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Adult-onset PYY overexpression in mice reduces food intake and increases lipogenic capacity

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ABSTRACT

Peptide YY (PYY) is best known for its important role in appetite regulation, but recent pharmacological studies have suggested that PYY is also involved in regulating energy balance and glucose homeostasis. However, the mechanism behind the regulation of these parameters by PYY is less clear. Here, by utilising an inducible transgenic mouse model where PYY overexpression is induced in adult animals (PYYtg) and release of mature PYY peptides is controlled by endogenous machineries, we show that elevating PYY levels leads to reduced food intake after a 24-h fast. Furthermore, PYYtg mice, although not significantly different from WT with respect to body weight, adiposity, lean mass, physical activity or energy expenditure, exhibited a significantly increased respiratory exchange ratio (RER), indicating decreased lipid oxidation and/or increased lipogenesis. Importantly, PYYtg mice showed a 25% reduction in liver protein levels of phosphorylated acetyl-CoA carboxylase (pACC) in the absence of changes in total ACC levels compared to those of WT mice. Moreover, liver protein levels of AMP-activated kinase (AMPK) in PYYtg mice were 25% lower than those of WT mice, consistent with a reduced pACC in these mice. These data suggest that elevation of PYY levels as seen after a meal can increase lipogenic capacity, which is likely a key contributor to the increased RER seen in PYYtg mice. In addition, PYYtg mice exhibited comparable insulin tolerance and oral glucose tolerance to those of WT, but showed a trend towards decreased insulin levels in response to an oral glucose challenge, indicating that PYY could improve insulin action. Taken together, these findings demonstrate that under physiological conditions, PYY reduces food intake while enhancing lipogenic capacity and insulin action, likely contributing to fuel assimilation in the postprandial state.

1. Introduction

Obesity and type 2-diabetes mellitus are two major current public health challenges. Gut-derived hormones play a crucial role in the regulation of body weight and glucose homeostasis, thus have been of major interest as possible targets for the treatment of obesity and type-2 diabetes. One of these hormones is peptide YY (PYY), which has been recognized as an important mediator of satiety and which may play a role in the aetiology of obesity and type-2 diabetes (Batterham et al., 2003; Roth et al., 2005; Boey et al., 2006a; le Roux et al., 2006).

PYY belongs to the neuropeptide Y (NPY) family, which includes NPY and pancreatic polypeptide (PP). All three peptides signal through a set of G-protein-coupled Y receptors (Y1, Y2, Y4, Y5 and y6), which are widely expressed centrally as well as peripherally (Blomqvist and Herzog, 1997; Zhang et al., 2011). PYY is primarily released from endocrine L cells of the lower gastrointestinal tract in proportion to the amount of calories ingested (Adrian et al., 1986; Batterham et al., 2002; Ekblad and Sundler, 2002). A recent study shows that PYY is also synthesized by cells in the taste buds of the tongue, and this has important implications for the induction of satiety (Acosta et al., 2011). In the circulation, two forms of PYY exist; 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 (DPPIV) (Mentlein, 1999; Unniappan et al., 2006). The conversion of PYY1-36 to PYY3-36 is

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enhanced after meal ingestion, with PYY3-36 contributing to approximately 37% of circulating total PYY in the fasted state and 63% in the fed state (Grandt et al., 1994). Importantly, PYY1-36 binds to all known Y receptors, albeit to each with differing affinity, whereas PYY3-36 preferentially binds to Y2 and to a lesser extent also Y5 receptors (Blomqvist and Herzog, 1997).

Acute peripheral administration of PYY3-36 has been demonstrated to reduce food intake in both lean and obese animals and humans (Batterham et al., 2002; Pittner et al., 2004; Koegler et al., 2005; le Roux et al., 2006; Field et al., 2010). In diet-induced obese rodents, PYY3-36 has also been shown to promote lipid oxidation and/or reduce lipogenesis (Adams et al., 2006; van den Hoek et al., 2007), as well as to enhance insulin-mediated glucose disposal (van den Hoek et al., 2004, 2007; Vrang et al., 2006), further demonstrating the potential therapeutic value of PYY3-36 in the treatment of obesity and type-2 diabetes. The mechanism underlying these effects of PYY3-36 on lipid metabolism and glucose homeostasis remains to be elucidated. However, with respect to effects on food intake, it has been shown that PYY3-36 acts on Y2 auto-receptors on NPY neurons in the arcuate nucleus of the hypothalamus to reduce NPY-ergic activity and thereby induce satiety (Batterham et al., 2002; Acuna-Goycolea and van den Pol, 2005).

In contrast to great research efforts investigating effects of PYY3-36, little attention has been paid to the role of PYY1-36, or the possible interactions between PYY1-36 and PYY3-36, in the regulation of energy balance and glucose homeostasis. PYY1-36 might be expected to have a broader and more complex profile of effects, since in addition to having a higher affinity for Y2 receptors than PYY3-36, it also has a high affinity for other Y receptors, such as the Y1 receptor, which elicits prominent but differential effects on energy balance and glucose homeostasis compared to Y2 receptor (Shi et al., 2010, 2011; Zhang et al., 2010b,c, 2011). For instance, lack of Y1 signalling promotes lipid oxidation (Henry et al., 2005; Zhang et al., 2010b), whereas lack of Y2 signalling appears to have opposite effects on oxidative fuel selection (Shi et al., 2010, 2011; Zhang et al., 2010c). Furthermore, Y1 receptor deficiency leads to pronounced hyperinsulinemia which is not observed in Y2 receptor deficient mice (Kushi et al., 1998; Sainsbury et al., 2003; Zhang et al., 2010b), suggesting that Y1 but not Y2 signalling plays an important role in regulating insulin secretion. Based on the differential effects of Y1 and Y2 signalling on energy and glucose homeostasis, and considering that PYY1-36 and PYY3-36 coexist in the circulation in varying ratios depending on nutritional status (Grandt et al., 1994), PYY1-36 and PYY3-36 may act in a co-ordinate or synergistic manner to regulate energy and glucose metabolism in fasted and postprandial states. Investigating the physiological actions of total PYY on energy balance and glucose homeostasis is important for the development of PYY-based anti-obesity and anti-diabetes therapies, particularly considering that nausea and vomiting are common side-effects associated with PYY3-36 treatment (Degen et al., 2005; Gantz et al., 2007; Sloth et al., 2007; le Roux et al., 2008).

