Critical role for peptide YY in protein-mediated satiation and body-weight regulation

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Summary

Dietary protein enhances satiety and promotes weight loss, but the mechanisms by which appetite is affected remain unclear. We investigated the role of gut hormones, key regulators of ingestive behavior, in mediating the satiating effects of different macronutrients. In normal-weight and obese human subjects, high-protein intake induced the greatest release of the anorectic hormone peptide YY (PYY) and the most pronounced satiety. Long-term augmentation of dietary protein in mice increased plasma PYY levels, decreased food intake, and reduced adiposity. To directly determine the role of PYY in mediating the satiating effects of protein, we generated *Pyy* null mice, which were selectively resistant to the satiating and weight-reducing effects of protein and developed marked obesity that was reversed by exogenous PYY treatment. Our findings suggest that modulating the release of endogenous satiety factors, such as PYY, through alteration of specific diet constituents could provide a rational therapy for obesity.

Introduction

The obesity epidemic has profound healthcare implications (Yach et al., 2006). Body weight is regulated by complex physiological mechanisms, and obesity results when there is an imbalance between food intake and energy expenditure (Barsh and Schwartz, 2002; Flier, 2004). Environmental factors interacting with genetic susceptibility have fueled the recent rise in obesity, with increased food availability and portion size, altered meal composition, and lower levels of physical activity playing key roles (Friedman, 2003; Hill and Peters, 1998). Despite significant increases in our understanding of energy homeostasis, safe and effective pharmacological treatments for obesity have remained elusive (Yanovski and Yanovski, 2002). A potential therapeutic strategy is to increase the satiating power of the diet by harnessing the endogenous mechanisms that suppress appetite and caloric intake.

The average Western diet derives 49% of energy intake from carbohydrate, 35% from fat, and 16% from protein (Henderson, 2003; USDA, 1994–1996). This differs considerably from the diet of our hunter-gatherer ancestors who obtained 19%–35% from protein, 28%–47% from fat, and 22%–40% from carbohydrate (Cordain et al., 2002). This observation suggests that reduced protein intake may be in part responsible for the high levels of diseases of affluence in modern Western populations (Cordain et al., 2002). Indeed, high-protein content meals have been shown to increase satiety and decrease food intake (Latner and Schwartz, 1999; Lejeune et al., 2006; Porrini et al., 1997), resulting in both improved weight loss and weight loss maintenance (Dumesnil et al., 2001; Skov et al., 1999; Westerterp-Plantenga et al., 2004). However, it remains unclear how increased dietary protein increases satiety.

The gut-endocrine system plays a major role in regulating ingestive behaviour, and a number of gut-derived hormonal signals have been characterized (Badman and Flier, 2005). Ghrelin, a stomach-derived hormone, increases hunger and food intake. Cholecystokinin (CCK) released from the duodenum acts as a short-term satiety signal. Glucagon-like peptide 1 (GLP-1) and oxyntomodulin, derived from the proglucagon gene and released from gut-endocrine L cells, decrease food intake in rodents and humans (Badman and Flier, 2005). Peptide YY3-36 (PYY3-36), an agonist at neuropeptide Y2 receptors (NPY2R), and also produced by L cells, reduces hunger and food intake in rodents and in lean and obese humans (Batterham et al., 2002, 2003a; Degen et al., 2005). The precise sites of action of PYY3-36 remain to be clarified. Electrophysiological, intra-arcuate administration and NPY2R antagonist studies suggest that PYY3-36 acts directly in the hypothalamic circuits that regulate energy homeostasis, but additional work has implicated brainstem and vagal inputs (Abbott et al., 2005a, 2005b; Acuna-Goycolea and van den Pol, 2005; Challis et al., 2003; Halatchev and Cone, 2005; Halatchev et al., 2004). Meal-associated gut hormone release depends on the amount and composition of the food consumed. The satiating effects of protein require the gut-endocrine axis, as suppression of food intake is observed only when protein is administered enterally (Walls et al., 1991). Gut hormones could mediate the differential satiation produced by protein, fat, and carbohydrate. We therefore undertook studies in normal and obese humans and mechanistic rodent studies to investigate this hypothesis.

Results

Enhanced-protein diets cause the greatest satiation and PYY release in humans

To determine the effect of meal macronutrient composition on hunger scores and plasma concentrations of PYY, ghrelin, and GLP-1, age-matched normal-weight and obese male volunteers participated in a randomized, three-way crossover study (Table 1). Fasted subjects consumed an isocaloric meal, high in one macronutrient: high-protein, high-fat, or high-carbohydrate (Table S1). The high-protein diet caused the greatest reduction in hunger in both normal and obese subjects (Figures 1A and 1B), as previously reported (Latner and Schwartz, 1999; Lejeune et al., 2006; Porrini et al., 1997). In normal-weight but not obese subjects, the high-fat meal was more satiating than the high-carbohydrate meal. The high-protein meal resulted in the greatest increment in both total plasma PYY and integrated PYY levels in both groups, although post-meal levels were lower in obese subjects (Figures 1C and 1D and Table S2). High-fat meals caused greater total PYY release than high-carbohydrate meals in normal but not obese subjects. Satiety and plasma PYY levels thus varied in parallel.

PYY circulates in both PYY1-36 and PYY3-36 forms, but the latter has the most potent anorectic effects when administered peripherally (Chelikani et al., 2005). In fasted normal-weight and obese subjects, PYY3-36 constituted ~65% of total circulating PYY, but plasma PYY3-36 concentrations were significantly lower in the obese group compared with the normal-weight group (fasting PYY3-36; normal-weight group = 22.7 ± 2.2 pmol/l, obese group = 15.8 ± 1.3 pmol/l, $p < 0.05$). In response to the different meals, the temporal plasma profiles and integrated levels of PYY3-36 mirrored those of total PYY in both groups (Figures 1E and 1F) and varied in parallel with satiety. Neither plasma-active ghrelin levels nor plasma-active GLP-1 levels showed differential responses to protein, fat, or carbohydrate intake (Figures S1A–S1D and Table S2). Leptin levels were unchanged during the study. The high-carbohydrate diet caused the greatest release of insulin in both subject groups (Table S2). These findings suggested that PYY could mediate the satiating effects of protein in humans. We therefore developed a rodent experimental model in which to investigate this possibility.

High-protein diets reduce caloric intake and increase PYY release in mice

High-protein diets have been demonstrated to increase satiety and reduce food intake in rodents without causing conditioned taste aversion (Bensaid et al., 2003). We utilized two isocaloric high-fat diets, either with normal-protein (HFNP) or high-protein (HFHP), and two isocaloric low-fat diets, with normal-protein (LFNP) or high-protein (LFHP), for our studies (Table S3). In a randomized four-way crossover study, when fasted C57Bl/6 mice were re-fed with each of the different diets, increasing the protein content of both the low-fat and high-fat diets significantly decreased ad libitum calorie intake (Figure 2A). Furthermore, the two high-protein diets (HFHP and LFHP) caused greater PYY release than the two normal-protein diets (Figure 2B and integrated AUC: HFNP = 157.1 ± 6.6 pmol.8 hr, HFHP = 182.2 ± 5.2 pmol.8 hr, LFNP = 138 ± 6.8 pmol.8 hr, LFHP = 196.2 ± 7.3 pmol.8 hr. LFNP versus LFHP $p < 0.001$, HFNP versus HFHP $p < 0.01$). In contrast, there was no differential effect of the different meals on the suppression of either active ghrelin or desacyl ghrelin (Figures S2A and S2B). These findings were consistent with our human studies validating the use of mice as a model.

