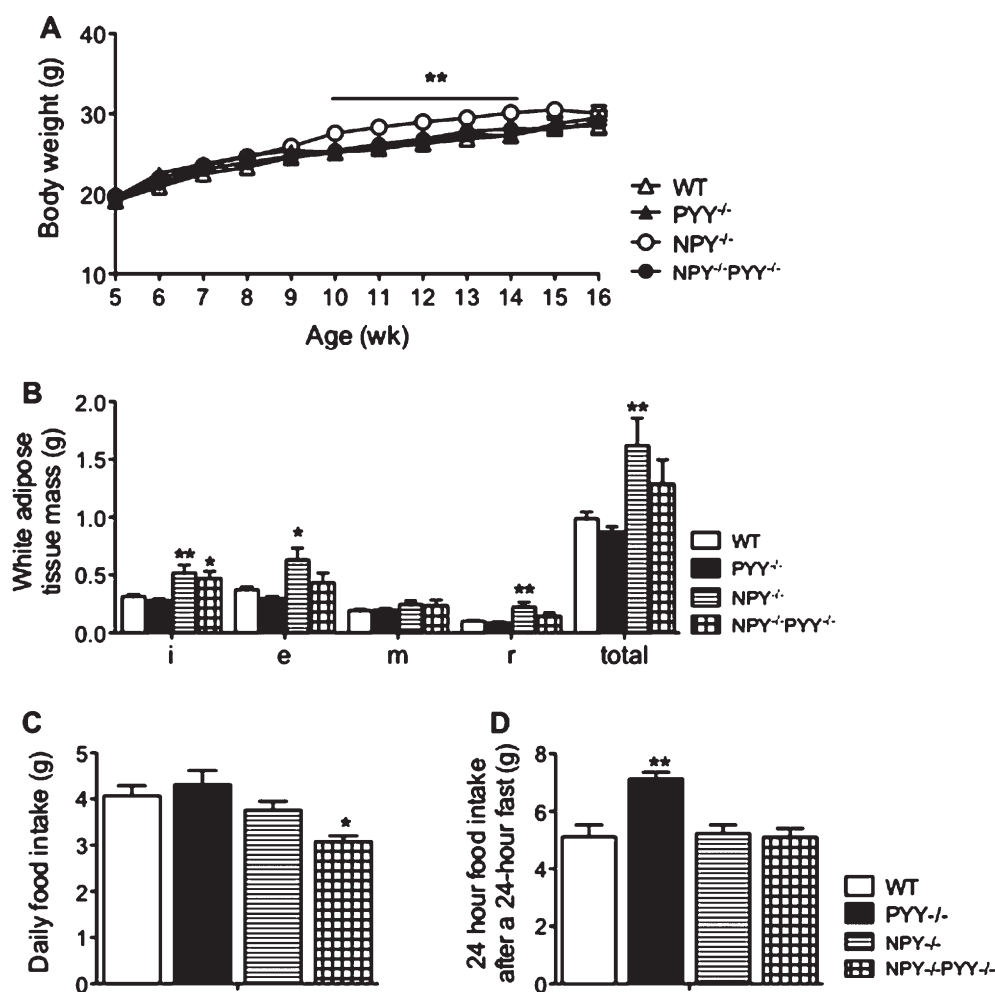
Figure 1. Effects of neuropeptide Y/peptide YY (NPY/PYY) single and double deletion on body weight (A), adiposity (B), daily food intake (C) and 24-h food intake after a 24-h fast (D). Male NPY$^{-/-}$, PYY$^{-/-}$, NPY$^{-/-}$PYY$^{-/-}$ and wild type (WT) mice were used. Adiposity (B) was assessed by weighing individual dissected white adipose depots, namely the inguinal (i), epididymal (e), mesenteric (m) and retroperitoneal (r) depots, as well as the combined weight of these depots (total). Data are means $\pm$ s.e.m. of 6–10 mice per group. *p < 0.05, **p < 0.001 versus WT mice.

Table 1. Adiposity, daily food intake, 24-h fasting-induced food intake, weight loss and post-fasting body weight recovery during 24 h re-feeding in male WT, PYY$^{-/-}$, NPY$^{-/-}$ and NPY$^{-/-}$PYY$^{-/-}$ mice at 15 weeks of age.