Understanding the physiological effects of total PYY on energy and glucose homeostasis is difficult to achieve by pharmacologically administrating PYY1-36, PYY3-36 or in combination, since pharmacological approaches are normally complicated by dosage, carrier and delivery route, and – more importantly – because the ratios of PYY1-36 to PYY3-36 in the circulation show circadian changes (Grandt et al., 1994). Interestingly, a previous study shows that modest germline PYY overexpression induces marked resistance to diet-induced obesity and significantly attenuates the metabolic syndrome of genetically obese *ob/ob* mice (Boey et al., 2008). However, compensatory changes may occur during development in germline genetically manipulated mouse models, with high levels of PYY during embryogenesis actually being lethal (Yuzuriha et al., 2007). Thus, to investigate the physiological role of PYY in the regulation of energy balance and glucose homeostasis in the absence of possible compensatory changes, we generated a conditional PYY transgenic mouse model in which PYY overexpression is induced in adulthood, and in which post-translational modification of PYY1-36 to PYY3-36 is achieved by the host's own regulatory machinery. Moreover, the single extra copy of the PYY transcript in these transgenic mice is only processed to mature and functional protein in cells and tissues that have endogenous machineries to process pre-proPYY peptide, and is released due to endogenously-controlled neuronal, hormonal and meal nutrient stimulation (Onaga et al., 2002). Thus in this model, PYY overexpression is expected to occur only in tissues endogenously producing PYY or similar peptides. Thus, these PYY transgenic mice are expected to maintain the normal regulation of PYY expression in response to physiological stimuli, but with a larger change in magnitude relative to wild type mice after induction in adult animals, thus providing more definitive answers as to the physiological role played by PYY in the regulation of food intake, energy metabolism and glucose homeostasis.

2. Materials and methods

2.1. 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. Male mice were used for all experiments. Mice were housed under conditions of controlled temperature (22 °C) and illumination (12-h light cycle, lights on at 07:00 h). 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) and were given water *ad libitum* unless otherwise stated.

To generate an adult-onset PYY over-expression model, heterozygote transgenic mice carrying an additional copy of the mouse PYY gene that is separated from a CMV promoter by a loxP-flanked stop cassette (PYYlox/+), were crossed with homozygous Cre transgenic mice (ROSACre, Jackson Laboratory, Bar Harbor, ME, USA), in which expression of Cre-recombinase is driven by the *Rosa26* promoter in a tamoxifen-inducible manner (Hayashi and McMahon, 2002). The resultant animals of this cross consist of both tamoxifen-inducible over-expressing PYY transgenic mice (PYYlox/+,ROSACre/+) and non PYY overexpressing littermate controls (WT,ROSACre/+). Tamoxifen treatment of (PYYlox/+,ROSACre/+) mice activates Cre-recombinase expression that in turn removes the transcriptional stop cassette between the *CMV* promoter and the *PYY* gene, thereby allowing expression of the additional copy of the PYY gene in tamoxifen-responsive tissues. Upon tamoxifen treatment, (PYYlox/+,ROSACre/+) and (WT,ROSACre/+) mice will be referred to as PYYtg and WT, respectively. Tamoxifen (i.p. 100 mg/kg) was injected on two separate occasions 3 days apart into mice at 8 weeks of age. Metabolic studies on these mice as described below commenced at least 4 weeks after the induction of PYY expression.

2.2. Immunohistochemical analysis

Pancreatic tissues obtained from wild type, $PYY^{-/-}$ and Rosa–PYYtg mice were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), processed and embedded in paraffin and cut at 5 μm sections. Sections were deparaffinised, rehydrated and incubated in 1% H_2O_2 in ethanol for 20 min to block endogenous peroxidase activity. Sections were then rinsed in PBS and incubated with

10% horse serum in PBS for 20 min in order to reduce non-specific binding. Subsequently sections were incubated overnight at 4 °C in hydration chambers with an in-house-generated monoclonal antibody against PYY. Slides were then rinsed in PBS before incubation for 30 min at room temperature with biotinylated goat anti-mouse IgG antibody (whole molecule) (diluted 1:500 in PBS) (Sigma–Aldrich Inc. Saint Louis, MO, USA). Sections were then rinsed in PBS and incubated with ExtrAvidin-Peroxidase (1:250) (Sigma–Aldrich Inc. Saint Louis, MO, USA) for 30 min at room temperature. Sections were washed in PBS and treated with diaminobenzidine (DAB + Substrate Chromogen System, DAKO Corp, Carpinteria, CA, USA) for 5 min. Slides were rinsed in water, counterstained with haematoxylin and dehydrated through to xylene before coverslipping.

2.3. Induction of PYY overexpression and food intake, body weight and tissue analyses

PYY over-expression was induced by i.p. injection of tamoxifen (100 mg/kg) in PYYtg and WT littermate mice at 8 weeks of age. 4 weeks after the induction of PYY over-expression (at 12-weeks of age), spontaneous daily and 24 h fasting-induced food intake were examined using a metabolic caging system (Techniplast, Buguggiate, VA Italy), where pellet food was used as that was given in the home cage. Mice were transferred from group housing on soft bedding to individual cages with paper towel bedding and allowed to acclimatize for 2 nights. Basal daily food intake was determined as the average of duplicate readings taken over 2 consecutive days. For fasting-induced food intake, mice were fasted for 24 h and food intake was determined at 2, 4, 8, 24 and 48 h after the re-feeding. At 17 weeks of age, animals were culled between 1200–1500 h by cervical dislocation followed by decapitation. White adipose tissue depots (right inguinal, right epididymal, mesenteric and right retroperitoneal) and the entire interscapular brown adipose tissue depot were removed and weighed as published previously (Sainsbury et al., 2002; Zhang et al., 2010b).