Table 1. Baseline characteristics of the normal-weight and obese human subjects studied

	Normal-weight	Obese	P value
Age (years)	28.4 ± 2.6	32.2 ± 4.1	> 0.5
BMI (kg/m^2)	23.0 ± 0.8	37.7 ± 2.4	< 0.001
Body fat (%)	15.6 ± 1.5	40.4 ± 2.9	< 0.001
Leptin (ng/ml)	1.26 ± 0.24	17.47 ± 1.92	< 0.001
Glucose (mmol/l)	5.26 ± 0.19	5.13 ± 0.17	> 0.05
Insulin (pmol/l)	25.6 ± 3.6	110.5 ± 11.2	< 0.001

Blood parameters are for the fasted state. Values represent mean ± SEM, and n = 9 for obese group and n = 10 for normal-weight group.

Long-term high-protein diets reduce weight gain and enhance PYY synthesis and secretion in mice

We next determined the long-term effects of the different macronutrient diets on adiposity and plasma levels of gut hormones. Mice were randomized to four groups, each with ad libitum access to one of the diets for 16 weeks. In combination with either high-fat or low-fat, increased dietary protein resulted in significantly less weight gain, less white adipose tissue, and lower plasma leptin levels compared to the corresponding isocaloric normal-protein diets (Figures 2C, 2D, and S2C). Moreover, high-protein diets resulted in higher fasting and fed plasma levels of PYY than the corresponding normal-protein diets and an equivalent pattern in both colonic and ileal *Pyy* mRNA expression (Figures 2E, 2F, and S2D). In contrast, while plasma-active ghrelin, desacyl ghrelin, and stomach *ghrelin* mRNA were lower in the fed state in all groups, no differences between the high-protein and normal-protein dietary regimens were observed (Figures S2E and S2F).

In the arcuate hypothalamic nucleus, orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) and anorexigenic pro-opiomelanocortin (POMC) neurons respond to gut hormones and adiposity signals (Barsh and Schwartz, 2002; Flier, 2004; Acuna-Goycolea and van den Pol, 2005; Batterham et al., 2002; Cowley et al., 2003). We therefore determined the effects of the different diets on hypothalamic mRNA expression of *Npy*, *Agrp*, and *Pomc*. High-protein resulted in enhanced reduction of hypothalamic *Npy* and *Agrp* mRNA in the fasted to fed transition (Figures 2G and 2H). No difference in hypothalamic *Pomc* mRNA levels was seen between the four diets, although appropriate alterations in the fasted to fed transition were apparent (Figure S2G). Together, these results showed that high-protein diets reduced adiposity, enhanced PYY synthesis and secretion, and caused greater reduction in hypothalamic expression of *Npy* and *Agrp* mRNA. These findings suggested that PYY could mediate both the satiating effects of protein and the associated reduction in long-term adiposity.

Deletion of *Pyy* in mice causes hyperphagia and obesity

To investigate whether PYY plays a primary role in mediating the satiating effects of protein, we deleted the entire coding region of *Pyy* in mice using CreloxP techniques. We did not leave a selectable marker or an expression cassette in the *Pyy* locus and avoided using homology arms that impinged upon the adjacent pancreatic polypeptide (*Ppy*) gene (Figure 3A). We also

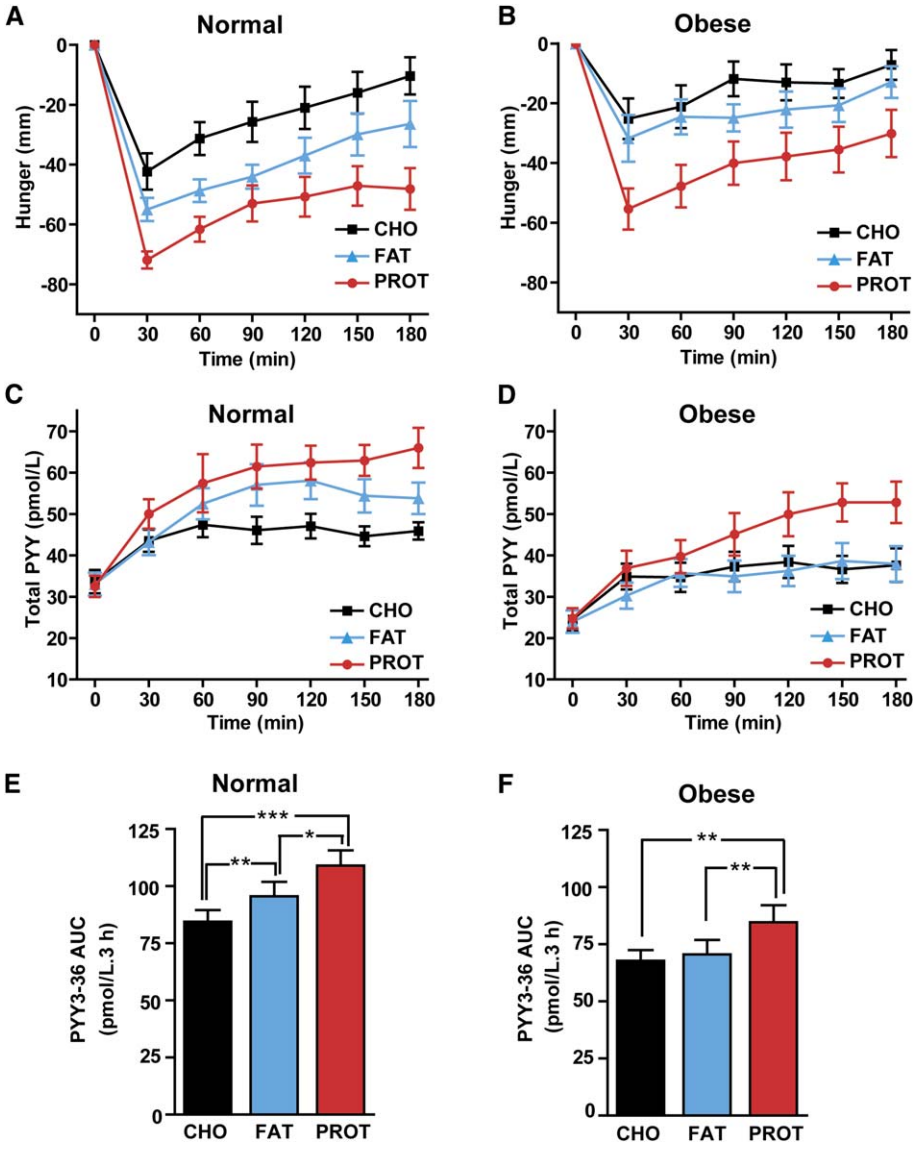

Figure 1. Hunger scores and PYY levels in normal-weight and obese human subjects following high-protein, high-fat, and high-carbohydrate isocaloric meals

A and B) Time course of subjective visual analog scale ratings (mm change from baseline) for hunger in normal-weight subjects (**A**) and obese subjects (**B**) after ingestion at time 0 of isocaloric meals.

C and D) Time course of plasma PYY levels in normal-weight subjects (**C**) and obese subjects (**D**) after ingestion at time 0 of isocaloric meals.