	WT	PYY$^{-/-}$	NPY$^{-/-}$	NPY$^{-/-}$ PYY$^{-/-}$
WATi (% BW)	1.19 ± 0.07	1.06 ± 0.04	1.72 ± 0.17**	1.64 ± 0.14*
WATe (% BW)	1.41 ± 0.08	1.13 ± 0.08	2.08 ± 0.27*	1.48 ± 0.21
WATm (% BW)	0.725 ± 0.044	0.754 ± 0.035	0.824 ± 0.081	0.808 ± 0.126
WATr (% BW)	0.378 ± 0.025	0.324 ± 0.027	0.731 ± 0.109**	0.496 ± 0.072*
WAT total (% BW)	3.73 ± 0.20	3.31 ± 0.14	5.36 ± 0.62*	4.42 ± 0.52
BAT (% BW)	0.313 ± 0.013	0.341 ± 0.019	0.347 ± 0.018	0.316 ± 0.020
24-h fasting-induced weight loss (g)	3.68 ± 0.12	5.22 ± 0.19**	4.08 ± 0.16	4.22 ± 0.16
24-h fasting-induced weight loss (% pre-fasting BW)	13.38 ± 0.39	16.63 ± 0.60*	15.68 ± 0.47*	5.18 ± 0.54 (p = 0.06 vs. WT)
24-h post-fasting BW recovery (% pre-fasting BW)	98.36 ± 0.60	99.96 ± 0.56	96.72 ± 0.47#	96.86 ± 0.73#
Fasting serum glucose concentration (mM)	5.89 ± 0.42	9.16 ± 0.79*	5.41 ± 0.40	8.33 ± 0.73*
Fasting serum insulin (pM)	62.3 ± 16.2	256 ± 36.1**	47.9 ± 4.9	69.6 ± 10.8
Non-fasting blood glucose concentration (mM)	8.54 ± 0.24	9.59 ± 0.39*	8.1 ± 0.17	9.63 ± 0.31*