2.4. Determination of metabolic rate, respiratory exchange ratio and physical activity

Energy metabolism was studied in 15 week-old male PYYtg, $PYY^{-/-}$ and wild type mice. Metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH, USA) as described previously (Zhang et al., 2010b). Briefly, mice were housed individually in specially built Plexiglas cages ($20.1 \times 10.1 \times 12.7$ cm). Temperature was maintained at 22 °C with airflow of 0.6 l/min. Mice were singly housed for at least 3 days prior to transferring into plexiglass cages and were acclimatized to the cages for 24 h before recordings commenced. Mice were subsequently monitored in the system for 24 h. Pellet food was used when mice were in metabolic chambers, and excess amount food was given prior to the data collection so that no food needs to be re-introduced during the recording. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured every 27 min. The respiratory exchange ratio (RER) was calculated as the quotient of VCO_2/VO_2, with 100% carbohydrate oxidation giving a value of 1, and 100% fat oxidation giving value of 0.7 (Frayn, 1983; Ferrannini, 1988). Energy expenditure (kcal heat produced) was calculated as Calorific Value (CV) $\times$ VO_2, where CV is $3.815 + 1.232 \times$ RER (McLean and Tobin, 1987). 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.

2.5. Analysis of body composition

Upon completion of indirect calorimetry and physical activity measurements, animals were anesthetized 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 the tail were excluded from DEXA analyses. Fat mass at the anterior abdominal region between the xiphisternal joint to the inferior end of the rib cage was analyzed from DEXA scans as the indication of the hepatic fat mass, since liver is the major organ residing in the anterior abdominal cavity (Cook, 1965).

2.6. Oral glucose tolerance test

Oral glucose tolerance test was performed in a subset of male PYYtg and wild type mice at 18–20 weeks of age. An oral glucose bolus was given as a glucose jelly, which was consumed voluntarily by mice within 1 min. To achieve this, 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 (Cox et al., 2010; Zhang et al., 2010a). Mice were fasted at 2 pm prior to testing day for 24 h and were then given an oral glucose bolus (3 g/kg body weight) at 2 pm on the testing day. 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 glucose and insulin levels as described below. Glucose tolerance curves for glucose and insulin are presented as absolute values. Area under the glucose or insulin concentration curves between 0 and 120 min after glucose ingestion were calculated, subtracting glucose or insulin concentrations prior to glucose ingestion.

2.7. Intraperitoneal insulin tolerance test

A subset of PYYtg and wild type male mice at 18–20 weeks of age were used for this study. Food was removed from cage hoppers at 8.30 h, and 5–6 h later a dose of insulin (0.75 IU/kg body weight, 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 determination of blood glucose concentration using a glucometer (Accu-Check Go, Roche).

2.8. Serum analyses

Serum insulin levels were measured using an RIA kit from Mercodia (Uppsala, Sweden). Serum glucose in samples collected during oral glucose tolerance tests was determined by a glucose oxidase kit (Trace Scientific, Melbourne Australia).

2.9. Western blotting

Western blotting was performed on liver and quadriceps muscle samples following procedures described previously (Zhang et al., 2010b) in order to determine protein levels of key enzymes involved in lipid metabolism. Briefly, liver and quadriceps muscles were collected from 20 week-old WT and PYYtg mice, snap frozen in liquid nitrogen and stored at −80 °C. Subsequently, liver and powdered muscle samples were resuspended in radioimmunoprecipitation

assay buffer (PBS, pH–7.5; 1% nonident NP-40; 0.5% sodium deoxycholate; and 0.1% SDS), supplemented with protease and phosphatase inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na_3VO_4, 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 antibodies against carnitine palmitoyltransferase-1 (CPT-1) (Alpha Diagnostic Intl., San Antonio, Texas, USA), AMP-activated kinase (AMPK, Cell Signalling Technology, Danves, MA, USA), phospho-Ser79 acetyl-CoA carboxylase (pACC) and total acetyl-CoA carboxylase (tACC) (Cell Signalling Technology, Danvers, MA, USA). Immunolabelled bands were quantitated by densitometry. Relative protein levels of the mutant mice as a percentage of that of control mice are presented.

2.10. Statistical analyses

All data are expressed as means ± SEM. 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 different genotypes were assessed by 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 covariates. The adjusted means of energy expenditure at a common lean mass for the comparison were generated by ANCOVA. Multiple food intake measurements after a 24-h fast were analysed by repeated-measures ANOVA. Statistical analyses were performed with SPSS for Mac OS X, version 16.0.1 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Adult-onset PYY overexpression reduces food intake

Transgenic mice carrying an additional copy of the mouse PYY gene, separated from a CMV promoter by a loxP-flanked stop cassette (PYYlox/+), were crossed with mice carrying the gene for Cre recombinase. The Cre recombinase gene was under the control of a tamoxifen-sensitive promoter (ROSACre), leading to the generation of (PYYlox/+,ROSACre/+) mice. Overproduction of PYY in adult mice was achieved by intraperitoneal injection of tamoxifen (100 mg/kg) into 8 week-old (PYYlox/+,ROSACre/+) mice on two separate occasions, 3 days apart.

In order to confirm successful deletion of the stop cassette in front of the additional PYY gene, we employed PCR on genomic DNA isolated from liver tissue at 3 weeks after tamoxifen treatment, using primers that only amplify a product when the stop cassette has been removed. Production of a fragment of the expected size was only obtained in the tamoxifen-treated mice but not in the saline control treated mice (data not shown). We also performed immunohistochemistry using a monoclonal antibody specific to PYY in order to detect elevated PYY levels in target tissues such as the pancreas. The specificity of this antibody is demonstrated by the lack of PYY staining in pancreas tissue obtained from $PYY^{-/-}$ mice (Fig. 1A). Importantly, the cells stained for PYY in pancreatic tissue from tamoxifen-induced (PYYlox/+,ROSACre/+) mice were much darker and significantly greater in number compared to those from (WT,ROSACre/+) control pancreatic tissue (Fig. 1A), confirming the successful induction of PYY overexpression.