E and F) Area under curve (AUC) for PYY3-36 release in normal-weight subjects (**E**) and obese subjects (**F**) after ingestion at time 0 of isocaloric meals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PROT = high-protein; CHO = high-carbohydrate; FAT = high-fat. All values represent group mean ± SEM, n = 10 for normal weight subjects, and n = 9 for obese subjects.

generated mice that retained the *Pyy* gene flanked by loxP sites but lacked the neomycin cassette ($Pyy^{lox/lox}$ mice). *Pyy* null mice, backcrossed four times onto a C57Bl/6 background, had undetectable levels of circulating PYY and colonic *Pyy* mRNA (Figure 3F). Importantly, expression of *Ppy* was normal (Figure 3G). $Pyy^{lox/lox}$ mice on the same genetic background had normal PYY levels (data not shown). *Pyy* null mice were hyperphagic, displaying increased daily cumulative food intake and increased re-feeding in response to an overnight fast (Figures 4A and 4B). From 5 weeks of age, *Pyy* null mice of both sexes weighed significantly more than littermates on normal chow and displayed increased body size (Figures 4C and 4D and data not shown). Ten-week-old *Pyy* null mice on normal chow were 37% ± 5% heavier (n = 16, $p < 0.001$) than wild-type (wt) littermate controls. MRI scanning revealed a 237% ± 40% increase in total body fat, with increased internal and subcutaneous fat (Figures 4E–4G and Figures S3A and S3B). Plasma leptin levels were also increased relative to control littermates (Figure S3C). In contrast, $Pyy^{lox/lox}$ mice, which like the *Pyy* null mice retain the 129Sv genomic region around the *Pyy* locus, but had normal *Pyy* expression, displayed no body weight or food intake phenotypes and were indistinguishable from wt littermates (Figure S3D and data not shown). These findings strongly suggest that the obesity phenotype is due to the loss of *Pyy* and not due to the presence of an additional obesity gene linked to the *Pyy* locus.

The marked obesity phenotype resulting from the deletion of *Pyy* suggests that PYY is a physiologically relevant component of the mechanisms regulating energy homeostasis. We therefore reasoned that *Pyy* null mice would be hypersensitive to the acute effects of PYY3-36 and that long-term PYY3-36 replacement would reverse the obesity phenotype. Acute dose-response studies examining feeding responses over 24 hr revealed that *Pyy* null mice were markedly hypersensitive to the anorexigenic effects of exogenous PYY3-36 at all doses examined compared to wt littermates (Figures 4H–4L). For example, administration of 1 μg/100 g of body weight of PYY3-36 to *Pyy* null mice produced a similar reduction in food intake as 10 μg/100 g of body weight of PYY3-36 caused in control mice, a 10-fold increase in sensitivity (Figures 4H–4L).

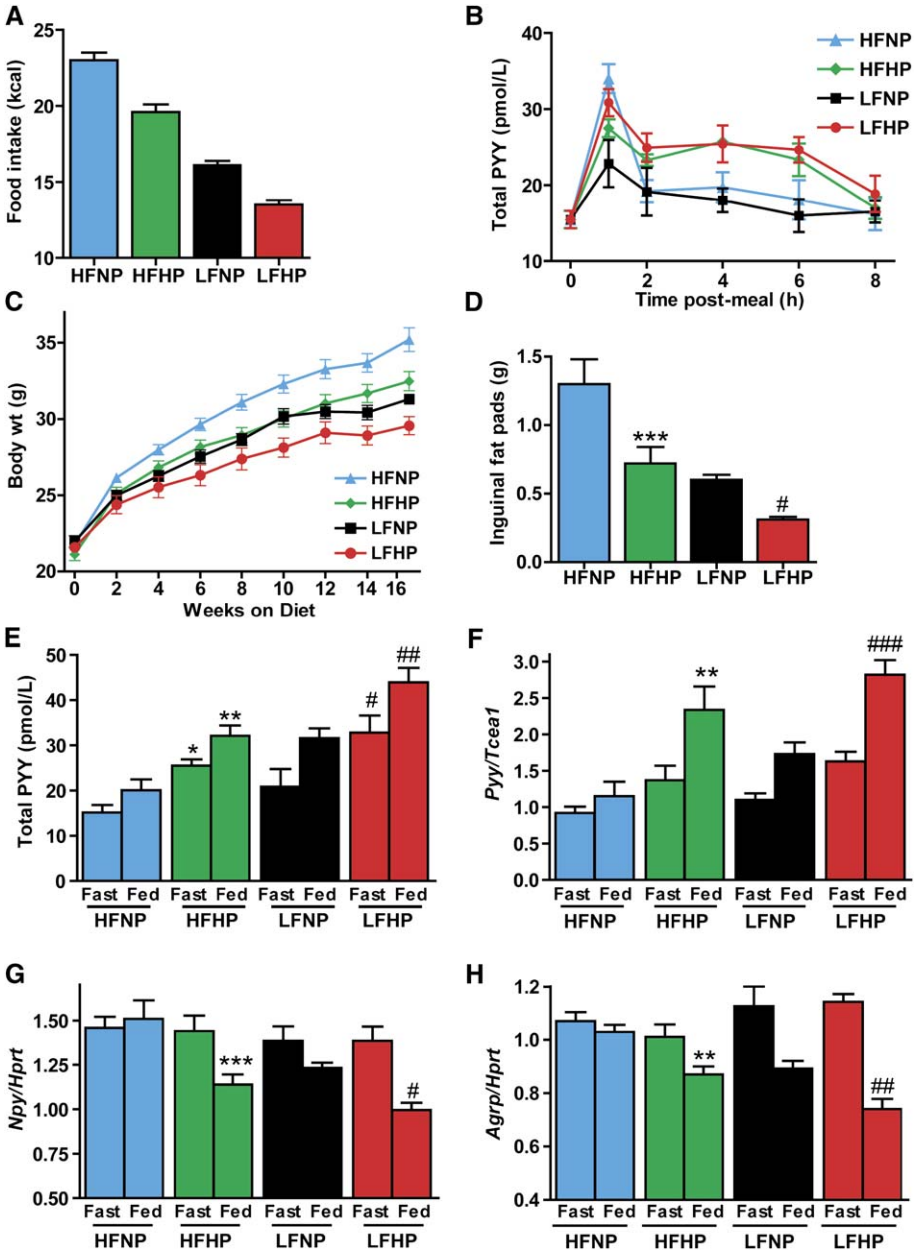

Figure 2. Effect of high-protein diets on food intake, PYY profiles, body weight, and hypothalamic neuropeptide expression in mice

For all panels: high-fat normal-protein (HFNP: blue symbols), high-fat high-protein (HFHP: green symbols), low-fat normal-protein (LFNP: black symbols), and low-fat high-protein (LFHP: red symbols).

A) 24 hr caloric intake in 8- to 10-week-old male C57Bl/6 mice exposed to different composition diets, n = 30 per diet. All diets are significantly different from each other $p < 0.01$.

B) Plasma PYY profiles in 10-week-old male C57Bl/6 mice after ingestion of different isocaloric meals, n = 6 per diet.

C) Body weight profiles of male C57Bl/6 mice on different diets for 16 weeks, n = 20 per diet.

D) Inguinal fat pad weights in male C57Bl/6 mice after 16 weeks on different diets, n = 10 per diet.

E) Fasted (fast) and fed plasma PYY levels in male C57Bl/6 mice after 16 weeks on different diets, n = 10 per diet.

F) Fasted (fast) and fed colonic *Pyy* mRNA levels in male C57Bl/6 mice after 16 weeks on different diets, n = 10 per diet. *Pyy* mRNA levels are expressed as relative *Tcea1*.

G and H) Fasted (fast) and fed hypothalamic *Npy* mRNA levels (**G**) and *Agrp* mRNA in male C57Bl/6 mice after 16 weeks on different diets, n = 10 per diet. *Npy* and *Agrp* mRNA levels are expressed as relative *Hprt*. All values represent group mean ± SEM.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for HFNP versus HFHP, #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ for LFNP versus LFHP.