Mass of white adipose tissue depots, namely the inguinal (WATi), epididymal (WATe), mesenteric (WATm) and retroperitoneal (WATr) white adipose depots, as well as the summed (total) weight of these depots, as well as that of the brown adipose tissue (BAT), are presented as a percentage of body weight (% BW). Data are means ± s.e.m. of 8–10 mice per group. NPY, neuropeptide Y; PYY, peptide YY; WT, wild type.
*p < 0.05; **p < 0.001 versus WT; #p < 0.05 versus PYY$^{-/-}$ group.
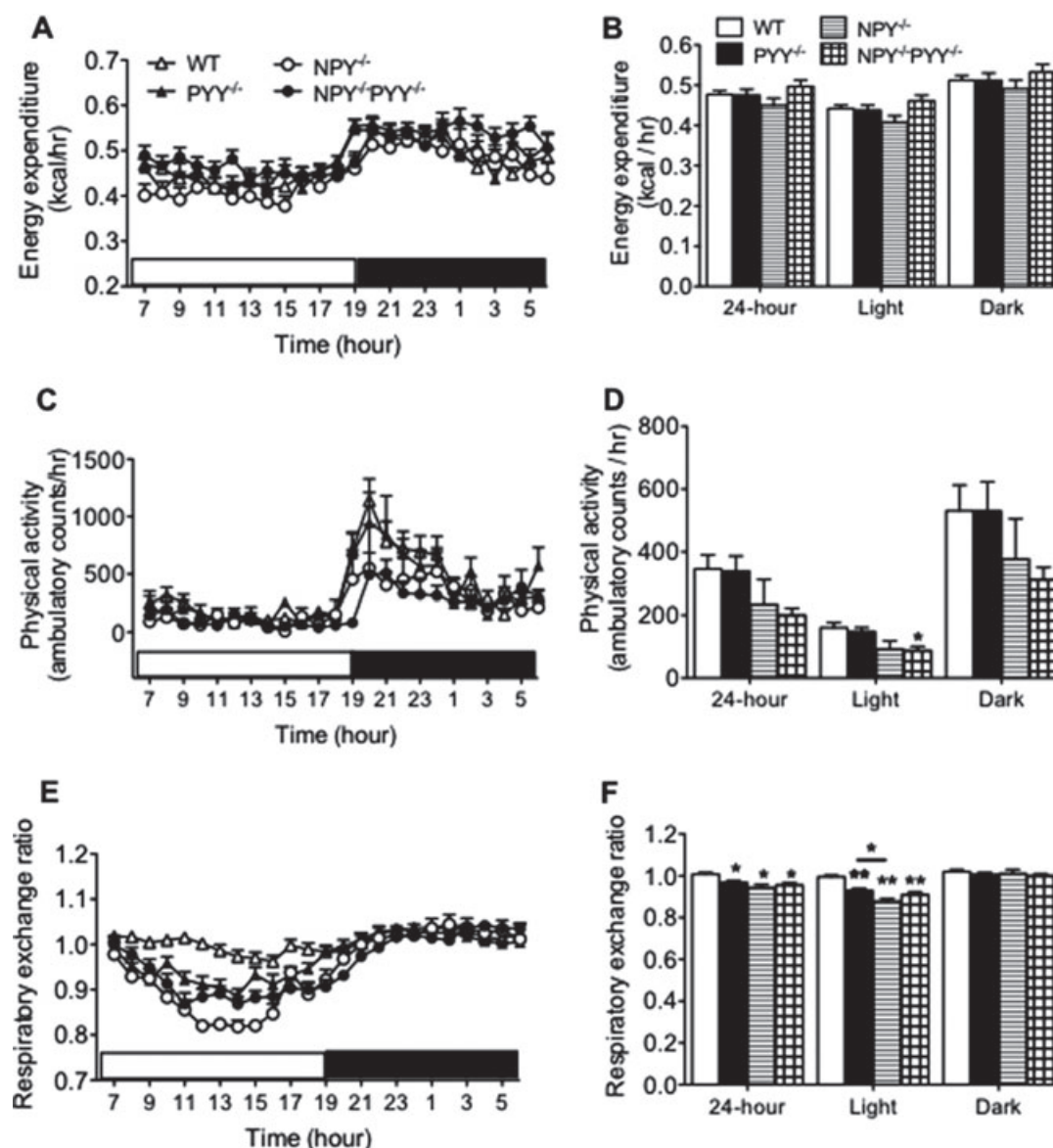
Figure 2. Effects of neuropeptide Y/peptide YY (NPY/PYY) single and double deletion on energy expenditure (A and B), physical activity (C and D) and respiratory exchange ratio (E and F). Male NPY$^{-/-}$, PYY$^{-/-}$, NPY$^{-/-}$PYY$^{-/-}$ and wild type (WT) mice were used. A, C and E represent the time course for each parameter over 24 h, with open and filled horizontal bars indicating the light and dark phases, respectively. B, D and F represent averages for 24 h, light and dark phases for each parameter. For comparison of energy expenditure by analysis of covariance, the common lean mass was 21.13 g. Data are means ± s.e.m. of 6−10 mice per group. *p < 0.05, **p < 0.001 versus WT mice or the comparison indicated by horizontal bars.

or double NPY/PYY deletion exhibited significantly lower RER than WT controls, particularly during the light phase (figure 2E, F), suggesting an important role of NPY and PYY in the regulation of substrate utilization, with lack of either ligand leading to increased lipid oxidation and/or decreased lipogenesis. Moreover, the reduction in RER relative to WT mice was more pronounced in NPY$^{-/-}$ than in PYY$^{-/-}$ mice, significantly so during the light period, suggesting that NPY may exert a greater effect on oxidative fuel selection than PYY. Interestingly, whereas NPY or PYY deficiency each result in a decreased RER, double ablation of NPY and PYY did not lead to an additive reduction in RER, implying that PYY and NPY may regulate oxidative fuel selection via partly shared

mechanisms. Taken together, these results show that NPY and PYY are each important players in the regulation of oxidative substrate metabolism, and may exert this regulation via some common pathways.