Prior to tamoxifen injection at 8 weeks of age, body weight was comparable between (PYYlox/+,ROSACre/+) and littermate control (WT,ROSACre/+) mice (Fig. 1B). Upon injection of tamoxifen to induce PYY over-expression, PYYtg mice showed a similar growth curve to that of WT mice (Fig. 1B). Whole body lean and fat mass determined by DEXA scan at 7 weeks after the induction of PYY overexpression (when mice were 15 weeks old) were comparable between PYYtg and WT mice (Fig. 1C). Moreover, weights of the dissected individual and total summed weight of these individual white adipose tissue depots, namely the inguinal, epididymal, mesenteric and retroperitoneal white adipose tissue depots, were not significantly different between the transgenic and the WT groups (Fig. 1D), consistent with whole body adiposity results obtained by DEXA scan (Fig. 1B). Interestingly, spontaneous daily food intake tended to be decreased in PYYtg compared to WT littermates (4.20 ± 0.21 versus 4.62 ± 0.13 g/day for PYYtg and WT mice, respectively, data are mean ± SEM of 6–13 mice per group, $P = 0.098$). Importantly, after a 24 h fast, PYYtg mice consumed significantly less food than WT mice during the 48 h re-feeding period ($P < 0.05$ for PYYtg versus WT mice by repeated ANOVA measures, data are mean ± SEM of 6–13 mice per group) (Fig. 1E), demonstrating the important role of PYY in the regulation of food intake. Taken together, these results show that whereas over-expressing PYY during adulthood does not significantly alter body weight or adiposity, it reduces food intake, particularly under conditions associated with increased appetite, namely after fasting.

3.2. Adult-onset overexpression of PYY increases respiratory exchange ratio

To investigate the role of PYY in the regulation of energy metabolism, we performed indirect calorimetry with concurrent measurement of physical activity in PYYtg and WT mice. Physical activity (Fig. 2A), energy expenditure (Fig. 2C) and respiratory exchange ratio (Fig. 2E), an index of oxidative fuel source, showed circadian rhythms in both PYYtg and WT mice, with increased levels in all parameters during the dark phase. Moreover, the circadian patterns as well as the values of physical activity and energy expenditure were similar between PYYtg and WT mice (Fig. 2A–D). Interestingly, however, PYYtg mice showed a significantly higher respiratory exchange ratio during the light period compared to WT mice (Fig. 2E and F), suggesting decreased lipid oxidation and/or increased lipogenesis in mice with elevated PYY levels.

3.3. Adult-onset overexpression of PYY increases lipogenic capacity

To elucidate pathways via which PYY increases respiratory exchange ratio, we examined protein expression of key molecules involved in the control of lipid metabolism by Western blotting. We determined the liver protein levels of total acetyl-CoA carboxylase (tACC), the enzyme catalysing the regulatory step for fatty acid synthesis, as well as the hepatic level of phosphorylated ACC (pACC), since the activity of ACC is negatively regulated by phosphorylation at ser-79/218 (Ruderman et al., 2003). Whereas there was no significant difference in the liver protein levels of tACC between PYYtg and WT mice, the protein levels of pACC were reduced by 25% in PYYtg compared to WT mice (Fig. 2G), suggesting a higher level of active ACC enzyme and thus an increased lipogenic capacity in mice with elevated PYY levels. Moreover, consistent with the significant reduction in ACC phosphorylation by PYY overexpression, liver protein levels of AMP-activated protein kinase (AMPK), the enzyme that phosphorylates and inactivates ACC, was 25% lower in PYYtg compared to WT mice (Fig. 2G). Interestingly, the fat mass determined by DEXA scan at the anterior abdominal region where liver resides was not significantly different between PYYtg and WT mice (Fig. 2H), indicating the increased lipogenic capacity by PYY overexpression may not lead to an increased hepatic adiposity. To investigate whether the capacity for fatty acid oxidation was also increased by PYY