For the chronic replacement experiments we adopted two treatment strategies. Since PYY3-36 is normally released episodically in response to food intake, we first used an intermittent treatment protocol. Mice were treated once a day before the dark phase feeding period with 5 μg/100 g of body weight of PYY3-36, a dose that inhibited cumulative 24 hr food intake in *Pyy* null but not control mice (Figures 4K and 4L). In addition, we implanted mice with osmotic mini-pumps continuously delivering PYY3-36 at a dose previously demonstrated to cause weight loss in mice with diet-induced obesity (Pittner et al., 2004). To assess whether replacement of PYY3-36 could both prevent weight gain and cause weight loss in markedly obese mice we used *Pyy* null mice at two ages: 12-week-old mice, which were still gaining weight, and 22-week-old mice, with marked but relatively stable obesity. After 15 days of once daily PYY3-36 injections, *Pyy* null mice lost approximately 20% of their body weight (Figure 5A). In contrast, saline-treated *Pyy* null, saline-treated wt mice and PYY3-36-treated wt animals had stable weight profiles (Figure 5A). Within one week of stopping PYY3-36 replacement *Pyy* null mice regained more than 60% of the body weight they had lost, demonstrating that the re-removal of PYY resulted in the reappearance of the obesity phenotype (weight change in the 7-day period post-study: previously saline-treated wt mice = 0.99 ± 0.19 g, previously PYY3-36-treated wt mice = 0.96 ± 0.25 g, previously saline-treated *Pyy* null mice = 1.54 ± 0.27 g, previously PYY3-36-treated *Pyy* null mice = 7.89 ± 0.4 g, $p < 0.001$). In the chronic osmotic mini-pump studies, PYY3-36 treatment for 21 days in 12-week-old *Pyy* null mice resulted in weight reduction and the prevention of the weight gain seen in saline-treated animals of the same genotype, normalizing the body weight of *Pyy* null mice to that of saline-treated wt littermates (Figure 5B). This weight loss was associated with a reduction in inguinal fat pad weight (Figure 5C). Treatment of obese weight-stable 22-week-old *Pyy* null mice

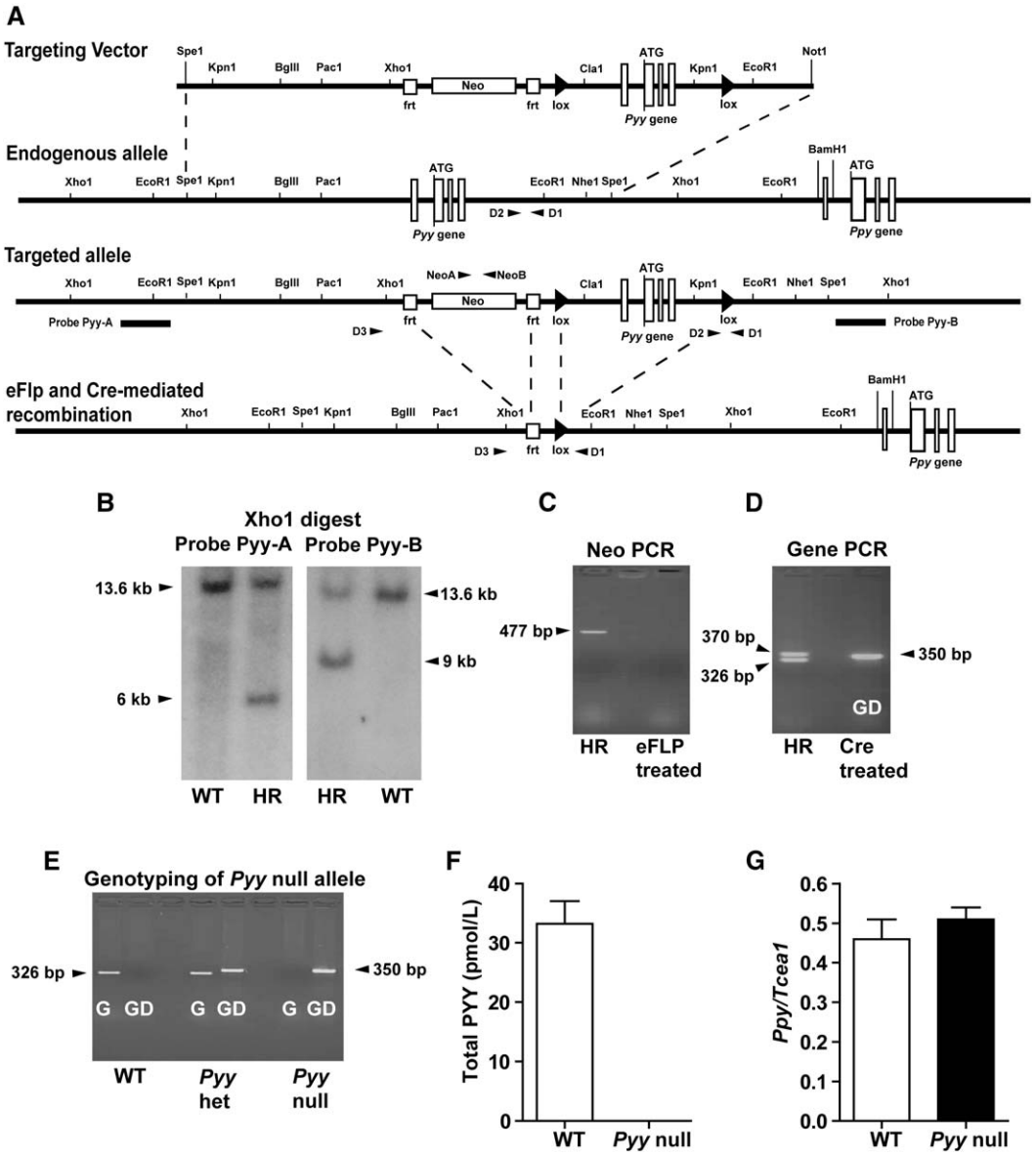

Figure 3. Targeting of the *Pyy* locus and lack of PYY peptide in *Pyy* null mice

A) Targeting construct design, restriction map of the *Pyy* and *Ppy* loci, and the *Pyy* locus after homologous recombination and after deletion of the neomycin cassette and *Pyy*-coding region.

B) External probes Pyy-A and Pyy-B were used to distinguish the homologously recombined (HR) from the wild-type (wt) locus after Xho1 digest of ES cell DNA.

C) e-Flp-mediated deletion of the neomycin cassette was confirmed by PCR using primers NeoA and NeoB.

D) Cre-mediated deletion of the *Pyy*-coding region was confirmed using primers D1, D2, and D3. The 326 bp and 370 bp products obtained with D1 and D2 demonstrate the presence of the wt and floxed *Pyy* alleles. The 350 bp product indicates the gene deletion (GD) of the *Pyy* coding region between the loxP sites.

E) Genotyping of the *Pyy* null allele using primers D1, D2, and D3 to identify wt mice with the intact *Pyy* gene (G), *Pyy* heterozygote (het), and gene-deleted (GD) *Pyy* null mice.

F) Absence of PYY peptide in the plasma of fed *Pyy* null mice, n = 8 per genotype.

G) Equivalent pancreatic polypeptide (*Ppy*) mRNA expression in pancreases from male wt and *Pyy* null mice, n = 8 per genotype. Data presented are mean ± SEM.

with PYY3-36 resulted in a 24.3% ± 0.5% reduction in body weight compared to saline-treated *Pyy* null mice ($p < 0.001$) (Figure 5D). Taken together, the acute dose-response studies demonstrating increased sensitivity of *Pyy* null mice to exogenous PYY3-36 and the reversal of the obesity phenotype of *Pyy* null mice with chronic PYY3-36 treatment strongly suggest that their phenotype is due to the loss of *Pyy*, which acts as a physiologically relevant satiety hormone.