Effects of NPY and PYY Deletion on Glucose Homeostasis

Fasting serum glucose levels in PYY$^{-/-}$ and NPY$^{-/-}$PYY$^{-/-}$ mice were significantly elevated compared to those in WT mice (Table 1, figure 3A), and remained higher than WT values during the 120 min following an oral glucose bolus (p < 0.05 PYY$^{-/-}$ or NPY$^{-/-}$PYY$^{-/-}$ versus WT by repeated ANOVA;
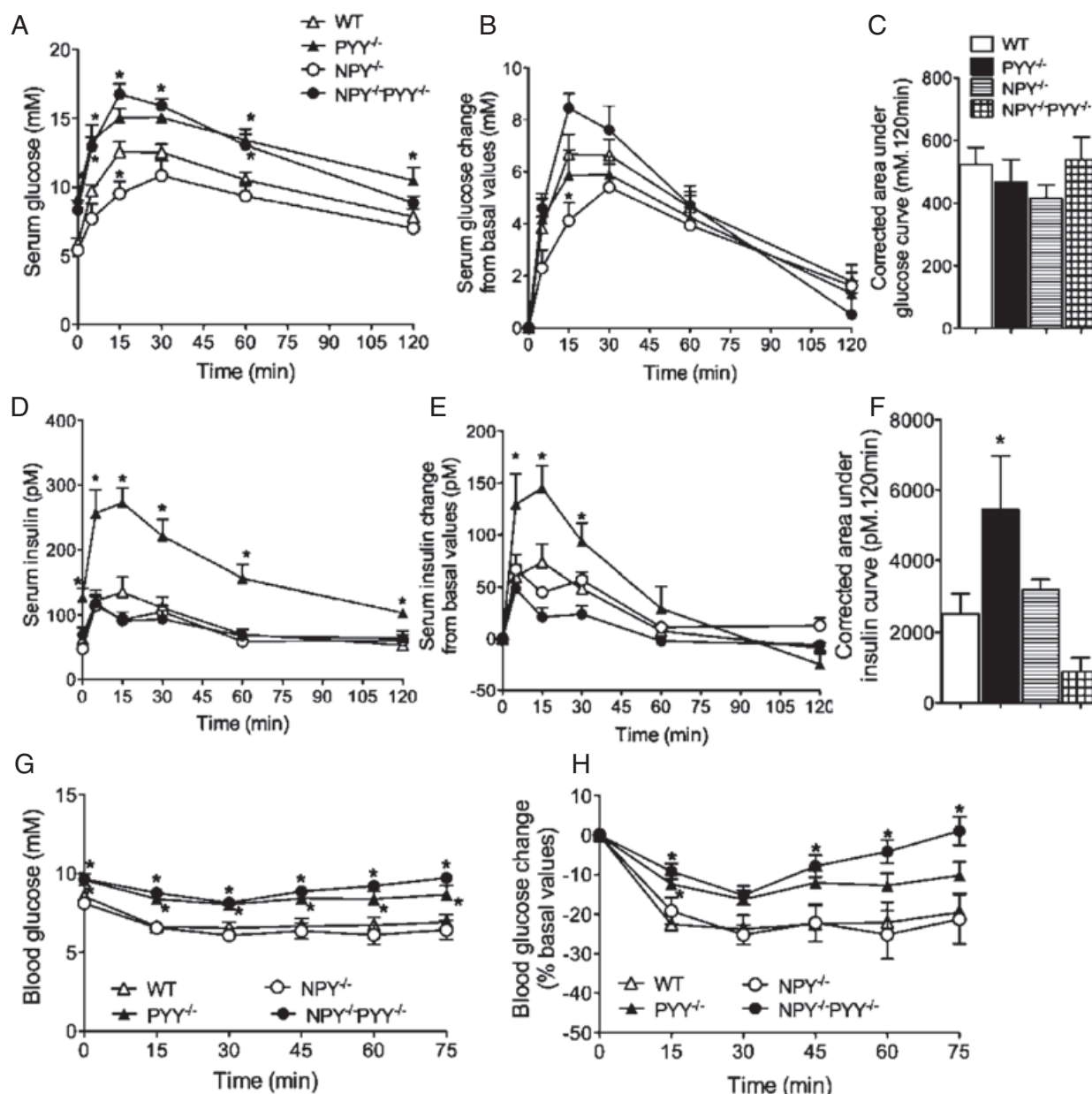
Figure 3. Effects of neuropeptide Y/peptide YY (NPY/PYY) single and double deletion on oral glucose tolerance and insulin tolerance. Male NPY$^{-/-}$, PYY$^{-/-}$, NPY$^{-/-}$PYY$^{-/-}$ and wild type (WT) mice were used. Serum glucose levels (A), baseline-corrected serum glucose levels (B) and area under the resultant glucose curve (C), serum insulin levels (D), baseline-corrected serum insulin levels (E) and area under the resultant insulin curve (F) during a 120-min oral glucose tolerance test (3 g/kg). Glucose was given as a glucose jelly that was voluntarily consumed by each mouse. Blood glucose levels (G) and the change in blood glucose levels as a percent of basal values (H) during a 75-min intraperitoneal insulin tolerance test (0.5 U/kg). Data are means ± s.e.m. of 6−10 mice per group. *p < 0.05 versus WT mice.