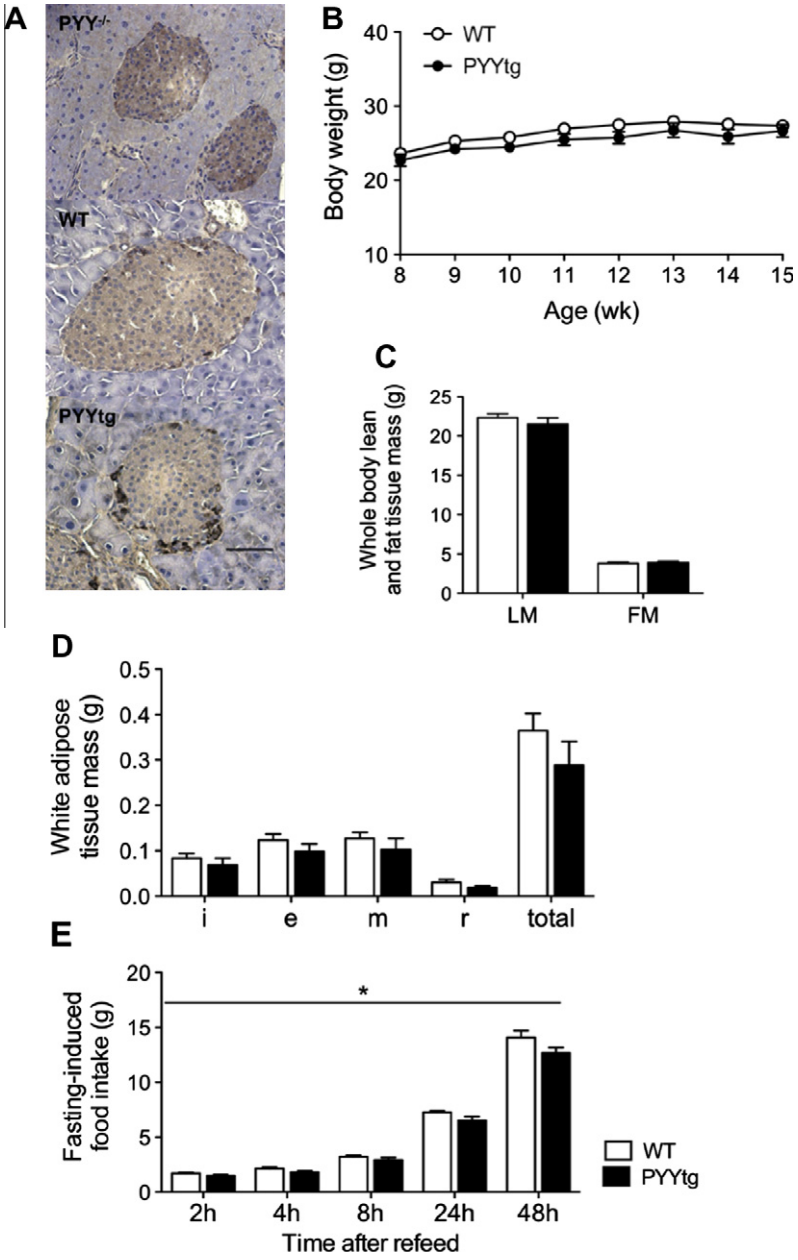

Fig. 1. Effects of adult-onset PYY overexpression on body weight, body composition and fasting-induced food intake. (A) Representative picture of immunohistochemical staining of PYY in pancreatic tissue from 18–20 week-old male wild type (WT), inducible PYY transgenic (PYYtg) and PYY knockout ($PYY^{-/-}$) mice. Scale bar in (A) represents 50 μm. (B) Body weight curve in WT and PYYtg mice. (C) Whole body fat mass (FM) and lean mass (LM) as determined by dual-energy X-ray absorptiometry in WT and PYYtg mice at 15 weeks of age. (D) Weights of individual and summed (total) white adipose depots, namely inguinal (i), epididymal (e), mesenteric (m) and retroperitoneal (r), from WT and PYYtg mice at 17 weeks of age. (E) Food intake after a 24-h fast in WT and PYYtg mice. Food intake measurements at 2, 4, 8, 24 and 48 h after re-feeding in (E) were analysed by repeated-measures ANOVA as indicated by the bar over the multiple measurements during the 48-h period. Data are means ± SEM of 6–8 mice per group. $^{*}P < 0.05$ versus WT mice.

overexpression, we determined the protein levels of carnitine palmitoyltransferase-1 (CPT-1), the mitochondrial transmembrane enzyme controlling entry of fatty acid into mitochondria and the rate-limiting enzyme for fatty acid oxidation (Saggerson and Carpenter, 1981; McGarry et al., 1983). There was no significant difference in the liver or muscle CPT-1 protein expression between PYYtg and WT mice (Fig. 2G), indicating unaltered fatty acid oxidative capacity by PYY overexpression. Taken together, these data show that PYY plays a critical role in regulating lipid metabolism, and that elevated PYY levels increase lipogenic capacity. This may be the major contributor to the increased respiratory exchange ratio seen in PYYtg versus WT mice.

3.4. Adult-onset PYY overexpression enhances insulin action

We performed intraperitoneal insulin tolerance tests and oral glucose tolerance tests in PYYtg and WT mice to investigate the effect of elevated PYY on glucose homeostasis. Blood glucose concentrations before insulin injection or oral glucose bolus were comparable between PYYtg and WT mice in both fasting and non-fasting states (fasting: 6.9 ± 0.29 and 7.3 ± 0.4 mM for WT and PYYtg mice respectively; non-fasting: 11.2 ± 0.33 and 10.8 ± 0.26 mM for WT and PYYtg mice, respectively; data are means ± SEM from 4–6 mice per group, ns). After insulin injection (0.75 U/kg), PYYtg mice showed similar blood glucose responses to that of WT mice

