Peripheral administration of $PYY_{3–36}$ produces conditioned taste aversion in mice

Ilia G. Halatchev[1] and Roger D. Cone[1,*]

[1]Vollum Institute and Center for the Study of Weight Regulation and Associated Disorders, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239
*Correspondence: cone@ohsu.edu

Summary

Peptide YY (PYY) is a postprandially released gut hormone. Peripheral administration of one form of the peptide PYY_{3-36} produces a short-term reduction in food intake in rodents. Initial reports suggested that effects of $PYY_{3–36}$ on food intake are mediated by increasing the anorexigenic drive from melanocortin neurons in the hypothalamic arcuate nucleus. However, more recent data have demonstrated that the anorexigenic activity of $PYY_{3–36}$ is not dependent on melanocortin ligands or their receptors in the CNS. We demonstrate here that the anorexigenic actions of $PYY_{3–36}$ are also not dependent on the vagus nerve, a common pathway of satiety signaling. Peripherally administered $PYY_{3–36}$ activates neurons in the area postrema and nucleus tractus solitarius, brainstem areas known to mediate effects of certain aversive stimuli. Furthermore, peripheral administration of $PYY_{3–36}$ causes conditioned taste aversion in mice. Thus, inhibition of food intake by $PYY_{3–36}$ may result in part from induction of an aversive response.

Introduction

Key sites in the brain, like the arcuate nucleus (ARC) of the hypothalamus and the nucleus tractus solitarius (NTS) of the brainstem, maintain energy homeostasis by integrating peripheral signals of energy availability with energy intake and expenditure. The energy normally used for expenditure comes in two forms: either as stored energy, in the form of glycogen and fat deposits, or as newly ingested energy from a meal. Leptin, an adipostatic signal, is known to relay information about the long-term energy reserves in adipocytes. Gut peptides, like cholecystokinin (CCK) and ghrelin, along with vagal afferent signals, relay satiety and hunger signals to regulate meal size and frequency.

CCK is the archetypal satiety peptide released postprandially from the gut. The proposed mechanism by which CCK causes satiety, under physiological conditions, is through activation of CCK-A receptors on the vagus (Ritter and Ladenheim, 1985). After activation, vagal afferents transmit the signal to the NTS, activating a broad array of NTS neurons to stimulate meal termination. However, at high concentrations, peripherally administered CCK causes vagally mediated taste aversion (Ervin et al., 1995; Verbalis et al., 1986) and activates neurons in the area postrema (AP) (Luckman, 1992), a circumventricular organ outside the blood-brain barrier (BBB) implicated in mediating the response to aversive stimuli (Miller and Leslie, 1994).

$PYY_{3–36}$ is yet another secreted gut peptide, first reported by two laboratories, to produce a short-lived inhibition of food intake in rats and mice and to reduce the rate of weight gain in rats (Batterham et al., 2002). This peptide has achieved additional attention due to the observation that continuous intravenous infusion of $PYY_{3–36}$ reduced 24 hr food intake and hunger scores in a small number of lean and obese human subjects, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2002; Batterham et al., 2003). However, following the initial report, 12 laboratories, in a single publication, reported a lack of anorexigenic activity for $PYY_{3–36}$ in rats and mice and also did not find a reduction in the rate of weight gain (Tschop et al., 2004). Since that time, five additional laboratories have repeated the original finding of acute inhibition of feeding by peripheral administration of $PYY_{3–36}$ in rats or mice (Adams et al., 2004; Challis et al., 2004; Chelikani et al., 2005; Cox and Randich, 2004; Pittner et al., 2004), and one laboratory has demonstrated inhibition of feeding in rhesus monkeys (Moran et al., 2005). Two additional observations may shed light on this discrepancy. First, careful acclimatization of mice appears to be required to see the effects of $PYY_{3–36}$ on acute food intake (Halatchev et al., 2004), suggesting that the effect of the peptide may be sensitive to a variety of stressors. Secondly, intravenous infusion of $PYY_{3–36}$ in rats for 3 hr, meant to mimic the postprandial rise in the peptide, potently inhibited food intake, while a 15 min dosing was much less efficacious, suggesting that the bolus intraperitoneal (i.p.) administration of the peptide used in most studies may be a much less effective route of administration (Chelikani et al., 2005). Indeed, 11 of the 12 studies in the negative report utilized i.p. administration; the single study using subcutaneous infusion showed a statistically significant inhibition of food intake for 3 days (Tschop et al., 2004). One study using subcutaneous infusion of the peptide has now reproduced the initial finding of a reduction of cumulative food intake and the rate of weight gain in a variety of normal and obese murine models (Pittner et al., 2004).

