PYY3-36 and pancreatic polypeptide reduce food intake in an additive manner via distinct hypothalamic dependent pathways in mice.

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Abstract

Objective
Peptide YY (PYY3-36) and pancreatic polypeptide (PP) potently inhibit food intake in rodents and humans, however, it is unclear whether they have any synergistic/additive interaction in decreasing food intake.
Design and Methods
Fasted WT, *Y2−/−*, *Y4−/−*, or *Y2Y4−/−* mice were i.p. administrated with saline, PYY3-36, and/or PP.
Results
Combined injection of PYY3-36 and PP reduces food intake in an additive manner was demonstrated in this study. This effect is mediated via Y2 and Y4 receptors, respectively. It was demonstrated that PYY3-36 and PP activate distinct neuronal pathways in the hypothalamus, as demonstrated by immunostaining for c-fos, which shows distinct patterns in response to either hormone. After PYY3-36 injection, neurons in the dorsal aspect of the arcuate nucleus (Arc), paraventricular nucleus, and dorso-medial nucleus of the hypothalamus (DMH) are activated with minimal responses seen in the ventro-medial nucleus of the hypothalamus (VMH) and lateral hypothalamic area (LHA) of WT mice. These effects are absent in *Y2−/−* mice. PP activates preferably the lateral aspect of the Arc, the DMH, VMH, and LHA in a Y4 receptor-dependent manner. Importantly, the expression pattern of c-fos immunoreactive neurons induced by combined treatment appears to be the sum of the effects of single treatments rather than a result of synergistic interaction.
Conclusions
These findings demonstrate that PYY3-36 and PP activate distinct pathways in the hypothalamus to reduce food intake in an additive manner.

Introduction

The gut–brain axis plays an important role in the regulation of food intake and targeting molecules within this axis have emerged as a promising strategy for treating obesity ([**1-3**]). Peptide YY (PYY) and pancreatic polypeptide (PP), both members of the neuropeptide Y (NPY) family, are released in response to food intake and are among the most effective satiety signals. PYY and PP signal through a set of G-protein-coupled Y receptors, Y1, Y2, Y4, Y5, and y6, are highly expressed in areas of the hypothalamus that are critical for the control of food intake and energy homeostasis ([**4, 5**]). PYY is primarily synthesized by endocrine L cells of the gut and is released into the circulation in two forms: PYY1-36 and the truncated form PYY3-36, which is the product of cleavage of PYY1-36 by dipeptidyl peptidase-IV (DPPIV) ([**6, 7**]). While PYY1-36 binds to all known Y receptors, albeit with lesser affinity to Y4 receptors, PYY3-36 predominantly binds to Y2 receptor and with reduced affinity also to Y5 receptors ([**4**]). It appears that obese individuals have a deficiency in circulating PYY ([**8, 9**]), indicating a potential role for PYY in the development of obesity. In keeping with this hypothesis, healthy people with a genetic predisposition for obesity—on account of a family history of type 2 diabetes—have lower

circulating PYY levels ([**10**]). In fact, peripherally administrated PYY3-36 reduces food intake in rodents and humans ([**3, 11, 12**]). Furthermore, over-expression of PYY leads to a significant reduction in fasting-induced food intake without altering body weight or adiposity ([**13**]). Centrally, PYY3-36 is thought to act on Y2 receptors expressed on NPY neurons in the hypothalamic arcuate nucleus (Arc) to reduce NPY-ergic activity ([**3, 14**]). Additionally, PYY3-36 was shown to increase hypothalamic expression of pro-opiomelanocortin (POMC) mRNA after *in vivo* administration and to activate Arc POMC neurons after incubation with hypothalamic slices *in vitro* ([**3**]), however, the mechanisms and pathways within the hypothalamus by which PYY3-36 inhibits food intake are unknown.

Similar to PYY, PP is released postprandially. PP is expressed in F-type cells of the pancreas and can act on all Y receptors, but it has the highest affinity for Y4 receptors ([**4**]). Short-term peripheral PP injection leads to a marked reduction in food intake in humans ([**15, 16**]). Peripherally administered PP also attenuates body weight gain, insulin resistance, and hyperlipidemia in genetically obese *ob/ob* mice ([**17**]). When over-expressed in mice, PP reduces food intake, body weight, and adiposity and increases energy expenditure ([**18**]). PP dose-dependently reduces food intake in freely fed and fasted mice, and this effect is mediated through Y4 receptors since the effect is greatly abolished in *Y4−/−* mice ([**19**]). PP is thought to mediate its effects primarily via actions on the brainstem ([**19**]). However, the hypothalamus also appears to be an important site of action of PP ([**19**]).

While PYY- and PP-mediated satiety pathways are both being investigated as anti-obesity targets, there is evidence to suggest that PYY and PP may be more effective at reducing food intake when administered together. We have shown that the preferred receptors for PYY3-36 and PP, Y2 and Y4 receptors, respectively, have a synergistic action on several important physiological parameters, notably food intake, body fat content, and bone density ([**20**]). Indeed, whereas *Y2−/−* or *Y4−/−* receptor knockout had some effects on these parameters, *Y2Y4−/−* receptor double knockout produced synergistically much greater effects on food intake and food efficiency. Since PYY3-36 is known to induce its satiety effects via Y2 receptors ([**3**]), and PP induces its satiety effects via Y4 receptors ([**3, 21**]), it is plausible that PYY3-36 and PP may produce a synergistic reduction of food intake. Thus, in this study, we investigated the anorexigenic effect of administration of PYY3-36 or PP, with special focus on the effect of the combined injection of PYY3-36 and PP, with subsequent examination of neuronal responses measured by c-fos immunoreactivity particularly in key hypothalamic regions.

Methods

Animals

All animal experiments were conducted in accordance with relevant guidelines and regulations. Mice were housed under conditions of controlled temperature (22°C) with a 12:12 h light-dark cycle (lights on at 07:00 h), and fed a standard chow diet (6% fat, 21% protein, 71% carbohydrate, Gordon's Specialty Stock Feeds, Australia) with *ad libitum* access to water. Five to six male age- and body weight-matched mice (WT, *Y2−/−*, *Y4−/−*, and *Y2Y4−/−*) all on a mixed C57/Bl6–129SvJ background were used, and generation of these mice has been previously described ([**20**]). All mice were fasted for 24 h prior to administration of PP and/or PYY3-36 in order to maximize effects of PP or PYY3-36 on food intake, as fasting has been shown to enhance the ability of other gut-derived satiety hormones to induce significant hypophagic effects ([**11**]).