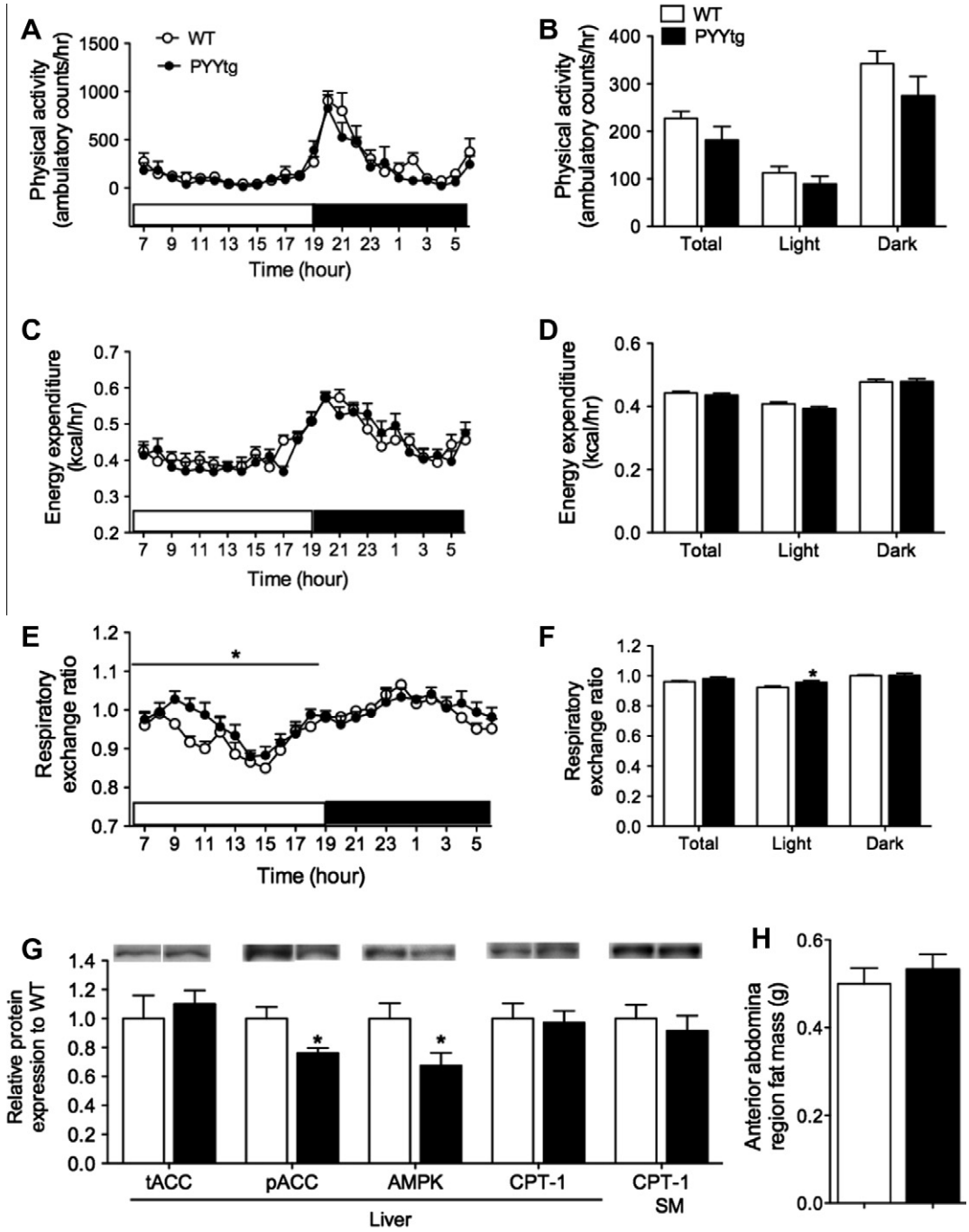

Fig. 2. Effects of adult-onset PYY overexpression on energy metabolism and protein levels of key enzymes involved in lipid metabolism and hepatic fat mass. Physical activity (A, B), energy expenditure (C, D) and respiratory exchange ratio (E, F) in male wild type (WT) and inducible PYY transgenic (PYYtg) mice at 15 weeks of age. 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 21.88 g. (G) Protein levels of total acetyl-CoA carboxylase (tACC), phospho-Ser79 acetyl-CoA carboxylase (pACC), AMP-activated kinase (AMPK) and carnitine palmitoyltransferase-1 (CPT-1) in liver, and protein levels of CPT-1 in skeletal muscle from 20 week-old male wild type (WT) and inducible PYY transgenic (PYYtg) mice. (H) Hepatic fat mass was determined by DEXA scan with region-of-interest frame located at the anterior abdominal region between the xiphisternal joint to the inferior end of the rib cage where liver resides. Data in (G) are normalized to WT values. Data are means ± SEM of 4–7 mice per group. $^{*}P < 0.05$ versus WT mice.

(Fig. 3A and B), suggesting an unaltered response to insulin by PYY overexpression, at least at the dose used. Upon a voluntary oral glucose bolus, PYYtg mice exhibited a similar blood glucose excursion and a comparable area under the resultant glucose curve to WT mice (Fig. 3C and D). Interestingly however, serum insulin concentrations after the oral glucose bolus tended to be lower in the PYYtg mice (Fig. 3E), with the area under the resultant insulin curve showing a trend to be decreased in PYYtg compared to WT mice ($P = 0.1$) (Fig. 3F). Taken together, these results show that adult-onset PYY overexpression does not significantly alter insulin or glucose tolerance, however, it may enhance insulin action on target tissues.

4. Discussion

In this study, we used an adult-onset PYY over-expression mouse model and demonstrated that chronic elevation of PYY