PYY3-36 has been shown to decrease feeding when injected into the arcuate nucleus, alter expression of hypothalamic *Npy* and *Pomc* mRNA (Challis et al., 2003), and have acute electrophysiological effects on arcuate NPY and POMC neurons (Acuna-Goycolea and van den Pol, 2005; Batterham et al., 2002). Therefore, we examined the effects of *Pyy* deletion on expression of hypothalamic *Npy, Agrp*, and *Pomc* mRNA. Compared to wt littermate controls, *Pyy* null mice displayed increased levels of *Npy* mRNA in the fasted state and a failure to regulate *Npy* expression upon feeding (Figure 5E). Fasting levels of *Agrp* mRNA were similar in *Pyy* null and wt control mice but were significantly higher in *Pyy* null mice in the fed state

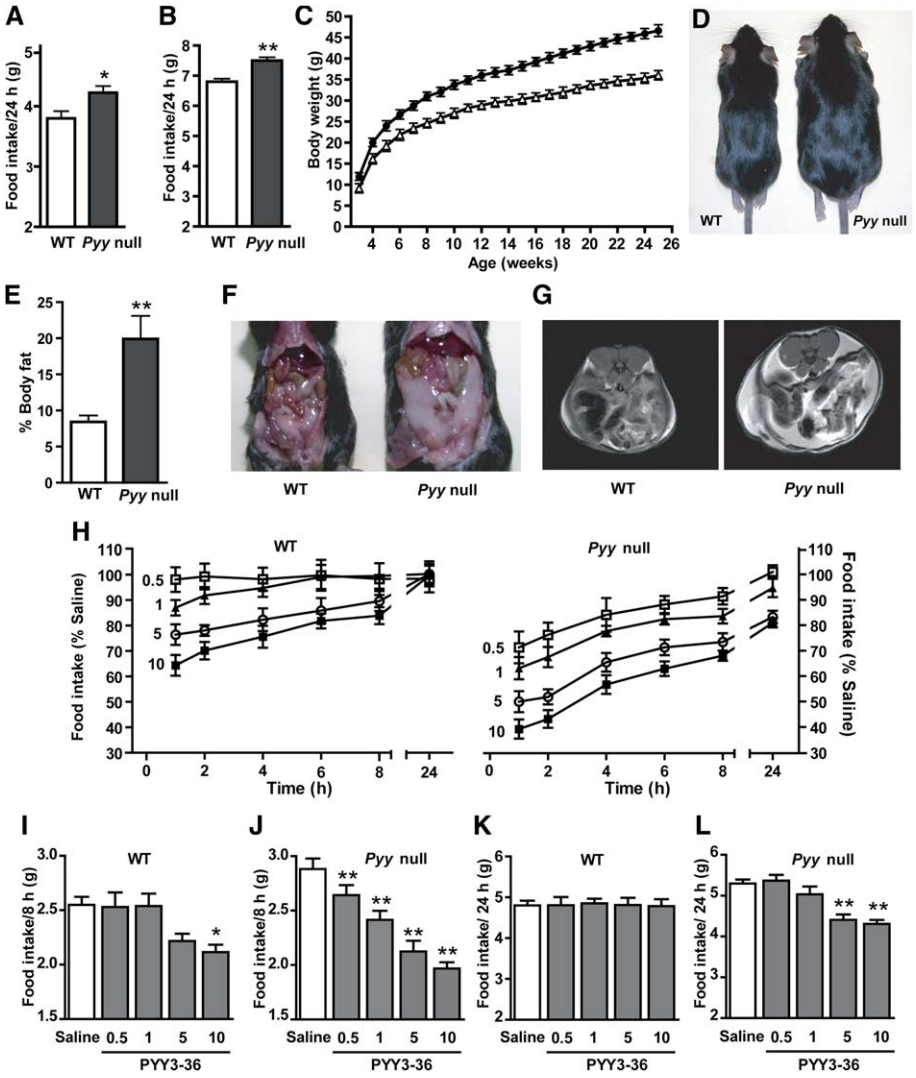

Figure 4. *Pyy* null mice display hyperphagia and obesity and have increased sensitivity to exogenous PYY3-36

A) 24 hr food intake in 8-week-old male wild-type control (wt) and *Pyy* null mice on regular chow, n = 12.

B) 24 hr food intake in 8-week-old male wt and *Pyy* null mice in response to 16 hr overnight fast, n = 12.

C) Body weight profiles of male wt (open triangles) and *Pyy* null mice (closed circles) on regular chow, n = 16 ($p < 0.05$ for all data points).

D) Typical external dorsal appearance of 10-week-old male wt and *Pyy* null mice.

E) Percentage body fat in 10-week-old male wt and *Pyy* null mice determined by MRI, n = 6.

F) Exposed ventral view of 10-week-old wt and *PYY* null mice illustrating increased body fat.

G) Representative cross-sectional MRI scans of 10-week-old male wt and *Pyy* null mice.

H–L) wt and *Pyy* null mice were fasted overnight then injected i.p with saline or PYY3-36 at the doses indicated (μg per 100 g body weight) and cumulative food intake measured, n = 12 per genotype for each dose. 8 hr cumulative food intake in wt (**I**) and *Pyy* null mice (**J**) and 24 hr cumulative food intake in wt (**K**) and *Pyy* null (**L**) mice. All data presented are mean ± SEM. *$p < 0.05$, **$p < 0.01$.

(Figure 5F). In *Pyy* null mice there was a trend to lower *Pomc* levels in the fed state compared to control animals, but no differences were observed in fasted mice (Figure S3E). These findings suggest that PYY regulates hypothalamic neuropeptide expression, but it remains unclear whether this is a direct effect or also involves other pathways such as vagal inputs or the brainstem.

Pyy null mice are resistant to the satiating and weight-reducing effects of high-protein diets

We used *Pyy* null mice to test the hypothesis that PYY is required for the satiating and weight-reducing effects of high-protein diets. Using a four-way crossover study design we examined the effects of the HFNP, HFHP, LFNP, and LFHP diets on 24 hr food intake in *Pyy* null mice, wt littermate controls, and $Pyy^{lox/lox}$ mice. wt littermates and $Pyy^{lox/lox}$ mice exhibited a similar feeding response to the four different diets to that seen in our experiments with C57Bl/6 mice, suggesting that genetic background did not influence this finding (Figures 2A, 6A, and 6B). When dietary protein was kept constant an increase in the carbohydrate content and decrease in fat content resulted in equivalent 25% reduction in caloric intake in *Pyy* null mice, wt littermate controls, and $Pyy^{lox/lox}$ mice (compare HFNP versus LFNP and HFHP versus LFHP) (Figures 6A–6C). These results demonstrate that the food intake response to alterations in dietary fat and carbohydrate do not require PYY. In contrast, while increasing the protein content of the low-fat (LFHP) and high-fat (HFHP) diets reduced ad libitum caloric intake in wt controls and $Pyy^{lox/lox}$ mice, the satiating effects of high protein were abolished in the *Pyy* null mice (Figures 6A and 6C). Moreover, in *Pyy* null mice the weight-reducing effects of the HFHP diet were absent (Figure 6D). These findings suggest that PYY is required for the satiating and weight-reducing effects of protein. Mice lacking *Npy2r*, which mediates the anorectic effects of peripherally administered PYY3-36 (Abbott et al., 2005b; Batterham et al., 2002; Scott et al., 2005), also failed to reduce their calorie intake in response to increased dietary protein while responding to changes in dietary fat and carbohydrate under equivalent protein conditions (Figure 6E).