data are means ± s.e.m. of 6−12 mice per group) (figure 3A). The glucose curves corrected for baseline values (figure 3B) and areas under the resultant glucose curves (figure 3C) from PYY$^{-/-}$ or NPY$^{-/-}$PYY$^{-/-}$ mice, however, were not significantly different from those of WT mice (figure 3B), suggesting an unaltered oral glucose tolerance in PYY$^{-/-}$ and NPY$^{-/-}$PYY$^{-/-}$ mice. In contrast, NPY$^{-/-}$ mice were not different from WT with regard to basal fasting serum glucose levels (figure 3A, Table 1), but had significantly lower serum

glucose concentrations than WT mice during the 120-min oral glucose tolerance test (p = 0.05 by repeated ANOVA) (figure 3A), albeit the glucose excursion from the baseline (figure 3B) and area under the resultant curve were not significantly different from those of WT mice (figure 3C). Regarding serum insulin, PYY$^{-/-}$ mice showed significantly higher serum insulin levels than WT mice, both in the fasting state (figure 3D, Table 1) and throughout the course of the glucose tolerance test (figure 3D, p < 0.001 by repeated ANOVA 0−120 min), which

resulted in a pronounced increase in these mice in insulin excursion from basal values (figure 3E, $p < 0.005$ by repeated ANOVA 0–120 min) and the area under the insulin curve (figure 3F). In contrast, $NPY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice were not significantly different from WT mice with regard to insulin concentrations in the fasting basal state (Table 1, figure 3D) or during the course of the glucose tolerance test (figure 3D). Furthermore, the insulin levels corrected for basal values (figure 3E) and area under the resultant insulin curve (figure 3F) was unaltered in $NPY^{-/-}$ but showed a trend to a decrease in $NPY^{-/-}PYY^{-/-}$ mice compared to WT. These data show that circulating insulin levels were not significantly altered by NPY single deletion, but were markedly increased in the fasting state and in response to oral glucose by PYY single deletion. Moreover, the effects of PYY deficiency on fasting and oral glucose-induced serum insulin concentrations were abolished by additional NPY ablation, showing critical interactions between NPY and PYY signalling in regulating insulin responses.

To investigate the effects of NPY and PYY deficiency on insulin action, we performed an intraperitoneal insulin tolerance test in WT, $NPY^{-/-}$, $PYY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice. Fasting blood glucose levels in $PYY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice were significantly elevated compared to that of WT mice (Table 1, figure 3G), and remained higher than WT values during the 75 min following insulin injection (0.5 U/kg) ($p < 0.05$ $PYY^{-/-}$ or $NPY^{-/-}PYY^{-/-}$ versus WT by repeated measures ANOVA; data are means ± s.e.m. of 6–12 mice per group) (figure 3G). Moreover, the changes in blood glucose levels as a percentage of the fasting basal values were less in $PYY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ compared to those of WT mice ($p = 0.086$ and $p < 0.05$ for $PYY^{-/-}$ versus WT and $PYY^{-/-}NPY^{-/-}$ versus WT, respectively, by repeated measures ANOVA; data are means ± s.e.m. of 6–12 mice per group) (figure 3H), suggesting impaired insulin action in mice with PYY single or NPY/PYY double deletions. In contrast, $NPY^{-/-}$ mice showed comparable blood glucose levels to those of WT mice at baseline (Table 1, figure 3G) and during the insulin tolerance test (figure 3G). The changes in blood glucose concentrations in response to the intraperitoneal insulin challenge were also similar between $NPY^{-/-}$ and WT mice (figure 3H), showing an unaltered insulin tolerance in mice with NPY single ablation. Taken together, these results show that lack of PYY leads to a marked reduction in the hypoglycaemic response to insulin, whereas NPY deficiency has no such effect. Moreover, the PYY deficiency-induced impairment in insulin action was not affected by the addition of NPY deletion, in contrast to the correcting effects additional deletion of NPY has on PYY deletion-induced increases in insulin levels.