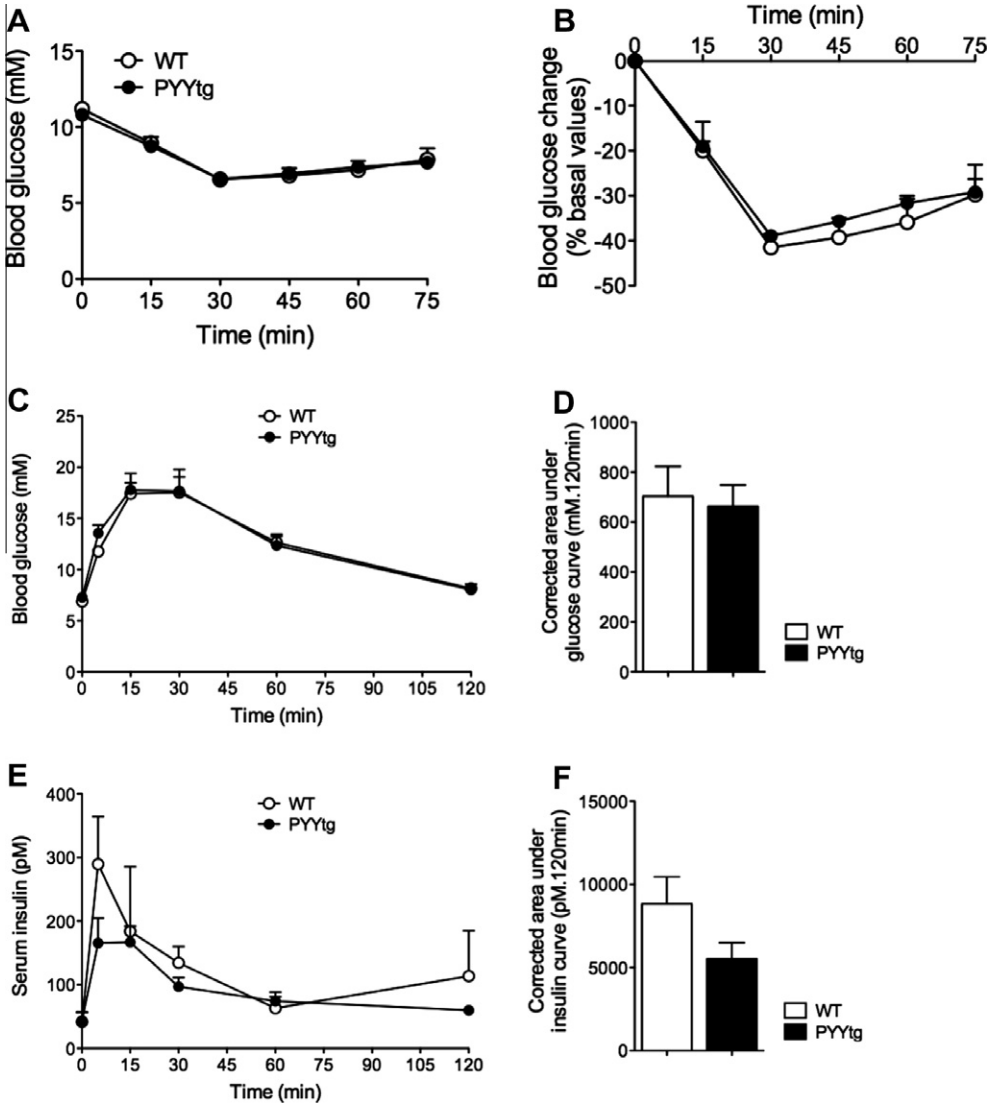

Fig. 3. Effects of adult-onset PYY overexpression on oral glucose and insulin tolerance. Blood glucose levels (A) and the change in blood glucose levels as a percent of basal values (B) during a 75-min intraperitoneal insulin tolerance test (0.75 U/kg) in male wild type (WT) and inducible PYY transgenic (PYYtg) mice at 18–20 weeks of age. Serum glucose levels (C) and area under the resultant glucose curve (D), 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 within 1 min after which t = 0 by each mouse. Data are means ± SEM of 4–6 mice per group. $^{*}P < 0.05$ versus WT mice.

levels has significant effects on energy homeostasis, since PYYtg mice exhibit significantly reduced fasting-induced food intake and increased respiratory exchange ratio. These results are indicative of decreased lipid oxidation and/or increased lipogenesis, albeit there were no significant changes in body weight, adiposity, physical activity or energy expenditure. The increased respiratory exchange ratio with PYY overexpression is likely due to increased lipogenic capacity, since PYYtg mice show significantly reduced phosphorylation of liver ACC, the enzyme catalysing a key regulatory step of fatty acid synthesis, and the activity of which is negatively regulated by phosphorylation (Ruderman et al., 2003). The capacity for lipid oxidation, on the other hand, may not be altered with PYY overexpression, since PYYtg and WT mice have comparable liver or muscle protein levels of CPT-1, the rate-limiting enzyme for lipid oxidation (Saggerson and Carpenter, 1981; McGarry et al., 1983). Moreover, although equally glucose tolerant, PYYtg mice show a trend towards reduced serum insulin levels in response to an oral glucose bolus, suggesting enhanced insulin action in peripheral tissues. Finally, it is important to note that these effects are being observed on a lean background and might be more pronounced under obesifying conditions.

Our data suggest that elevating PYY levels may have a long-term effect in reducing appetite, particularly in suppressing the enhanced appetite induced by a period of energy deficit. It is noteworthy that the effect of chronic peripheral PYY3-36 administration to reduce spontaneous food intake is transient, lasting only for the first few days of administration (Adams et al., 2006; Vrang et al., 2006; Ortiz et al., 2007; van den Hoek et al., 2007). Y receptor down-regulation due to constant stimulation by continuously elevated PYY is suggested to contribute to transient feeding responses to continuous administrations of PYY3-36 (Chelikani et al., 2006). Furthermore, and perhaps more importantly, pharmacological administration of PYY3-36 only targets Y2 receptors and does not affect Y1 receptor signalling, which is still a component of the endogenously-elicited physiological effects by PYY. In this regard, although not statistically significant, the lower spontaneous food intake observed in our PYYtg mouse model, where transgene PYY is expressed and secreted in the same

way as endogenous PYY, thus maintaining the normal meal-associated release rhythm and proportional production of full-length and truncated form of PYY peptides, suggests that the rhythmic release of PYY as well as a combined activation of both Y1 and Y2 receptors might be critical for a balanced and continuous control of food intake. In keeping with the former notion, recent studies show that administrating PYY3-36 intermittently in rats lead to a sustained reduction in daily food intake (Chelikani et al., 2006, 2007). Importantly, our data show a more pronounced reduction in food intake in PYYtg versus WT mice after a 24-h fast than in the non-fasted state, suggesting a potent action of PYY in suppressing hyperphagic responses induced by a period of negative energy balance. This finding has important clinical implications, since negative energy balance, a prerequisite for weight loss, is known to enhance hunger and is a significant predictor for subsequent weight regain (Sainsbury and Zhang, 2010, 2011). Thus, PYY-based anti-obesity therapy may be particularly effective during the weight maintenance period in post-obese people, by controlling hyperphagia. In addition, PYY released from the gut is also thought to act as an "ileal brake" that slows intestinal transit, thereby inducing satiety (Savage et al., 1987; Lin et al., 1996; Maljaars et al., 2008). This effect of PYY has recently been shown to involve interactions with NPY, which increases gastric emptying via central mechanism (Chen et al., 1997; Monnikes et al., 2000; Tough et al., 2011). These findings that PYY interacts with NPY to exert its anorexic effects are in accordance with the marked reduction in fasting-induced food intake observed in our PYYtg mice, since energy deficit, such as that induced by fasting, enhances hypothalamic NPY expression and NPY central actions (Sainsbury and Zhang, 2010).