Discussion

Peripheral administration of PYY3-36 to rodents, primates, and humans acutely decreases food intake (Batterham et al., 2002, 2003a; Chelikani et al., 2005, 2006; Degen et al., 2005; Koegler et al., 2005; Scott et al., 2005). Furthermore, chronic administration of PYY reduces adiposity in rodents (Chelikani et al., 2006;

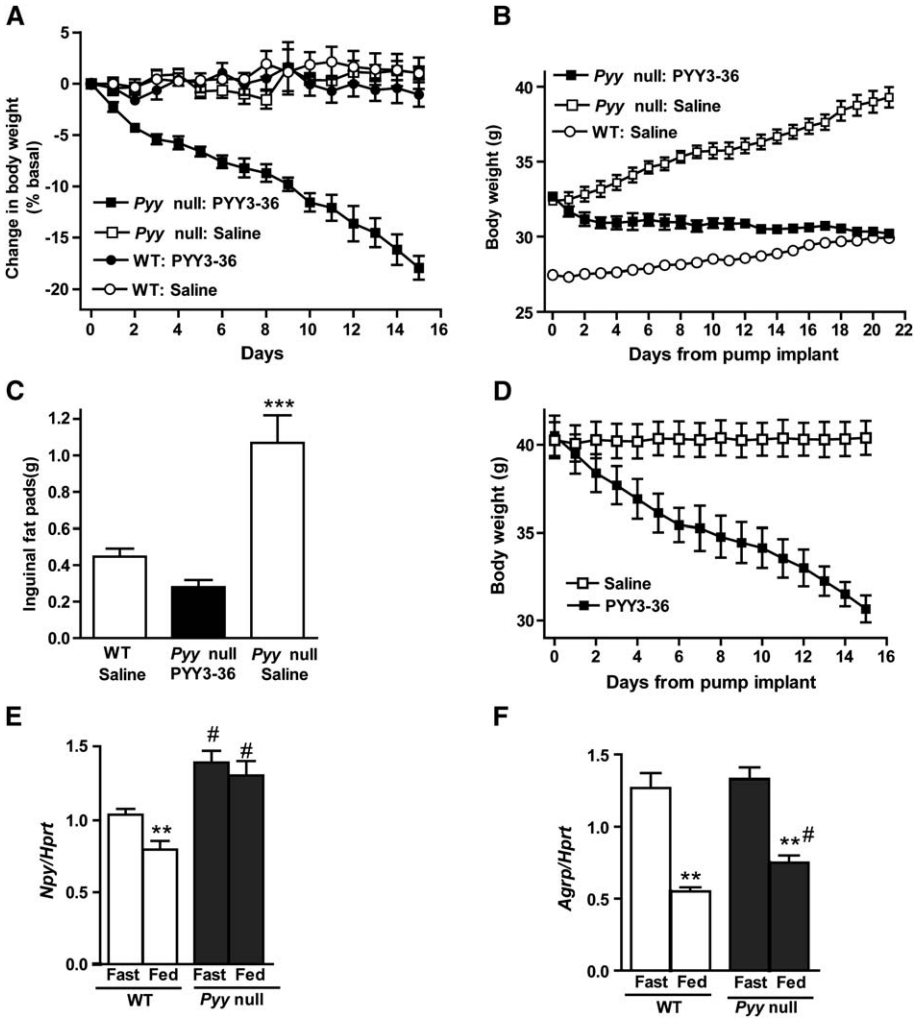

Figure 5. Effect of exogenous PYY3-36 on body weight in *Pyy* null mice

A) Effect of once daily i.p PYY3-36 (5 μg/100 g body weight) or saline on body weight in 20-week-old male wt and *Pyy* null mice. Results are expressed as percentage change of starting body weight, n = 8 per treatment group.

B) Effect on body weight of PYY3-36 (50 μg/100 g body weight/day) or saline administered continuously to 12-week-old male *Pyy* null or wt mice by mini-osmotic pump for 21 days, n = 12 per treatment group.

C) Inguinal fat pad weight after 21 days continuous treatment with PYY3-36 (50 μg/100 g body weight/day) or saline in male *Pyy* null or wt mice, n = 12 (saline v PYY3-36 treated *Pyy* null mice, ***$p < 0.001$).

D) Effect on body weight of PYY3-36 (50 μg/100 g body weight/day) or saline administered continuously to 22-week-old female *Pyy* null or wt mice by mini-osmotic pump for 15 days, n = 6 per treatment group.

E and F) Expression of hypothalamic *Npy* mRNA (**E**) and *AgRP* mRNA (**F**) in fasted (fast) and fed conditions in wt and *Pyy* null mice determined by RT-PCR. Results are expressed relative to *Hprt* mRNA, n =10 per group. All data presented are mean ± SEM. **$p < 0.01$ wt fed versus wt fasted, #$p < 0.05$ *Pyy* null versus wt.

Pittner et al., 2004). Despite these findings, the role of PYY as a negative regulator of energy homeostasis has been questioned (Tschop et al., 2004). Here we show that *Pyy* null mice are hyperphagic and develop marked obesity but are hypersensitive to exogenous PYY. Moreover, chronic treatment with PYY reverses their obesity phenotype. These findings provide compelling evidence that PYY is a physiologically relevant regulator of food intake and body weight.

The phenotype of our *Pyy* null mouse differs from that of two recently described models (Boey et al., 2006; Schonhoff et al., 2005). In contrast to our model, these mice were generated with gene replacement vector techniques and retain exogenous genetic material in the *Pyy* locus. The model of Schonhoff et al. expresses lacZ from the *Pyy* start site and also lacks expression of the pancreatic polypeptide gene (*Ppy*), which is adjacent to the *Pyy* gene. It is unclear why expression of the *Ppy* gene is disrupted, but one potential reason is that the homologous recombination event has interfered with the regulatory sequences required for *Ppy* expression. While pharmacological evidence has implicated PPY in energy homeostasis, the physiological role of this hormone is currently unclear (Batterham et al., 2003b). Combined disruption of *Pyy* and *Ppy* may therefore lead to compensatory changes in the mechanisms regulating energy homeostasis, making interpretation of the physiological role of PYY alone impossible in this model. The *Pyy* null mouse generated by Boey et al. retains both green-fluorescent protein and Cre recombinase expression cassettes within the *Pyy* locus. PPY is detectable by immunocytochemistry in this model but it is unclear if expression levels are normal. The expression of Cre recombinase within L cells and PYY-producing cells in the pancreatic islets may have adverse effects upon these cell types as recently it has been demonstrated that transgenic mice expressing Cre recombinase in β cells have defective glucose homeostasis (Lee et al., 2006). Differences in genetic background may also contribute to the phenotypic discrepancies. To delete the neomycin cassette, Schonhoff et al. used a deletor mouse on an FVB background (Schonhoff et al., 2005), which is an obesity-resistant strain when either on a high-fat diet or when carrying a transgene that causes obesity on a C57Bl/6 background (Chen et al., 2005; Ludwig et al., 2001). Boey et al. utilized C57Bl/6 mice to generate their model, but the precise genetic background studied is not stated. In contrast, we have backcrossed our *Pyy* null allele onto a C57Bl/6 background for analysis. Furthermore, we have studied the $Pyy^{lox/lox}$ mice that retain the *Pyy* locus and normal *Pyy* expression and have a similar genetic background to our *Pyy* null mice. $Pyy^{lox/lox}$ mice are indistinguishable from wt control littermates, further suggesting that the phenotype of our mice is not due to genetic background, the presence of an obesity gene linked to the *Pyy* locus, or a feature of our gene-targeting strategy. Our data robustly demonstrating