Discussion

This study represents the first investigation of the possible interactions between the neuronal peptide NPY and the gut hormone PYY in the regulation of energy balance and glucose homeostasis using mutant mouse models. We show that PYY deletion alone leads to increased fasting-induced food intake, enhanced fasting and oral glucose-induced serum insulin

levels, as well as an impaired insulin action in mice, effects not observed in mice with NPY single deletion. Interestingly, whereas it had no impact on the impairment of insulin action induced by PYY deficiency, NPY deletion blocked the effects of PYY deficiency on fasting-induced food intake and fasting and glucose-induced circulating insulin concentrations, suggesting that NPY signalling may be required for PYY's action on insulin secretion and fasting-induced hyperphagia. Moreover, NPY/PYY double deletion but not single deletion of either peptide resulted in a significant decrease in daily food intake, indicating interactive control by NPY and PYY on spontaneous food intake. Furthermore, NPY or PYY single deletion each result in significantly reduced RER during the light phase, with no additive effects observed with double deletion, showing that NPY and PYY may regulate oxidative fuel selection via partly shared mechanisms. Physical activity or energy expenditure, however, are not significantly altered by NPY and PYY single or double deficiencies. Taken together, these data show complex interactions between NPY and PYY in the regulation of various aspects of energy balance and glucose homeostasis, with NPY having a more controlling function over PYY action.

Both NPY and PYY are known to play important roles in regulating food intake, with hypothalamic NPY being orexigenic and postprandial release of PYY inducing satiety. Although the lack of effects of NPY or PYY germline deletion on spontaneous daily food intake observed here and reported elsewhere [20–23,32] suggest compensatory changes may have occurred, mice with NPY/PYY double ablation showed a significant reduction in spontaneous food intake, suggesting interactive control of spontaneous food intake by NPY and PYY. The nature of and mechanisms for this interaction are unclear. However, it seems possible that by removing PYY, the compensatory orexigenic mechanisms activated by the lack of NPY is abolished or diminished, or overcome by an activated anorexic mechanism. In keeping with the later possibility, PYY ablation has been shown to cause an increase in hypothalamic POMC expression [23]. Importantly, our results show that mice with PYY deletion exhibit significantly enhanced food intake after a 24-h fast, showing the important role of PYY in inhibiting hyperphagia induced by a period of energy deficit such as a 24-h fast. In addition, PYY may exert this effect by interacting with NPY-ergic pathways, as the greater hyperphagic response to fasting observed in PYY knockout mice is abolished by the addition of NPY ablation, suggesting that NPY is required for PYY's inhibitory effects on negative energy balance-induced hyperphagia. Interestingly, a period of energy deficit is known to enhance hypothalamic NPY expression, which is thought to be a key contributor to the hyperphagic response to negative energy balance [33]. Thus, PYY may exert its inhibition of energy deficit-induced hyperphagia by reducing NPY-ergic tone. In keeping with this, it has been proposed that PYY acts on Y2 auto-receptors on NPY neurons in the arcuate nucleus of the hypothalamus to reduce orexigenic drive [8,34]. The lack of expected reduction in fasting-induced food intake in $NPY^{-/-}$ mice relative to WT suggests possible compensatory changes induced by germline NPY deletion, such as increased expression of the orexigenic agouti-related peptide in the arcuate nucleus of the hypothalamus as reported previously [22].

However, the absence of PYY deficiency-induced inhibition of fasting-induced hyperphagia in mice on the NPY$^{-/-}$ background, in contrast to its effect in WT mice, shows the inability of PYY to interact with other feeding-regulatory pathways that might have been activated to compensate the absence of NPY, suggesting a specific interaction of PYY with NPY signalling to regulate the energy deficit-induced hyperphagic response. Taken together, our results suggest interactive control of spontaneous food intake by PYY and NPY. Moreover, PYY may play a critical role in regulating the hyperphagic response to energy deficit, and this regulation is likely to involve a specific interaction with NPY signalling.