Measurement of food intake in response to PYY3-36 and/or PP administration

WT, *Y2−/−*, *Y4−/−*, and *Y2Y4−/−* animals were single housed and acclimatized to daily i.p. injection of saline for one week prior to peptide administration. After acclimatization, mice were fasted for 24 h and received a 200 µl i.p. injection of either PP (Bachem AG, Bubendorf, Switzerland) at 200 µg/kg body weight (BW), PYY3-36 (GenWay Biotech, Inc., San Diego, CA) at 300 µg/kg BW, PYY3-36 and PP in combination—using the same dose as in the single injection—or 0.9% saline (10 ml/kg) simultaneously. Animals were given free access to chow diet directly following injection and food intake was measured over the 24 h following i.p. injection and re-feeding. Actual food intake was calculated as the weight of pellets taken from the food hopper minus the weight of food spilled in the cage. The weight of food spillage was determined after removing all feces and air-drying for one day to eliminate weight changes due to urine and water bottle drips.

Immunohistochemical determination of changes in hypothalamic c-fos or p-ERK1/2 expression in response to PP and/or PYY3-36 injection
After 24 h fasting, WT, *Y2−/−*, *Y4−/−*, or *Y2Y4−/−* mice were i.p. injected with either PP (200 μg/kg BW), PYY3-36 (300 μg/kg BW), the combination of PP and PYY3-36 at these same doses, or saline vehicle (200 μl) between 10:00 and 12:00 h. Immediately thereafter, mice were given access to food. At 30 min after i.p. injection and provision of food, the mice were anaesthetized and the brains were perfused with saline and then 4% paraformaldehyde (PFA). Brains were postfixed in 4% PFA, then placed in 30% sucrose overnight and cut at 30 μm, and immunohistochemistry was performed as described in Supporting Information Experimental Procedures. After cover slipping, 12 sections from each mouse were visualized and counted for c-fos or p-ERK1/2 immunoreactivity within the brain nuclei of interest, using a Zeiss Axioplan light microscope. Double-blind counting of the c-fos-positive and p-ERK1/2 positive neurons was conducted.

Determination of co-localization of c-fos and NPY neurons in NPYGFP mice
Transgenic mice expressing the green fluorescence gene (GFP) under the endogenous mouse NPY promoter (B6.FVB-Tg(Npy-hrGFP)1Lowl/J) were obtained from Jackson Laboratory ([**22**]). After 24 h fasting, NPYGFP mice were i.p. injected with PYY3-36 (300 μg/kg BW) between 10:00 and 12:00 h following refeeding. Thirty minutes after injection, these mice were perfused with 4% PFA as described earlier. Brain sections were incubated with the primary antibody, rabbit-anti-mouse c-fos (1:2,000 dilution, Santa Cruz) following the protocol described in Supporting Information Experimental Procedures. Sections were visualized for c-fos immunoreactivity in NPYGFP transgenic mice using a Zeiss Axiophot microscope (Carl Zeiss).

Statistical analyses
Two-way ANOVA were used to determine the significance of interactions and treatments (GraphPad Prism 5, Version 5.0a, GraphPad Software, Inc.). When there was a significant overall effect or interaction effect, Bonferroni *post-hoc* tests were performed to identify differences among means. *F* values and degrees of freedom between groups and within groups were reported. $P < 0.05$ was considered significant.

Results

Additive effect of peripherally administrated PYY3-36 and PP on food intake
I.p. injection of PYY3-36 or PP alone into WT mice significantly reduced fasting-induced food intake, as compared to saline-injected controls (Figure 1A). Interestingly, there was no difference in food intake between PYY3-36-injected and PP-injected mice, indicating an equal efficacy of PYY3-36 and PP in inhibiting food intake in fasted WT mice. Importantly, simultaneous injection with both PYY3-36 and PP led to a further reduction in food intake, when compared to the individual administration alone, to a level suggestive of an additive effect of PYY3-36 and PP on inhibiting food intake.

Whereas i.p. injection of PYY3-36 alone into WT mice induced a marked reduction in food intake, no such effect was observed in *Y2−/−* mice injected with PYY3-36 alone (Figure 1B), consistent with Y2 receptor signaling being required for PYY3-36 induced inhibition in food intake. On the other hand, PP injection alone into *Y2−/−* mice resulted in a similar reduction in food intake to that seen in WT mice, while the combination of PYY3-36 and PP administration into *Y2−/−* mice led to a food intake comparable to that of PP injection alone (Figure 1B), suggesting little involvement of Y2 receptors in the PP-induced anorexigenic action. On the other hand, PYY3-36 administration on its own was able to decrease food intake in *Y4−/−* mice to a level comparable to its effect in WT mice (Figure 1C compared to Figure 1A), indicating a lack of involvement of Y4 receptors in this process, whereas in the absence of Y4 receptors PP lost its capacity to reduce food intake (Figure 1C). *Y4−/−* mice injected with both PYY3-36 and PP showed a decreased food intake similar to that seen in PYY3-36 alone. Collectively, these data not only confirm that PYY3-36 and PP inhibit food intake via Y2 and Y4 receptor signaling, respectively, but also indicate that the anorexigenic actions of PYY3-36 and PP are likely mediated via distinct and additive mechanisms.

PYY3-36 and PP activate differential sets of neurons in the hypothalamic Arc
In order to understand the underlying mechanisms via which combined PYY3-36 and PP

treatment elicits anorexigenic actions, we tracked and compared changes in expression of c-fos, an early neuronal activation marker, in key hypothalamic nuclei that are critical in the regulation of energy homeostasis, in response to i.p. PYY3-36 and/or PP injection. Based on previous experiments ([**24**]), c-fos expression at 30 min post-injection was chosen to represent initial activation.