$PYY_{1–36}$ is secreted from L cells in the ileum and colon in response to ingested fatty acids and other nutrients (Adrian et al., 1985; Bottcher et al., 1986; Bottcher et al., 1993). In the circulation, $PYY_{1–36}$ is cleaved by dipeptidyl peptidase-IV (DPP-IV) to produce $PYY_{3–36}$ (Grandt et al., 1994). The PP fold, a common tertiary structure shared between $PYY_{3–36}$ and all other NPY family peptides, confers its specificity for the NPY

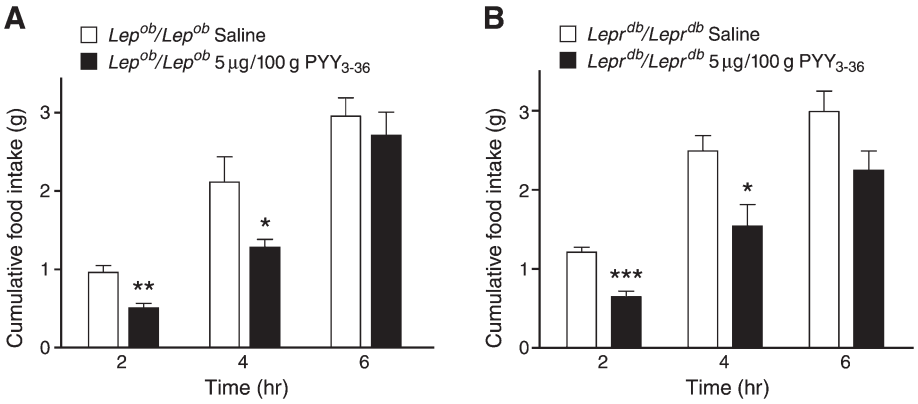

Figure 1. Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice respond equivalently to peripherally administered $PYY_{3–36}$ in a nocturnal feeding paradigm

A) Nocturnal feeding response of Lep^{ob}/Lep^{ob} mice to saline or a $PYY_{3–36}$ dose of 5 μg/100 g at 2, 4, and 6 hr post injection (n = 6, each group).

B) Nocturnal feeding response of $Lepr^{db}/Lepr^{db}$ mice to saline or a $PYY_{3–36}$ dose of 5 μg/100 g at 2, 4, and 6 hr post injection (n = 5, each group). Data is expressed as mean ± SEM, statistics by repeated measures two-way ANOVA followed by a Student's (two-tailed) t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

receptors 1–5 (Y1–Y5) (for review, see Keire et al. [2002]). $PYY_{3–36}$ has high-affinity binding restricted to the Y2 (Ki = 0.03–0.3 nM) and Y5 receptors (Keire et al., 2002).

Initially, $PYY_{3–36}$ was postulated to exert its anorexigenic effects through activation of ARC POMC neurons (Batterham et al., 2002). Using an in vitro slice preparation, $PYY_{3–36}$ was observed to reduce inhibitory postsynaptic potentials onto POMC neurons and increase the basal firing rate of these neurons, presumably via Y2 receptor on NPY neurons (Batterham et al., 2002). In this model, $PYY_{3–36}$ is proposed to have direct anorexigenic actions in the ARC, gaining access either through the median eminence, a circumventricular organ, or through the blood-brain barrier. Furthermore, peripheral administration of $PYY_{3–36}$ increased c-Fos immunoreactivity (IR), a marker of neuronal activation, in ~12% of POMC neurons in the ARC, compared to saline-treated mice (Batterham et al., 2002; Halatchev et al., 2004) .