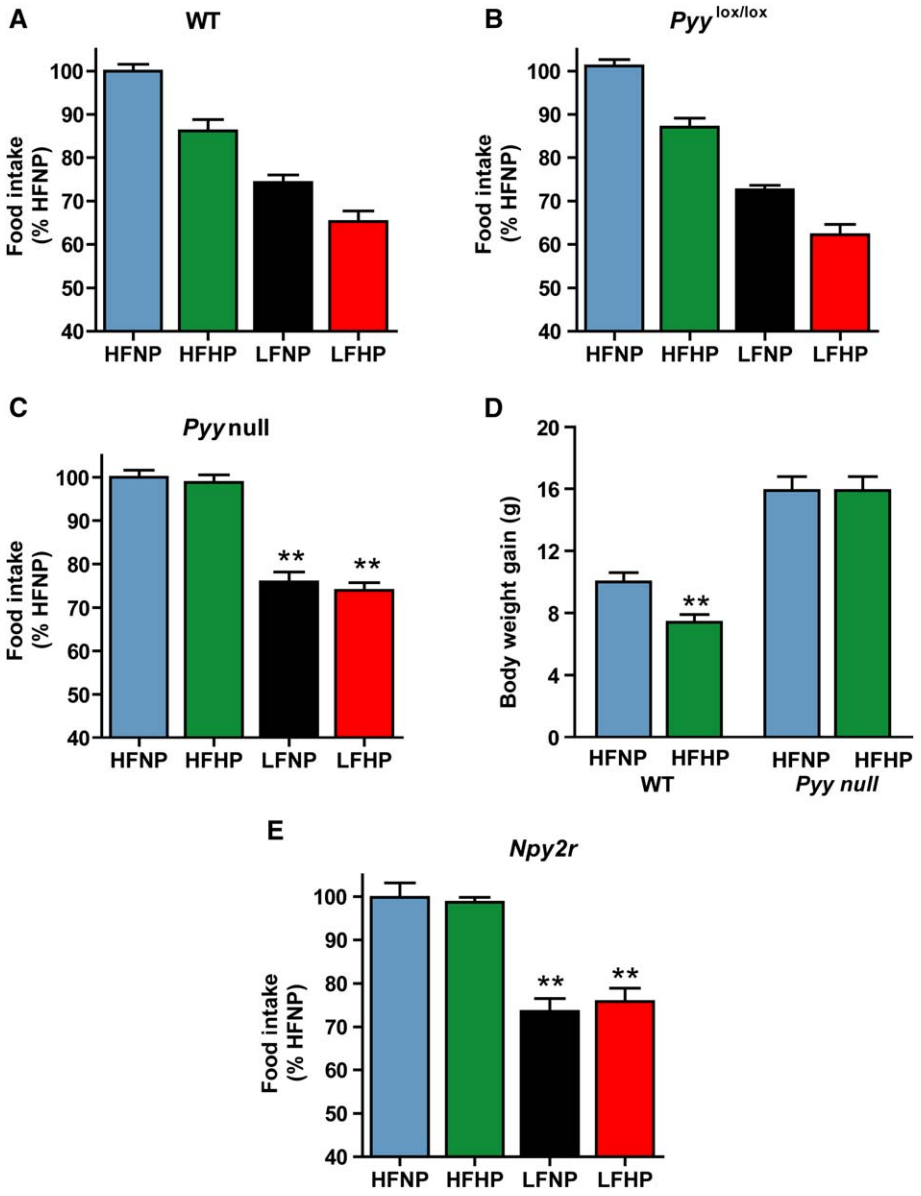

Figure 6. *Pyy* null mice resist the satiating effects of protein

For all panels: high-fat normal-protein (HFNP: blue bar), high-fat high-protein (HFHP: green bar), low-fat normal-protein (LFNP: black bars), and low-fat high-protein (LFHP: red bars). Food intake is expressed as a % of intake of the HFNP diet.

A) 24 hr cumulative food intake of different diets following an overnight fast in 8-week-old female wt mice, n = 18 per diet group; all diets are significantly different from each other, $p < 0.05$.

B) 24 hr cumulative food intake of different diets in 8-week-old female $Pyy^{lox/lox}$, n = 12 per diet group; all diets are significantly different from each other, $p < 0.05$.

C) Total 24 hr cumulative food intake of different diets following an overnight fast in 8-week-old female *Pyy* null mice. n = 17 per group, ** $p < 0.01$, high-fat diets versus low-fat diets.

D) Body weight gain in male wt and *Pyy* null mice with ad libitum access to HFNP or HFHP diets for 8 weeks, n = 8 per group, **$p < 0.01$, HFNP versus HFHP.

E) 24 hr cumulative food intake of different diets following an overnight fast in 8-week-old female *Npy2r* null mice, n = 12 per diet group, **$p < 0.01$ high-fat diets versus low-fat diets. All data presented are mean ± SEM.

that *Pyy* deficiency causes obesity that can be reversed by PYY replacement confirms the physiological role of this hormone in energy homeostasis.

Our studies revealed that fasting and postprandial levels of PYY were reduced in the obese subjects compared with the normal-weight subjects in agreement with previous studies in obese adults and children (Alvarez Bartolome et al., 2002; Batterham et al., 2003a; Korner et al., 2005; le Roux et al., 2006b; Roth et al., 2005; Stock et al., 2005). However, not all of these studies have demonstrated reduced levels in the fasted state (Korner et al., 2005; Stock et al., 2005). The reasons for this discrepancy have not been determined, but the duration of fasting before sampling affects plasma PYY concentrations (Chan et al., 2006). The mechanistic links between PYY and obesity are likely to be complex. The phenotype of our *Pyy* null mouse suggests that primary PYY deficiency causes obesity. In addition, our studies in mice with diet-induced obesity demonstrate that the development of obesity reduces PYY synthesis, fasting PYY levels, and PYY secretion in response to food intake. The finding that weight reduction in obese children results in increased PYY levels further suggests a reciprocal relationship between obesity and PYY (Roth et al., 2005).

Interestingly, in our current studies the high-fat meal resulted in a greater total PYY release than high-carbohydrate meal in the normal-weight subjects, but this effect was not seen in obese subjects. This suggests that in obese subjects PYY release in response to high-fat diets is attenuated. This observation may reflect the fact that we used equivalent caloric loads in normal and obese humans and therefore a relatively lower kcal/kg of body weight food intake in the obese subjects, which limits the comparisons that can be made between groups. However, recent studies using a 47% fat content meal have shown that for any given caloric load between 250 and 3000 kcal, peak plasma PYY concentrations were less in obese than normal-weight subjects (le Roux et al., 2006b). Indeed it was estimated that the caloric load required to evoke a given PYY response in the obese subjects was more than double that required in the normal-weight cohort (Young, 2006). Therefore, despite the limitations

of our studies, it is clear that the obese state is associated in both rodents and humans with reduced PYY levels in response to food intake. However, it remains uncertain how the obese state alters PYY levels, but potential mechanisms include the presence or absence of circulating factors that suppress or enhance PYY secretion or other alterations in L cell function (Young, 2006). In addition, such mechanisms may be specific to particular macronutrients. Taken together it is therefore likely that reduced PYY levels could play a dual role in the pathogenesis of obesity being both a primary driver while also being required for the persistence of obesity.

Previous studies have reported increased satiety and reduced hunger following ingestion of high-protein "meals." The majority of these have used liquid pre-loads, varying in caloric intake (250 kcal to 450 kcal) and protein content (43%–84%), and then assessed subsequent hunger and/or ad libitum caloric intake (Bowen et al., 2006; Latner and Schwartz, 1999; Poppitt et al., 1998). There is some limited evidence from human studies that these acute effects on satiety translate into longer-term benefits. For example, a 6 day intervention study examining the effects of a normal-protein (15%) low-fat American Heart Association diet compared with high-protein (31%) low-fat diet found that ad libitum caloric intake was reduced by 25% with the increased protein content diet (Dumesnil et al., 2001). In a randomized 6 month trial comparing ad libitum high-protein (25%) low-fat diet with a normal-protein (12%) low-fat diet for 6 months, Skov et al. demonstrated improved weight loss and fat mass loss with enhanced-protein diet, an effect attributed to reduced food intake (Skov et al., 1999). Furthermore, increasing the protein content of the diet from 15% to 18% during weight maintenance after weight loss has been shown to halve the amount of weight regained (Westerterp-Plantenga et al., 2004). Protein-rich diets caused the greatest satiation in our acute human and rodents feeding studies and the greatest reduction in weight gain in our chronic rodent diet studies, consistent with these published studies on the beneficial effects of dietary protein.