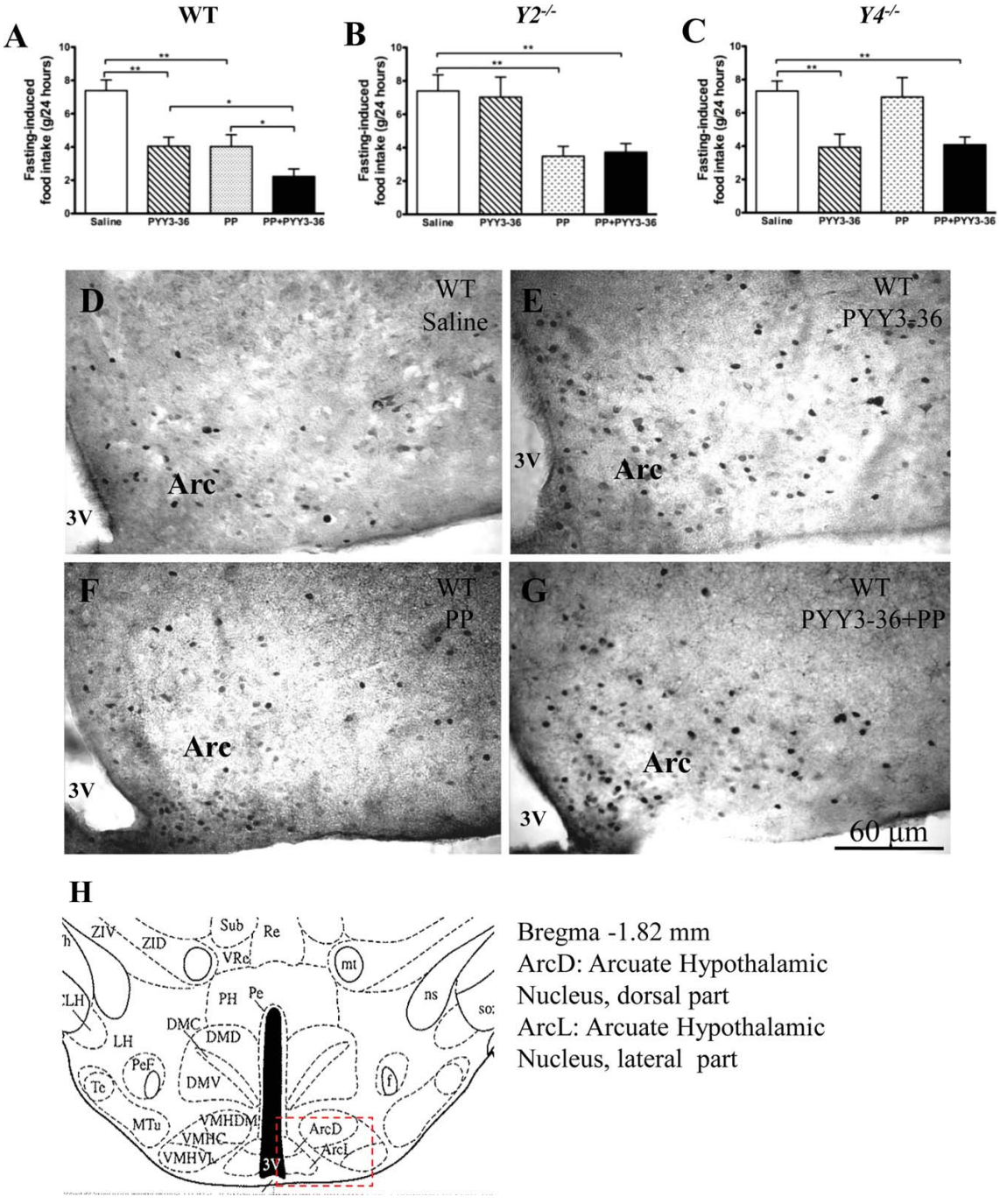

FIGURE 1 Effect of combined injection of PYY3-36 and PP on food intake and distinct pattern of c-fos immunoreactivity in the Arc of WT mice. Fasting-induced food intake was measured at 24 h after injection in WT (**A**), $Y2^{-/-}$ (B) or $Y4^{-/-}$ (C) mice. Data are means ± SEM of 5–6 mice per group. Interaction F (6, 54) = 6.173; Genotype F (2, 54) = 4.815; Treatment F (2, 54) = 25.70. * $P < 0.05$ and ** $P < 0.01$ versus saline-injected genotype-matched controls or the comparisons indicated by horizontal bars above. Photomicrograph of coronal sections from WT mice showing c-fos immunoreactivity at 30 min after i.p. injection of either saline (D), PYY3-36 (E), PP (F) or in combination (G). Schematic diagram (H) of dorsal aspect of the Arc of the hypothalamus (ArcD) and lateral aspect of the Arc of the hypothalamus (ArcL) (boxed area, adapted from permission from Ref. 23). Scale bar = 60 μm. Images are representative of five mice per group. Arc: arcuate nucleus, 3V: the third cerebral ventricle.

As shown in Table 1 and in Figure 1D and E, when WT mice were injected with PYY3-36 alone, significant increases in the number of c-fos immunoreactive neurons were detected in several hypothalamic regions, notably in the dorsal aspect of the hypothalamic arcuate nucleus (ArcD), which is known to also express NPY ([**25**]), with a few c-fos-positive neurons also found in the lateral aspect of the hypothalamic arcuate nucleus (ArcL). In contrast, PP injection alone induced a marked increase in c-fos immunoreactive neurons in the ArcL (Table 1, Figure 1D and F), which is an area that preferentially expresses POMC, with little induction of c-fos expression in the ArcD. Interestingly, dual administration of PYY3-36 and PP to WT mice led to a significant increase in the number of c-fos positive neurons in both the ArcD and ArcL (Table 1, Figure 1D and G), with the number of c-fos positive neurons being approximately equal to the sum of c-fos positive neurons induced by either peptide alone in the ArcD and ArcL (Table 1). However, when the combined treatment was applied to *Y2−/−* mice, a significant increase in the number of c-fos immunoreactive neurons was only detected in the ArcL, but not in the ArcD (Table 2, Figure 2A and B). This is consistent with the expression pattern seen with PP administration alone indicating the effect of PYY3-36 is abolished in the absence of Y2 receptors, with PP retaining its full capacity with intact Y4 receptor signaling. On the contrary, dual administration of PYY3-36 and PP to *Y4−/−* mice resulted in a marked increase in c-fos expression in the ArcD, but not in the ArcL (Table 2, Figure 2C and D). Importantly, *Y2Y4−/−* animals injected with PYY3-36 and PP displayed no significant neuronal activation in the Arc, with the c-fos expression level being similar to that seen in the saline-injected groups (Table 2, Figure 2E and F). Together, our data indicate that PYY3-36 and PP control food intake most likely by signaling through Y2 and Y4 receptor expressing neurons in the ArcD and the ArcL. However, further study using conditional hypothalamic-specific deletion mouse model will be necessary to confirm this assumption. Based on the expression pattern and the number of c-fos positive neurons after double treatment, one can assume that there is an additive action of PYY3-36 and PP in inducing neuron activity in the Arc via different pathways, rather than via synergistic responses on the same pathways.

TABLE 1 Number of c-fos immunoreactive neurons in the hypothalamus of wild type mice 30 minutes after *i.p.* injection of saline, PYY3-36, PP or both PYY3-36 and PP

Regions	Saline	PYY3-36	PP	PYY3-36+PP
Arc D	11±2	20±6[a]	12±4	24±5[a]
Arc L	8±3	10±3	19±3[a]	31±4[a]
PVN	23±5	51±9[a]	25±9	69±7[a]
DMH	23±5	32±6[a]	38±9[a]	62±11[ab]
VMHDM	11±3	12±7	19±4[a]	21±5[a]
VMHVL	12±4	14±4	24±8[a]	26±5[a]
LHA	19±3	28±9[a]	39±5[a]	42±7[a]

Data are means ± SEM of 5 mice per group. Interaction F (18, 122) = 8.835; Treatment F (3,112)=112.3; Brain regions F (6,112) = 73.6.
[a]$P<0.05$ versus saline- injected wild type mice.
[b]$P<0.05$ versus PYY3-36 or PP injection alone.
ArcD; dorsal aspect of the arcuate nucleus of the hypothalamus; ArcL: lateral aspect of the arcuate nucleus of the hypothalamus; PVN: paraventricular nucleus; DMH: dorso- medial nucleus of the hypothalamus; VMHDM: dorsomedial aspect of the ventromedial nucleus of the hypothalamus; VMHVL: ventrolateral aspect of the ventromedial nucleus of the hypothalamus; LHA: lateral hypothalamic area.