However, a number of recent reports have shown that this model may need to be reconsidered. Unexpectedly, in a nocturnal feeding and/or fast-induced refeeding paradigm, MC4-R knockout ($MC4\text{-}R^{-/-}$) (Halatchev et al., 2004), Agouti (Martin et al., 2004), and POMC knockout mice ($POMC^{-/-}$) (Challis et al., 2004) showed a reduction of food intake comparable to wild-type (wt) sex- and age-matched controls after peripheral administration of $PYY_{3–36}$. These studies demonstrated that melanocortin signaling is not necessary for the anorexigenic effects of $PYY_{3–36}$, and an alternative mechanism should be considered.

In this study, we sought to identify alternative mechanisms of action for $PYY_{3–36}$. We first tested if, like CCK, $PYY_{3–36}$ requires the vagus nerve for its actions or requires intact leptin signaling. Using c-Fos as a marker of neuronal activation, we also systematically examined brain regions activated by increasing peripheral doses of $PYY_{3–36}$ in order to identify other potential sites of action. Finally, we examined the ability of the peptide, at doses that reliably and reproducibly inhibit food intake, to induce conditioned taste aversion.

Results

Intact leptin signaling is not essential for the actions of $PYY_{3–36}$

Signals relaying information about long-term energy stores and immediately available energy are integrated at key sites in the brain (for review, see Wang et al. [2000]) or Ellacott and Cone [2004]). It has been shown previously, for example, that leptin and CCK can act synergistically to inhibit food intake (Barrachina et al., 1997; Emond et al., 1999; Matson et al., 1997). Furthermore, rodents with deficient leptin signaling are hyposensitive to peripherally administered CCK (McLaughlin and Baile, 1980; Niederau et al., 1997). To test the hypothesis that the anorexigenic effects of $PYY_{3–36}$ might be abolished in the absence of leptin signaling, we examined the ability of $PYY_{3–36}$ to reduce food intake in Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice, two models of obesity caused by a disruption in the ligand or the receptor, respectively, required for leptin signaling. In a nocturnal free-feeding paradigm, $PYY_{3–36}$ (5 μg/100 g) was injected i.p. in Lep^{ob}/Lep^{ob} and/or $Lepr^{db}/Lepr^{db}$ mice right before lights out (1900 hr), and their food intake was measured every 2 hr for 6 hr. $PYY_{3–36}$ transiently but significantly reduced food intake in Lep^{ob}/Lep^{ob} mice in the first 4 hr (Figure 1A). Lep^{ob}/Lep^{ob} mice treated with the peptide ate 47% ± 11% less food in the first 2 hr and 39% ± 15% less food in 4 hr compared to saline-treated controls (Figure 1A), levels nearly identical to the reduction seen in wt mice by the same dose of $PYY_{3–36}$ (Figure 2B). By 6 hr, there was no difference in food consumption between saline and $PYY_{3–36}$-treated Lep^{ob}/Lep^{ob} mice (Figure 1A). Likewise, i.p. $PYY_{3–36}$ significantly reduced food intake in $Lepr^{db}/Lepr^{db}$ mice for the same duration and to the same degree (Figure 1B). The peptide treated group of $Lepr^{db}/Lepr^{db}$ mice ate 46% ± 9% less food at 2 hr and 38% ± 15% less at 4 hr compared to saline-treated controls (Figure 1B), also a nearly identical reduction in food intake to wt mice by the same dose of $PYY_{3–36}$ (Figure 2B). As in Lep^{ob}/Lep^{ob} mice, the effect of $PYY_{3–36}$ in $Lepr^{db}/Lepr^{db}$ mice was transient; no difference in food consumption was observed at 6 hr (Figure 1B).