Our study shows that both NPY and PYY play important roles in the regulation of oxidative fuel selection, as both NPY$^{-/-}$ and PYY$^{-/-}$ mice exhibit significantly reduced RER, suggesting that activation of NPY or PYY signalling shifts substrate oxidation towards carbohydrate and/or promotes lipogenesis. Previous studies show that mice lacking Y1, Y2 or Y4 receptors exhibit similar reductions in RER. Moreover, activation of central Y1 or Y5 receptors by intracerebroventricular administration of an Y1 or Y5 receptor agonist increases RER. These studies suggest that multiple Y receptors, including Y1, Y2, Y4 and Y5, are likely involved in mediating the effects of NPY and PYY on oxidative fuel selection. Interestingly, NPY appears to exert a stronger effect than PYY on substrate selection, as lack of NPY leads to a greater reduction in RER than that induced by PYY deletion. NPY may exert this stronger effect on RER via activation of central Y receptor signalling. Importantly, we show that dual deletion of NPY and PYY does not lead to an additive effect on RER, suggesting that NPY and PYY may regulate oxidative substrate metabolism via some common—probably peripheral—pathways. In keeping with this, mice with conditional knockdown of peripheral Y1 receptors display significantly reduced RER, showing an important role of peripheral Y1 signalling in the regulation of oxidative fuel selection. It is interesting to note that peripheral administration of PYY3-36 is reported to increase circulating fatty acid levels and to decrease RER [35–38]. It is proposed that peripherally administered PYY3-36 has direct actions to promote lipolysis and mobilize fatty acids, which lead to enhanced fatty acid oxidation and decreased RER [35,38]. As PYY3-36 acts primarily on Y2 receptors, the effects of peripherally administered PYY3-36 on lipid metabolism and RER are likely to be mediated by peripheral Y2 signalling. In line with this, mice with conditional knockdown of peripheral Y2 receptors have increased RER [13]. Importantly, the decreased RER in PYY$^{-/-}$ mice observed in our study suggests that other peripheral Y receptors besides Y2 are likely to elicit effects on substrate oxidation opposing those elicited by lack of peripheral Y2 signalling. In line with this, peripheral administration of PYY1-36, which binds to all Y receptors, had no significant effects on fatty acid mobilization, in contrast to the marked effects of peripheral PYY3-36 administration [35,37]. In summary, our data show the important roles played by NPY and PYY in regulating oxidative fuel selection, and suggest that NPY and PYY may exert this regulation via partly shared common pathways in the periphery.

Consistent with a previous report [23], we observe that mice with PYY deficiency display marked hyperinsulinaemia and an enhanced insulin response to glucose, suggesting a strong inhibitory action of PYY on insulin secretion. This is in accordance with pharmacological studies showing significantly reduced glucose-stimulated insulin release by peripheral administration of PYY in mice [39], although these effects were not observed in humans [7,40]. In contrast, insulin levels in NPY$^{-/-}$ mice only exhibit very subtle changes compared to WT mice (i.e. the unaltered fasting serum insulin levels and a trend towards decreased insulin response to glucose challenge). NPY is known to have central effects on insulinaemia, with central elevation of NPY via intracerebroventricular injection resulting in marked hyperinsulinaemia and enhanced glucose-stimulated circulating insulin levels [41–43]. Interestingly, peripheral elevation of NPY in rats via intravenous infusion, to a level similar to that seen during muscular exercise or stressful situations in both animals and human, significantly reduces glucose-stimulated insulin secretion [44]. It is possible that lack of phenotopic changes in insulin levels in NPY$^{-/-}$ relative to WT mice may be due to the simultaneous removal of central NPY-mediated stimulation and peripheral NPY-mediated inhibition on insulin release. Further studies using tissue-specific NPY knockout models could help to elucidate this issue. However, considering the strong negative influence of PYY on insulin secretion, NPY$^{-/-}$ mice might be anticipated to have significantly lower fasting and glucose-induced circulating insulin levels due to the inhibitory tone imposed by PYY. However, this does not appear to be the case. In fact, our results show that the pronounced increases in serum insulin levels induced by PYY deficiency are abolished by NPY deletion, suggesting that PYY-mediated inhibition of insulin secretion requires the presence of NPY. One could hypothesize that the inhibitory effects of peripheral PYY and NPY may serve as the feedback mechanisms to counter-regulate central NPY-mediated stimulation on insulin release; thus without the stimulatory input from central NPY, the peripheral feedback pathway is silent. In keeping with negative feedback by PYY to central stimulation, peripheral administration of PYY at physiological doses has been shown to inhibit insulin release induced by neural stimulation, such as 2-deoxyglucose, a non-metabolized glucose analogue that elicits insulin release via central nervous activation [45,46]. Taken together, our data show a specific interaction between NPY and PYY in the regulation of fasting and oral glucose-induced serum insulin levels which may involve an inhibitory feedback from peripheral NPY and PYY to counter-regulate central NPY-mediated stimulation of insulin release.