In addition to assessment of induction of c-fos immunoreactivity, we also evaluated changes in immunoreactivity for phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2), a marker of activation of intracellular signaling cascades affected by G-protein coupled receptors, as further functional evidence that PYY3-36 and/or PP activate intracellular processes within the Arc. As shown in Table 3 in the ArcD of WT mice, compared to minor activation in saline injected controls, p-ERK1/2 was clearly induced at 30 min after PYY3-36 treatment alone, with minimal activation by PP injection alone, the pattern of activation being

consistent with that indicated by induction of c-fos expression. Interestingly, concomitant treatment with both peptides induced an increase in p-ERK1/2—positive neurons in the ArcD to a level that was comparable with that induced by injection of PYY3-36 alone, with only minor p-ERK1/2 activation seen in the ArcL (Table 3), indicating that PYY3-36 in the combination treatment is mainly responsible for the neuronal activation seen in the ArcD. On the other hand, in the ArcL of WT mice, PP induced a significant increase in p-ERK1/2 immunoreactive neurons (Table 3), whereas PYY3-36 was only able to elicit a low level of activation in p-ERK-positive neurons. Similar to the c-fos expression in the ArcL of WT, dual administration of both PYY3-36 and PP induced a neuronal expression pattern in the ArcL that was comparable to that induced by PP injection alone, again with only a minor activation contributed by PYY3-36 (Table 3), suggesting that PP, but very little PYY3-36, contributes to the neuronal activation in the ArcL in the combined treatment.

TABLE 2 Number of c-fos immunoreactive neurons in the hypothalamus of *Y2*$^{-/-}$, *Y4*$^{-/-}$ or *Y2Y4*$^{-/-}$ mice 30 minutes after *Lp*. injection of saline or combined PYY3-36 and PP

	Y2$^{-/-}$		*Y4*$^{-/-}$		*Y2Y4*$^{-/-}$				
Regions	Saline	PYY3-36+PP	Saline	PYY3-36+PP	Saline	PYY3-36+PP	Interaction F value	Genotype F value	Treatment F value
Arc D	8 ± 1	10 ± 3	7 ± 1	19 ± 4[a]	10 ± 2	12 ± 3	F (2, 24) = 5.712	F (2, 24) = 3.054	F (1,24) = 12.15
Arc L	8 ± 2	26 ± 3[a]	9 ± 1	8 ± 2	6 ± 2	11 ± 2[a]	F (2, 24) = 17.11	F (2, 24) = 18.49	F (1,24) = 30.91
PVN	18 ± 3	25 ± 4	13 ± 3	37 ± 8[a]	19 ± 3	24 ± 5	F (2, 24) = 7.303	F (2, 24) = 1.074	F (1,24) = 28.73
DMH	19 ± 3	44 ± 5[a]	11 ± 2	29 ± 7[a]	22 ± 3	27 ± 4	F (2, 24) = 4.795	F (2, 24) = 6.529	F (1,24) = 37.95
VMHDM	11 ± 2	28 ± 3[a]	17 ± 3	23 ± 4	9 ± 2	15 ± 4	F (2, 24) = 3.067	F (2, 24) = 6.039	F (1,24) = 22.52
VMHVL	9 ± 1	15 ± 2[a]	10 ± 3	21 ± 3[a]	11 ± 3	17± 3	F (2, 24) = 1.719	F (2, 24) = 2.132	F (1,24) = 33.41
LHA	21 ± 2	34 ± 5[a]	19 ± 3	24 ± 2	22 ± 3	28 ± 4	F (2, 24) = 1.084	F (2, 24) = 2.497	F (1,24) = 11.49

Data are means ± SEM of 5 mice per groups.
[a]***P***<0.05 versus saline-injected genotype-matched mice.
[b]***P***<0.05 versus PYY3-36 or PP injection alone.
Arc; arcuate nucleus of the hypothalamus; PVN: paraventricular nucleus; DMH: dorso-medial nucleus of the hypothalamus; VMHDM: dorsomedial aspect of the ventro-medial nucleus of the hypothalamus; VMHVL: ventrolateral aspect of the ventro-medial nucleus of the hypothalamus; LHA: lateral hypothalamic area.

Since the majority of c-fos and p-ERK induction in the Arc in response to PYY3-36 appears to be located in the ArcD area, which is known to have a high density of NPY-expressing neurons, we next aimed to determine the degree of activation of these NPY-ergic neurons by injecting PYY3-36 into a transgenic mouse model expressing GFP under control of the endogenous NPY promoter (NPYGFP). In the Arc, 63% of neurons exhibiting c-fos immunostaining at 30 min after injection also showed immunostaining for NPY (Figure 2G). Interestingly, some c-fos positive neurons—indicated by red fluorescence in Figure 3—were not co-localized with NPY-expressing neurons—indicated by green fluorescence—in the ArcD, the brain region that we have demonstrated to be activated by PYY3-36. These findings suggest that PYY3-36 controls food intake by acting on both NPY-containing and non-NPY neurons in the Arc.

PYY3-36 and PP activate neurons in other key hypothalamic regions

In addition to the effects on the Arc, we also examined the effects of PYY3-36 and/or PP injection on the expression of c-fos in other key hypothalamic regions that are critical in the regulation of food intake and energy homeostasis, including the paraventricular nucleus (PVN), dorso-medial nucleus of the hypothalamus (DMH), ventro-medial nucleus of the hypothalamus (VMH), including both the dorsomedial (VMHDM) and the ventrolateral divisions (VMHVL), and the lateral hypothalamic area (LHA). I.p. injection of PYY3-36 into WT mice induced a marked increase in c-fos immunoreactivity in the PVN (Table 1, Figure 3A and B). By contrast, PP injection did not induce significant c-fos expression in the PVN (Table 1, Figure 3A and C), whereas injection of both PYY3-36 and PP resulted in a similar c-fos expression pattern to that seen after PYY3-36 injection alone (Table 1, Figure 3A, B, and D). Lack of Y2 receptors, as in *Y2−/−* or *Y2Y4−/−* mice, but not lack of Y4 receptors, as in *Y4−/−* mice, blocked the effect of combined treatment to activate c-fos in the PVN (Table 2, Figure 4A–F), demonstrating the critical involvement of Y2 receptors in PYY3-36-mediated activation of the PVN.