Our studies aimed at understanding the effects of macronutrients on satiation and body weight in humans demonstrated that high-protein meals caused the greatest reduction in hunger coupled with greatest increase in PYY compared to fasting levels in both groups. However, the peak and AUC PYY levels obtained in the obese group were less than those observed in the normal-weight group, and this correlated with a smaller reduction in hunger scores in the obese subjects. In our rodent studies, long-term exposure to high-protein diets was associated with elevated *Pyy* expression and PYY plasma levels and reduced weight gain. Critically, *Pyy* null mice in both acute feeding studies and in long-term dietary manipulation studies were resistant to the satiating and anti-obesity effects of high-protein diets. In contrast, altering the fat and carbohydrate content of the diets while keeping the protein content constant resulted in a similar change in feeding in C57Bl/6, $Pyy^{lox//lox}$, *Pyy* null, and their wt littermates, suggesting that deletion of *Pyy* specifically affects the satiating effects of protein.

There may be an evolutionary explanation for why protein is the most satiating of the macronutrients. It has been estimated that hunter-gatherer diets contained significantly higher protein content than most modern diets (Cordain et al., 2000). As far as can be judged, hunter-gatherer societies were largely protected from the diseases of affluence (Cordain et al., 2000). The ready availability of carbohydrate-rich grains and cereals has been a recent development in human nutrition with the onset of organized agriculture. Many of the physiological systems that regulate food intake were probably established and may function better under lower-carbohydrate and higher-protein dietary conditions. This might explain the effects of protein on satiation and PYY release and the marked phenotype caused by the deletion of the *Pyy* gene.

Bariatric surgery, a highly effective but invasive means of inducing weight loss, is associated with elevated PYY levels, which may underlie the weight-reducing effects of this procedure (Korner et al., 2005; le Roux et al., 2006a; Morinigo et al., 2006). However, the mechanisms by which such surgical interventions alter gut hormone profiles are unknown. Indeed, little is known about the mechanism by which nutrients normally stimulate gut hormone release. Increasing PYY release to enhance the satiating effects of meals, perhaps by harnessing endogenous physiological systems, is an attractive therapeutic option for obesity. Strategies could include enhancing meal protein content within healthy limits or the development of protein-mimetics. In summary, our current studies have established the physiological role of PYY as a regulator of energy homeostasis and demonstrated that it mediates the satiating and weight-reducing effects of dietary protein.

Experimental procedures

Human studies

Ten healthy normal-weight and ten obese male volunteers were recruited by advertisement. Exclusion criteria were the use of medications and presence of any medical or psychiatric illnesses or food allergies. Subject characteristics are presented in Table 1. All subjects gave written informed consent for the study, and approval was obtained from the University College London (UCL) research ethics committee. A randomized three-way crossover study was performed, with each subject being studied on three separate occasions at weekly intervals. One obese subject dropped out due to an unrelated illness. On the day prior to each test meal, subjects consumed a similar meal between 1900 hr and 2000 hr and then fasted and drank only water. After arrival at the investigation center at 1000 hr, a cannula was inserted into an ante-cubital arm vein and subjects relaxed for 30 min before the start of the study protocol. Subjects completed validated visual analog scale (VAS) questionnaires to assess hunger (Flint et al., 2000). Blood samples were taken and VAS assessed at baseline, immediately before the meal, and then at 30 min intervals until 3 hr post-meal. On each attendance at time zero, subjects were given the test meals, in random order, and instructed to finish this within a 25 min period. Isocaloric high-protein, high-fat, and high-carbohydrate test meals were studied (Table S1). For further details of the test meals and assays used see Supplemental Data.

Mouse studies

Mice were maintained on a 12 hr light/dark cycle (lights on at 0700 hr) under constant temperature with free access to water and standard mouse chow (Teklad Global 2018) and housed in specific-pathogen free facilities. All in vivo studies were performed in accordance to the Home Office Animal Procedures Act (1986) and London, UK, and UCL, UK ethical guidelines. C57Bl/6 mice were purchased at 6 weeks of age from Charles River UK Ltd (Margate, Kent, United Kingdom). Food and mouse weights were measured using a Sartorius BP610 balance. High-fat normal-protein (HFNP), high-fat high-protein (HFHP), low-fat normal-protein (LFNP), and low-fat high-protein (LFHP) diets were purchased from Research Diets (Research Diets Inc., New Brunswick New Jersey). All four diets were marked with different tasteless food coloring and their composition is presented in Table S3.

Targeting of the mouse *Pyy* gene

Pyy null mice were generated using standard CreloxP technology (see Supplemental Data).

Dietary studies in mice

For the diet studies, 6-week-old male C57Bl/6, *Pyy* null mice, *Pyy*$^{lox/lox}$ mice, and *Npy2r* null mice and wt littermate controls were singly housed. To allow acclimatization to the study protocol, mice were fasted and re-fed each of the different diets (HFNP, HFHP, LFNP, LFHP) on four different occasions, in a random order. Mice from each genotype were then divided into four weight-matched groups and underwent a randomized four-way crossover study. On each study day mice were fasted overnight for 14 hr and then re-fed with one of the 4 different diets and ad libitum food intake measured. For gut hormone release studies, mice were habituated to eat 4 kcal of each of the different diets in a 1 hr period after an overnight fast. Mice were randomized into HFNP, HFHP, LFNP, and LFHP groups and were culled at 1, 2, 4, 6, and 8 hr post-re-feed. For long-term diet studies, four groups of mice were allowed ad libitum access to one of the four diets for 16 weeks. Mice were weighed weekly and culled either after an overnight fast or in the fed state. For details of tissue harvesting and assays used refer to Supplemental Data.

PYY3-36 replacement studies

Pyy null mice and wt littermates were singly housed and acclimatized to saline intra-peritoneal (i.p.) injections and to a fasting and re-feed protocol. Each genotype was then randomized into groups, fasted overnight, and then injected i.p with saline or PYY3-36 (at doses indicated in the figure legends) at 0800 hr and food re-weighed at 1, 2, 4, 6, 8, and 24 hr post-injection. For chronic i.p. injection studies, *Pyy* null and control mice were acclimatized to being singly housed and to i.p. injections prior to being randomized to PYY3-36 treatment or saline. Mice received a single i.p. injection of PYY3-36 (5 μg/100 g body weight) or saline at the beginning of the dark-phase feeding period for 14 days and body weight was monitored daily. For the continuous infusion studies, Alzet osmotic pumps (model 2004, Charles River UK Ltd) were loaded with phosphate-buffered saline (PBS) or PYY3-36 dissolved in PBS so as to deliver 500 μg/kg/day. After 48 hr incubation in sterile PBS at 37°C to initiate delivery, pumps were implanted subcutaneously between the scapulae in mice anaesthetized with isoflurane. Wounds were closed with surgical staples. Food intake and body weight were subsequently monitored every 24 hr in the early light phase for 3 weeks. At the end of the study mice were terminally anaesthetized and inguinal fat pads were dissected and weighed.

Statistics

For all studies, results are expressed as mean ± SEM. Values for integrated area under the curve (AUC) hormone concentrations versus time were calculated above zero concentration by using the trapezoid rule. Within-subject comparisons were made using repeated-measures analysis of variance (ANOVA) with Newman Keul's post-hoc tests. Comparisons between normal weight and obese groups were made using nonpaired Student's t test. Significance was set at $p < 0.05$.

Supplemental data

Supplemental data include additional experimental procedures, three tables, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/3/223/DC1/.

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