Our data also demonstrate that PYY is an important player in the regulation of oxidative substrate metabolism, with increasing PYY levels leading to an increased respiratory exchange ratio. It is worth to note that an increase in food intake can potentially enhance respiratory exchange ratio. Since the non-locomotion activity of a mouse in the metabolic chamber may be suggestive of feeding, we conducted an analysis of non-locomotive counts (subtracting the ambulatory counts from the total counts of photobeam breaks) as a measure of non-locomotion activity to examine whether an increased feeding behaviour in PYYtg versus WT mice could be identified during the light period when the increased respiratory exchange ratio was observed. This analysis showed a comparable non-locomotion activity between PYYtg and WT mice (data not shown), lending no support to the possible contribution of an increased light-phase feeding to the increase in light-phase respiratory exchange ratio in the PYYtg mice. Importantly, our results show that this effect may have been caused by an enhanced lipogenic capacity in our PYYtg mice. As fatty acid oxidative capacity seems unaltered by PYY overexpression, as indicated by the comparable liver and muscle CPT-1 protein levels between PYYtg and WT mice, decreased lipid oxidation seems unlikely to have contributed to the increased respiratory exchange ratio observed in PYYtg mice. PYY overexpression may enhance respiratory exchange ratio and lipogenic capacity by activating Y1 signalling, since lack of Y1 signalling either in the germline, or only in peripheral tissues of adult mice, results in significant decreases in respiratory exchange ratio (Zhang et al., 2010b). Interestingly, in contrast to Y1 signalling-mediated effects, activation of Y2 signalling promotes lipid oxidation and/or inhibition of lipogensis, since conditional knockdown of Y2 signalling in the periphery of adult mice leads to significantly increased respiratory exchange ratio (Shi et al., 2011). This is in accordance with pharmacological studies showing that peripheral administration of PYY3-36, which preferentially activates Y2 receptors, mobilizes fatty acids and enhances fatty acid oxidation (Adams et al., 2006; van den Hoek et al., 2007). Our finding that PYY overexpression leads to significantly increased respiratory exchange ratio suggests that the lipogenic activity mediated by PYY1-36 via Y1 signalling is more potent than the lipolytic activity mediated by PYY1-36 and PYY3-36 via Y2 signalling. Considering that PYY is released postprandially and remains elevated during the absorptive phase (Adrian et al., 1986; Ekblad and Sundler, 2002), the lipogenic and anti-lipolytic properties of total PYY may facilitate nutrient incorporation and storage.

Glucose and insulin tolerance were not significantly altered in our PYYtg compared to WT mice. It is worth to note however that the 24-h fasting period prior to oral glucose administration may have led to underestimation of any potential effect of PYY overexpression on glucose clearance, since it was recently shown that the duration of fasting has a significant impact on fasting and glucose-induced circulating glucose levels, and an impaired glucose tolerance induced by high fat diet feeding in mice was more apparent with a 6-h versus a 24-h fast regime (Andrikopoulos et al., 2008). Interestingly, serum insulin levels in PYYtg mice showed a trend towards decreased levels after an oral glucose bolus relative to WT mice, suggesting enhanced insulin action. In keeping with reduced insulin output and improved insulin action by PYY transgenic overexpression, mice with PYY deficiency exhibit marked elevations in glucose-stimulated serum insulin levels and glucose output without changes in circulating glucose excursions compared to WT mice (Boey et al., 2006b). Moreover, low serum PYY levels are associated with increased insulin secretion and insulin resistance in humans (Boey et al., 2006a). Pharmacological studies show that during hyperinsulinemic–euglycemic clamp, peripheral administration of PYY3-36 enhances insulin-stimulated glucose uptake in muscle and adipose tissue in mice fed a high fat diet, suggesting a mechanism involving Y2 signalling (van den Hoek et al., 2004, 2007). It is interesting to note that this PYY-induced enhancement in insulin-stimulated glucose uptake in peripheral tissues may synergize with its lipogenic action, and would be in keeping with the notion that postprandial release of PYY facilitates nutrient uptake and storage. Importantly, our results showing that elevating PYY levels in mice in adulthood may enhance insulin action are consistent with a long-term role of PYY in the regulation of glucose homeostasis and insulin sensitivity, and lend further support to the notion that PYY-based therapies have the therapeutic potential to treat type 2 diabetes (van den Hoek et al., 2004, 2007; Boey et al., 2006a,b).

In the current study where all mice are fed on a chow diet, mice with adult-onset PYY over-expression are not significantly different from WT mice with regards to body weight, body composition, energy expenditure or physical activity. Importantly, however, the reduction in food intake and increase in lipogenic capacity may negate each other, resulting in the unaltered body weight and adiposity despite hypophagia in PYYtg mice. Interestingly, a previous study showed that germline PYY transgenic mice on a high fat diet, or crossed onto the genetically obese *ob/ob* background, showed reduced adiposity and improved glucose tolerance compared to control mice (Boey et al., 2008). These differences were associated with a significant increase in rectal temperature and resistance to diet-induced inhibition of hypothalamo-pituitary-thyroid axis activity (Boey et al., 2008). These changes, however, were not seen between groups fed on a chow diet (Boey et al., 2008). Thus it seems possible that obesogenic conditions, either diet- or genetically-induced, may activate stronger anti-obesity and anti-diabetes properties of PYY that are not seen under normal conditions. Future studies challenging these adult-onset PYY over-expressing mice with obesogenic conditions may shed further light into the therapeutic potential of PYY-based anti-obesity and anti-diabetes treatment.

In summary, we show that adult-onset PYY overexpression leads to a significant reduction in fasting-induced food intake, a significant increase in respiratory exchange ratio in association

with a marked increase in lipogenic capacity, and a strong trend towards decreased glucose-stimulated insulin secretion without changing glucose excursion, suggesting improved insulin action. These results demonstrate the important roles played by PYY under physiological conditions in regulating energy balance and glucose homeostasis.

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