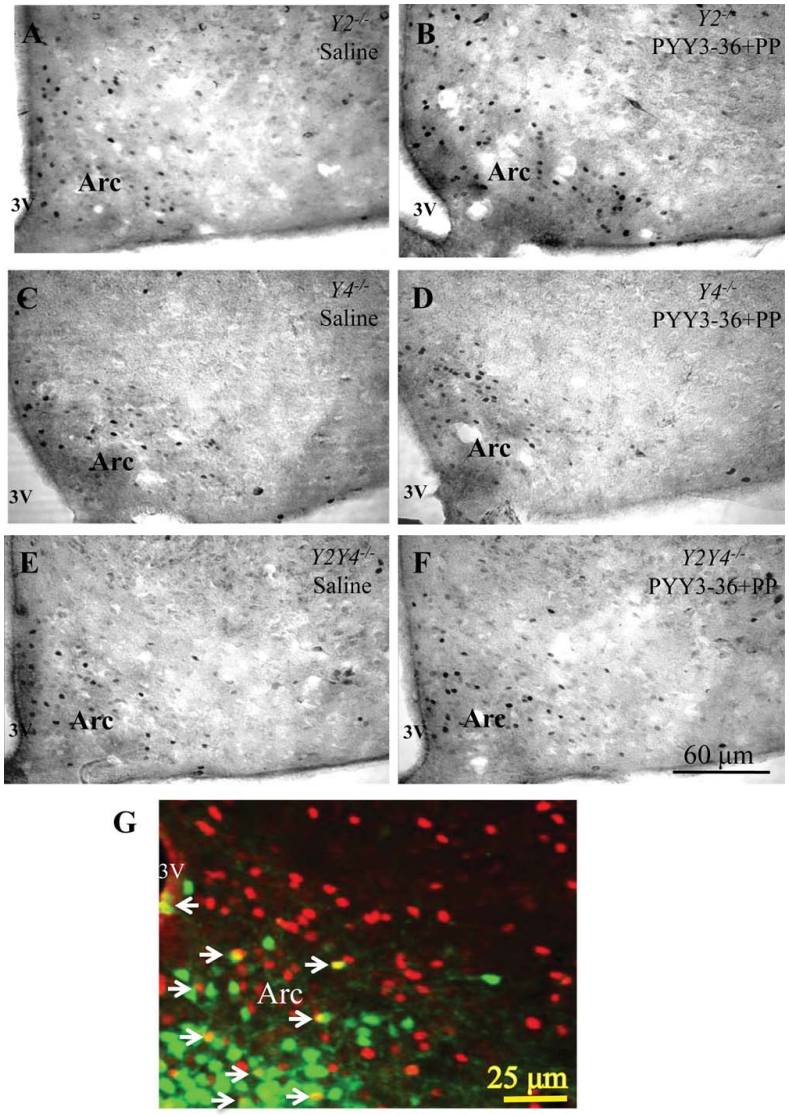

FIGURE 2 Distinct expression pattern of c-fos immunoreactivity in the Arc of $Y2^{-/-}$, $Y4^{-/-}$ or $Y2Y4^{-/-}$ mice and co-localization of c-fos positive neurons with NPY neurons in the Arc of WT mice. Photomicrograph of brains from $Y2^{-/-}$ (**A, B**), $Y4^{-/-}$ (C, D), or $Y2Y4^{-/-}$ (E, F) showing c-fos immunoreactivity at 30 min after i.p. injection of combined PYY3-36 and PP. Scale bar = 60 μm. (G) Co-localization of c-fos positive neurons with NPY neurons. Red staining indicates c-fos positive neurons only, with green-staining indicating NPY neurons expressing GFP, and yellow staining (with white arrow) indicates neurons expressing both c-fos and NPY. Scale bar = 25 μm. Images are representative of five mice per group. Arc: acuate nucleus, 3V: the third cerebral ventricle.

TABLE 3 p-ERK1/2 expression in the Arc of wild type mice 30 minutes after *i.p.* injection of saline, PYY3-36, PP, or both PYY3-36 and PP

	Saline	PYY3-36	PP	PYY3-36+PP
ArcD	4±1	11±2[a]	6±2	15±3[a]
ArcL	3±1	5±2	10±2[a]	16±3[ab]

Data are means ± SEM of 5 mice per groups. Interaction F (3,32)=10.96; Brain regions F (1,32) = 0.4089; Treatment F (3,32) = 25.6.
[a]***P***<0.05 versus saline-injected wild type mice.
[b]***P***<0.05 versus PYY3-36 or PP injection alone.
ArcD; dorsal aspect of the arcuate nucleus of the hypothalamus; ArcL: lateral aspect of the arcuate nucleus of the hypothalamus.

In the DMH of WT mice, the number of c-fos immunoreactive neurons after combined PYY3-36 and PP administration was close to the sum of that induced by either PYY3-36 or PP injection alone (Table 1, Figure 5A–D). In either *Y2−/−* or *Y4−/−* mice, compared to basal c-fos expressing neurons in saline-injected mice, double injection still led to a significant increase in c-fos positive neurons (Table 2). However, in the absence of Y2 and Y4 receptors, as in *Y2Y4−/−* mice, the number of c-fos positive neurons in combined treatment was not significantly different from that of saline-injected *Y2Y4−/−* control mice (Table 2), demonstrating that both Y2 and Y4 receptors are required for the additive effects of PYY3-36 and PP to activate neurons in the DMH.