Interestingly, like its effects on circulating insulin levels described above, NPY in the central nervous system and in the periphery appear to have differential effects on insulin action. Central elevation of NPY causes insulin resistance in skeletal muscle but enhances insulin responsiveness in the white adipose tissues [43]. In contrast, peripheral administration of NPY via intravenous infusion increases insulin-stimulated glucose uptake in skeletal muscle, contributing to the significantly increased whole-body glucose disposal, albeit having no effect on insulin action in adipose tissue [44]. It is possible that

central and peripheral actions of NPY on glucose homeostasis may balance each other, with no major phenotypic changes in glucose tolerance or insulin sensitivity induced by germline NPY deletion. In contrast, our data show that $PYY^{-/-}$ mice display a significant impairment in the hypoglycaemic response to insulin, suggesting impaired insulin action and/or quicker counter-regulatory responses in mice lacking PYY. The oral glucose tolerance of $PYY^{-/-}$ mice was not significantly different from that of WT. However, the 24-h fasting period prior to oral glucose administration may have led to underestimation of any potential negative effects of PYY deficiency on glucose clearance, as it was recently shown that the duration of fasting has a significant impact on fasting and glucose-induced circulating glucose levels, and the impairment in glucose tolerance due to a high-fat diet can be different in mice fasted for 6 h compared to those fasted for 24 h [47]. Peripheral administration of PYY3-36 has been shown to improve insulin sensitivity in diet-induced obese mice, in association with increased insulin-mediated glucose uptake in skeletal muscle and white adipose tissue depots [38,48,49]. Whereas so far there is no report on the effects of peripherally administered PYY1-36 on insulin-mediated glucose disposal, considering PYY1-36 has the same pharmacological profile to NPY [10], it is conceivable that peripheral administration of PYY1-36 may elicit the same effects on glucose metabolism as those seen with intravenous administration of NPY (i.e. an enhanced insulin-mediated glucose disposal) [44]. Moreover, the markedly elevated fasting serum glucose levels in $PYY^{-/-}$ versus WT mice may reflect increased hepatic glucose production in these animals. In conjunction with the simultaneous elevation in fasting serum insulin levels, the hyperglycaemia after a 24-h fast in $PYY^{-/-}$ mice may suggest hepatic insulin resistance. Thus, circulating PYY, either the full-length (PYY1-36) or truncated form (PYY3-36), appears to facilitate insulin-mediated glucose uptake in peripheral tissues as well as potentially also suppressing hepatic glucose output. Importantly, the significant impairment in the response to insulin observed in $PYY^{-/-}$ mice suggests a physiological role for PYY in regulating insulin action. Furthermore, our results show that $PYY^{-/-}$ and $NPY^{-/-}PYY^{-/-}$ mice have equally impaired hypoglycaemic responses to insulin, suggesting that the PYY deficiency-induced impairment in insulin action cannot be corrected by NPY deletion, in contrast to the correcting effects of NPY deletion on PYY deficiency-induced hyperinsulinaemia. This indicates that NPY and PYY may exert their control on insulin action via separate pathways. Taken together, these data show an important role of PYY in the regulation of insulin action, and that this regulation may be achieved via NPY-independent mechanisms.

In summary, this work reveals significant interactions between neuronal NPY and the gut hormone PYY in the regulation of food intake, oxidative substrate selection and circulating insulin levels, both in the fasted state and in response to oral glucose. NPY signalling appears to be required for PYY signalling-mediated control of energy deficit-induced hyperphagia, basal insulin levels and glucose-stimulated insulin responses, whereas substrate utilization may be regulated by NPY and PYY via partly shared common pathways. Moreover, our data show that PYY has important roles in the regulation of insulin action and this regulation may be achieved without interacting with NPY signalling.

Conflict of Interest

L. Z., A. S. and H. H. designed this study. A. N., I.-C. L., E. Y., S. R., B. S., R. E., S. L., Y.-C. S. and P. B. conducted/collected the data. L. Z. and E. Y. analysed the data. L. Z., A. S. and H. H. wrote the manuscript.

All the authors have no competing interest.

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