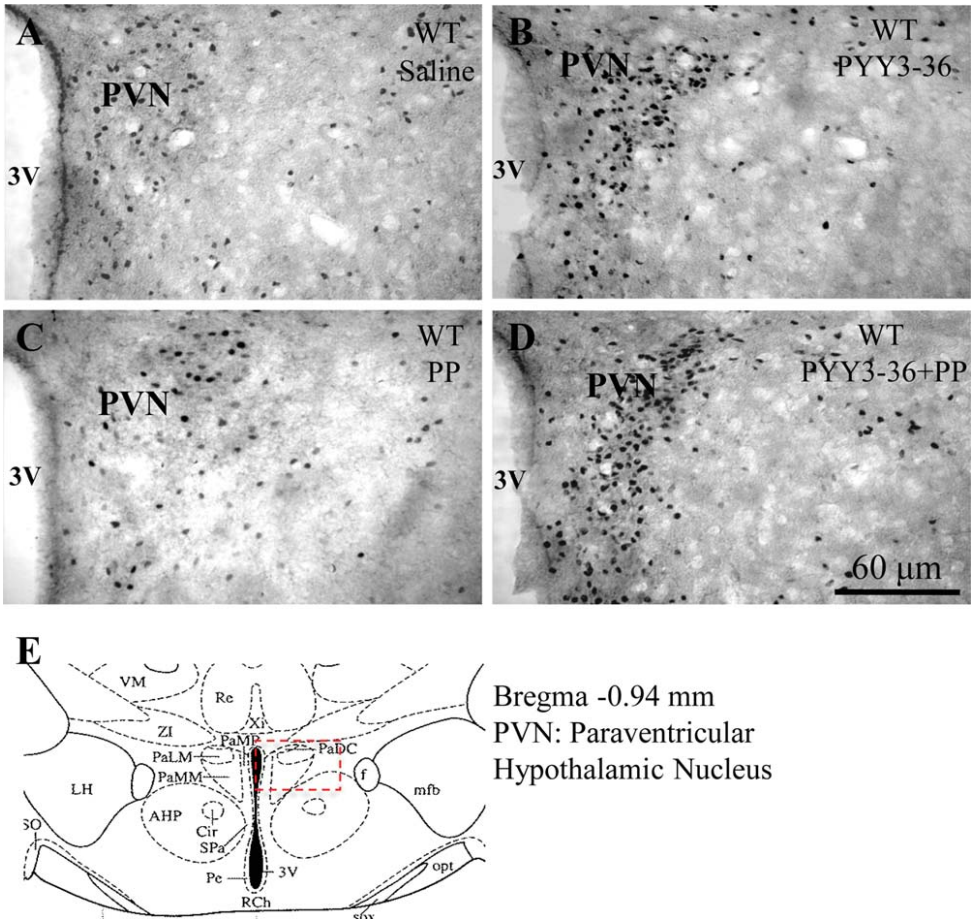

FIGURE 3 I.p. injection of PYY3-36 and/or PP induced a distinct pattern of c-fos immunoreactivity in the PVN of WT mice. Photomicrograph of coronal sections from WT mice showing c-fos immunoreactivity at 30 min after i.p. injection of either saline (**A**), PYY3-36 (**B**), PP (**C**), or in combination (**D**). Schematic diagram (**E**) of the PVN (boxed area, adapted from permission from Ref. 23). Scale bar = 60 μm. Images are representative of five mice per group. PVN: paraventricular nucleus of the hypothalamus, 3V: the third cerebral ventricle.

In the VMH of WT mice, PYY3-36 injection alone had no effect on c-fos immunoreactivity, whereas PP induced a significant increase in the number of c-fos immunoreactive neurons in both the VMHDM and VMHVL (Table 1). The combined administration of PYY3-36 and PP led to a similar increase in the number of c-fos immunoreactive neurons to that induced by PP injection alone (Table 1). This effect in the VMHDM was mediated by Y4 but not Y2 receptors, as no increases in c-fos immunoreactivity in response to PYY3-36 and PP injection was observed in the VMHDM of *Y4−/−* or *Y2Y4−/−* mice, while the response remained in *Y2−/−* mice (Table 2). Interestingly, in the VMHVL, it appears that other receptors besides Y2 and Y4 may be involved in the PYY3-36 and PP-mediated induction of c-fos immunoreactivity, because the response remains intact in Y2 or Y4 receptor single knockout mice.

Furthermore, in the LHA of WT mice, both PYY3-36 and PP injection, either alone or in combination, significantly increased the levels of c-fos immunoreactivity (Table 1). This effect is likely to be mediated by Y4 but not Y2 receptors, as it was completely abolished in *Y4−/−* and *Y2Y4−/−* but not in *Y2−/−* mice (Table 2).

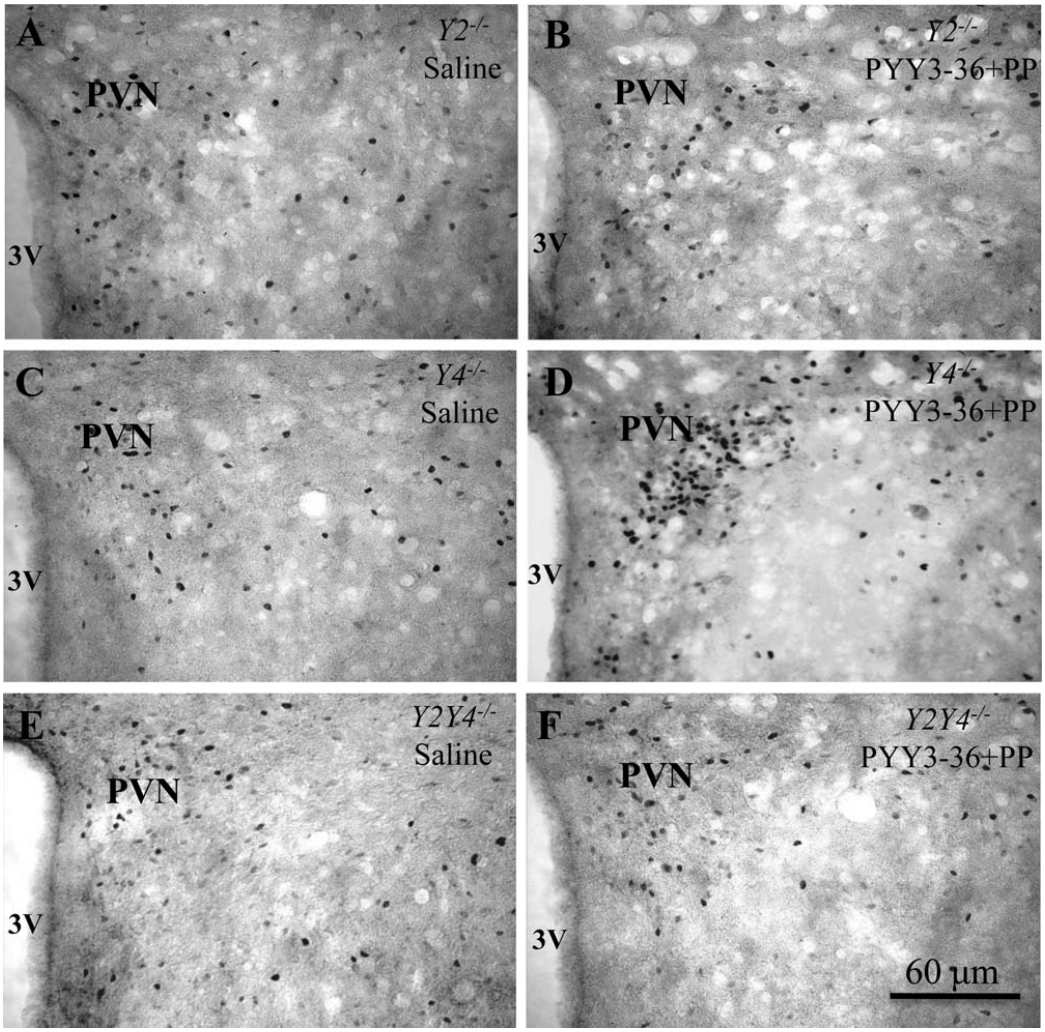

FIGURE 4 Distinct expression pattern of c-fos in the PVN of $Y2^{-/-}$, $Y4^{-/-}$, or $Y2Y4^{-/-}$ mice. Photomicrograph of brains from $Y2^{-/-}$ (**A, B**), $Y4^{-/-}$ (**C, D**), or $Y2Y4^{-/-}$ (**E, F**) showing c-fos immunoreactivity at 30 min after i.p. injection of both PYY3-36 and PP. Scale bar = 60 μm. Images are representative of five mice per group. PVN: paraventricular nucleus of the hypothalamus, 3V: the third cerebral ventricle.

Discussion

In this study, we demonstrate that the satiety peptides PYY3-36 and PP have additive actions to decrease food intake, and that these additive effects are likely mediated by the distinct neuronal pathways activated by signaling through Y2 and Y4 receptors. Indeed, we found that PYY3-36 and PP activate hypothalamic nuclei that are critical in the regulation of appetite and satiety regulation, as demonstrated by c-fos or p-ERK activation. Moreover, some of these hypothalamic nuclei or their subdivisions were activated either by PYY3-36 or PP, providing an explanation for the additive anorexigenic effects of these peptides.

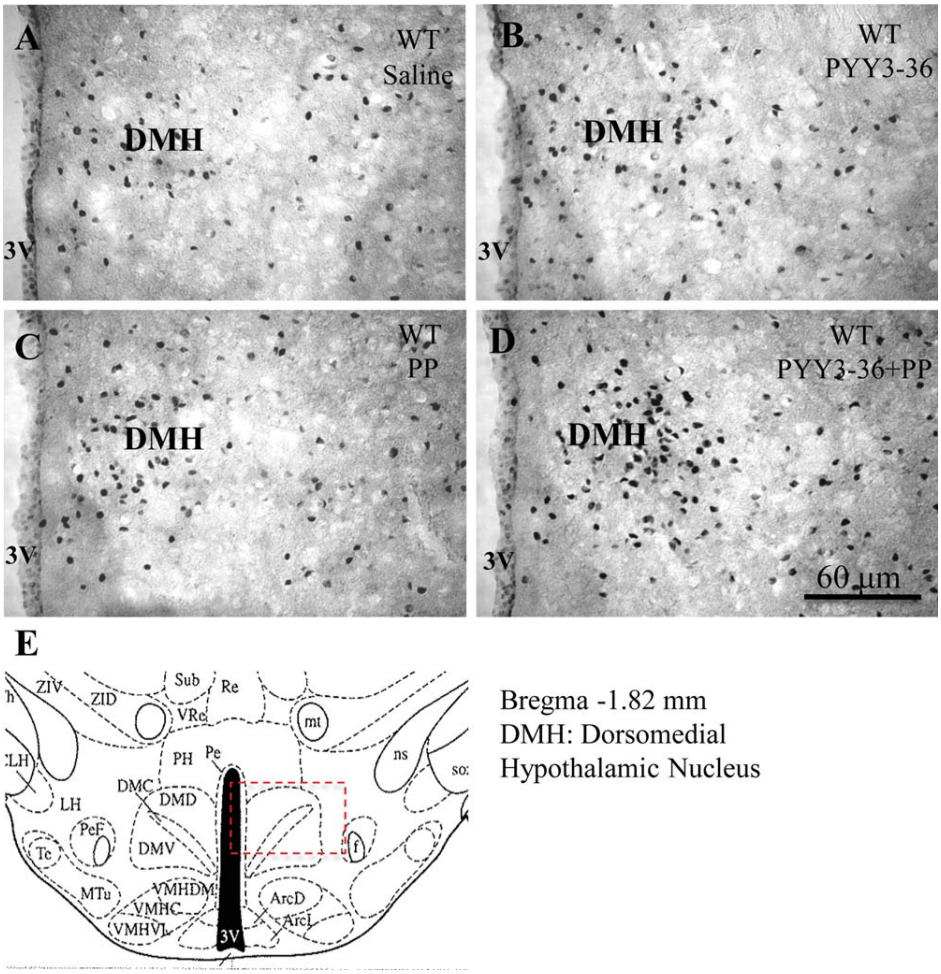

FIGURE 5 I.p. injection of PYY3-36 and/or PP induced a distinct pattern of c-fos immunoreactivity in the DMH of WT mice. Photomicrograph of coronal sessions from WT mice showing c-fos immunoreactivity at 30 min after i.p. injection of either saline (**A**), PYY3-36 (**B**), PP (**C**), or in combination (**D**). Schematic diagram (**E**) of the DMH (boxed area, adapted from permission from Ref. 23). Scale bar = 60 μm. Images are representative of five mice per group. DMH: dorsomedial nucleus of the hypothalamus. 3V: the third cerebral ventricle.

Our observation that the Y2 receptor mediates the anorexigenic and central actions of PYY3-36 but not PP, and that the Y4 receptor mediates these actions of PP but not PYY3-36 is consistent with their high affinities for the Y2 and Y4 receptor, respectively ([**4, 21**]). Comprehensive analysis of mRNA expression of these Y-receptors in the rat has shown that they are abundantly expressed in neurons of the hypothalamus, with particularly high density in the Arc ([**26**]). However, in the mouse brain, as shown here, it appears that there is a clear distinction in expression pattern between the Y2 and Y4 receptors within the Arc, with the former being more highly concentrated in the ArcD and the latter being more abundantly expressed in the ArcL. This species difference in Y-receptor location between rat and mouse is not too surprising considering that the rat genome is missing the Y6 receptor, a fully functional receptor in the mouse ([**27**]).

The slight difference in expression of Y2 and Y4 receptors within the Arc also coincides with the distribution of different neuronal populations in this area, with—for example—neurons expressing NPY and agouti related peptide (AgRP) being more prevalent in the ArcD, whereas neurons expressing POMC and cocaine amphetamine-related transcript (CART) are more common in the ArcL ([**25**]). Furthermore, within the Arc it has been shown that a large proportion of Y2 receptors are co-localized with NPY neurons, and that Y2 receptors within the Arc likely act as auto-receptors on NPY-ergic neurons to inhibit the synthesis and release of NPY

([**3, 28, 29**]). Consistent with this overlap of Y2 expression with NPY/AgRP neurons in the ArcD and their role as auto-receptors, we have recently shown that lack of Y2 receptors causes an increase in NPY mRNA and protein expression ([**29**]). Importantly, our current findings also demonstrate that some but not all of the ArcD neurons activated by i.p. PYY3-36 are co-localized with NPY, suggesting that PYY3-36-induced control of food intake is, at least in part, mediated by direct action on Y2 auto-receptors on NPY-ergic neurons in the ArcD, and also raising the possibility that PYY3-36 may bind to Y2 receptors located on non-NPY-ergic neurons in the ArcD to subsequently reduce appetite via other pathways.

Importantly, this work also shows that Y2 receptors and Y4 receptors are not only critical for PYY3-36- or PP-induced c-fos activation in the Arc but also in other hypothalamic nuclei, including the PVN, DMH, VMH, and LHA. Since the Arc innervates virtually the entire hypothalamus ([**30**]), and since these second-order neurons are not readily accessible to blood-borne PYY3-36 or PP, it is likely that PYY3-36 and PP predominantly act on Y2 or Y4 receptors in the Arc and this in turn leads to activation of distinct subsets of downstream neurons located in these key hypothalamic nuclei that regulate appetite.

Of note there was a previous study by Neary et al. also investigating the effects of double treatment with PP and PYY3-36 on food intake, which did not find a significant additive effect on food intake ([**31**]); however the mechanism behind that was not explored. Importantly, there are a few differences in the design and analysis between the previous and our study.
Firstly, the mice used had a different genetic background (C57BL/6 versus C57/B16–129SvJ in our study). Secondly, the dose of the peptides was different with a considerable lower dose used by Neary et al. Thirdly, and probably most critical, it is not clear from the methods whether spillage has been taken into account when calculating actual food consumed. Therefore, these differences between the two studies make direct comparisons difficult.

In summary, we demonstrate that combined PYY3-36 and PP injection decreases food intake in an additive manner. Our finding also highlights the fact that multiple pathways have arisen during evolution to control this important physiological process of controlling appetite to guarantee effective supply of nutrition to the organism. Although the long-term efficacy for appetite control and weight reduction and the potential side effects of combination treatment is yet to be determined, this prominent additive effect on inhibiting food intake highlights the potential therapeutic value of a combination approach that targets multiple Y receptors and/or multiple neuronal pathways to decrease food intake and subsequently reduce body weight.

References

1. Boggiano MM, Chandler PC, Oswald KD, et al. PYY3-36 as an anti-obesity drug target. Obes Rev 2005;6:307-322.

2. Holzer P, Reichmann F, Farzi A. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides 2012;46:261-274.

3. Batterham RL, Cowley MA, Small CJ, et al. Gut hormone PYY3-36 physiologically inhibits food intake. Nature 2002;418:650-654.

4. Blomqvist AG, Herzog H. Y-receptor subtypes-how many more? Trends Neurosci 1997;20:294-298.

5. Lin S, Boey D, Herzog H. NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides 2004;38:189-200.

6. Renshaw D, Batterham RL. Peptide YY: a potential therapy for obesity. Curr Drug Targets 2005;6:171-179.

7. Unniappan S, McIntosh CH, Demuth HU, Heiser U, Wolf R, Kieffer TJ. Effects of dipeptidyl peptidase IV on the satiety actions of peptide YY. Diabetologia 2006;49: 1915-1923.

8. Alvarez Bartolome M, Borque M, Martinez-Sarmiento J, et al. Peptide YY secretion in morbidly obese patients before and after vertical banded gastroplasty. Obes Surg 2002;12:324-327.

9. Batterham RL, Cohen MA, Ellis SM, et al. Inhibition of food intake in obese subjects by peptide YY3–36. N Engl J Med 2003;349:941-948.

10. Boey D, Heilbronn L, Sainsbury A, et al. Low serum PYY is linked to insulin resistance in first-degree relatives of subjects with type 2 diabetes. Neuropeptides 2006;40:317-324.

11. Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S. Acute effects of PYY3-36 on food intake and hypothalamic neuropeptide expression in the mouse. Biochem Biophys Res Commun 2003;311:915-919.

12. Halatchev IG, Ellacott KL, Fan W, Cone RD. Peptide YY3–36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology 2004;145:2585-2590.

13. Shi YC, Hammerle CM, Lee IC, et al. Adult-onset PYY overexpression in mice reduces food intake and increases lipogenic capacity. Neuropeptides 2012;46: 173-182.

14. Acuna-Goycolea C, van den Pol AN. Peptide YY(3–36) inhibits both anorexigenic proopiomelanocortin and orexigenic neuropeptide Y neurons: implications for hypothalamic regulation of energy homeostasis. J Neurosci 2005; 25:10510-10519.

15. Batterham RL, Le Roux CW, Cohen MA, et al. Pancreatic polypeptide reduces appetite and food intake in humans. J Clin Endocrinol Metab 2003;88:3989- 3992.

16. Jesudason DR, Monteiro MP, McGowan BM, et al. Low-dose pancreatic polypeptide inhibits food intake in man. Br J Nutr 2007;97:426-429.

17. Asakawa A, Inui A, Yuzuriha H, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. Gastroenterology 2003;124: 1325-1336.

18. Ueno N, Inui A, Iwamoto M, et al. Decreased food intake and body weight in pancreatic polypeptide-overexpressing mice. Gastroenterology 1999;117:1427-1432.

19. Lin S, Shi YC, Yulyaningsih E, et al. Critical role of arcuate Y4 receptors and the melanocortin system in pancreatic polypeptide-induced reduction in food intake in mice. PLoS One 2009;4:e8488.

20. Sainsbury A, Baldock PA, Schwarzer C, et al. Synergistic effects of Y2 and Y4 receptors on adiposity and bone mass revealed in double knockout mice. Mol Cell Biol 2003;23:5225-5233.

21. Sainsbury A, Shi YC, Zhang L, et al. Y4 receptors and pancreatic polypeptide regulate food intake via hypothalamic orexin and brain-derived neurotropic factor dependent pathways. Neuropeptides 2010;44:261-268.

22. van den Pol AN, Yao Y, Fu LY, et al. Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. J Neurosci 2009;29:4622-4639.

23. Franklin KB, Paxinos G. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press; 1997.

24. Johnen H, Lin S, Kuffner T, et al. Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. Nat Med 2007;13: 1333-1340.

25. Smith MS. Lactation alters neuropeptide-Y and proopiomelanocortin gene expression in the arcuate nucleus of the rat. Endocrinology 1993;133:1258-1265.

26. Parker R, Herzog H. Localization of Y-receptor subtype mRNAs in rat brain by digoxigenin labeled in situ hybridization. Neuropeptide Y Protocols 2000;153: 165-183.

27. Burkhoff A, Linemeyer DL, Salon JA. Distribution of a novel hypothalamic neuropeptide Y receptor gene and it's absence in rat. Brain Res Mol Brain Res 1998;53:311-326.

28. King PJ, Williams G, Doods H, Widdowson PS. Effect of a selective neuropeptide Y Y(2) receptor antagonist, BIIE0246 on neuropeptide Y release. Eur J Pharmacol 2000;396:R1-R3.

29. Shi YC, Lin S, Wong IP, et al. NPY neuron-specific Y2 receptors regulate adipose tissue and trabecular bone but not cortical bone homeostasis in mice. PLoS One 2010;5:e11361.

30. Fetissov SO, Kopp J, Hokfelt T. Distribution of NPY receptors in the hypothalamus. Neuropeptides 2004;38:175-188.

31. Neary NM, McGowan BM, Monteiro MP, et al. No evidence of an additive inhibitory feeding effect

following PP and PYY3-36 administration. Int J Obes (Lond) 2008;32:1438–1440.