The vagus nerve is not essential for the inhibition of feeding by $PYY_{3–36}$

Gut peptides implicated in the acute regulation of hunger and satiety, such as ghrelin and CCK, have been shown to require the vagus nerve for their actions (Date et al., 2002; Smith et al., 1985). We postulated that $PYY_{3–36}$, a postprandially released gut peptide, may also require the vagus nerve to mediate its anorexic activity. To test this hypothesis, we injected $PYY_{3–36}$ or saline i.p. in wt mice with both vagus nerves surgi-

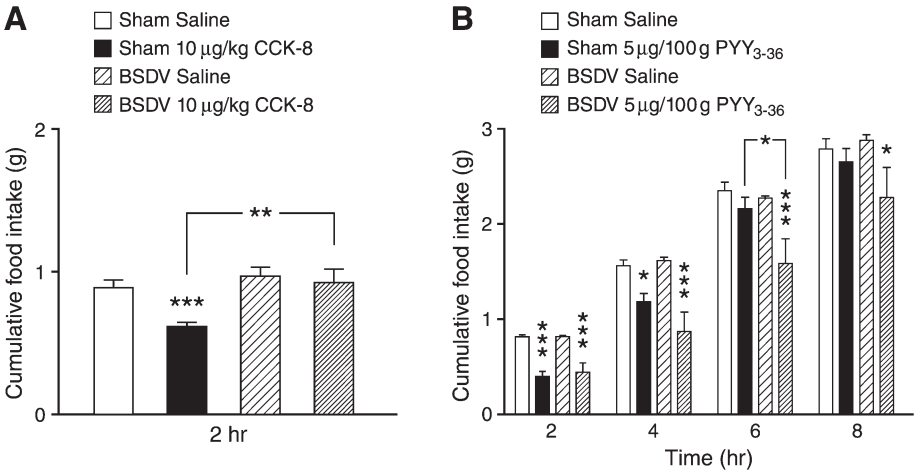

Figure 2. Intact vagus nerves are not essential for the actions of peripherally administered PYY_{3-36} in a nocturnal feeding paradigm

A) Nocturnal feeding responses of Sham and BSDV mice to i.p. CCK-8 dose of 10 μg/kg at 2 hr post injection (Sham saline and CCK-8, n = 10; BSDV saline and CCK-8, n = 6).

B) Nocturnal feeding responses of Sham and BSDV mice to i.p. PYY_{3-36} dose of 5 μg/100g at 2, 4, 6, and 8 hr post injection (Sham saline and CCK-8, n = 10; BSDV saline and CCK-8, n = 6). Data is expressed as mean ± SEM, statistics by Student's (two-tailed) t test for (**A**) and repeated measures two-way ANOVA followed by one-way ANOVA with Bonferroni's tests posthoc for each time point in (**B**), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cally transected below the level of the diaphragm (BSDV mice) and compared their responses to animals whose vagus nerves were surgically isolated and separated but not transected (Sham mice). We chose a nocturnal free-feeding paradigm to assay the effects of PYY_{3-36} on food intake because we found it eliminated the reduction of food intake in vagotomized mice that occurs due to stress in a fast-induced refeeding paradigm. Using CCK-8, we demonstrated that both vagus nerves were completely transected in BSDV mice and that Sham mice did not have their vagus nerves accidentally cut (Figure 2A). CCK-8 (10 μg/kg i.p.) significantly reduced 2 hr nocturnal food intake in the Sham surgical group but not in the BSDV group (Figure 2A).

We also tested the ability of PYY_{3-36} (5 μg/100 g) injected i.p. immediately before lights out (1900 hr) to reduce food intake in Sham and BSDV mice by measuring cumulative food intake every 2 hr for 8 hr. PYY_{3-36} reduced food intake to the same extent, 54% ± 7% in Sham and 46% ± 13% in BSDV mice in the first 2 hr, as compared to saline-matched controls for each surgical group (Figure 2B). However, PYY_{3-36} showed a significantly different effect across time with different surgical treatments (Sham versus BSDV), as assayed by a repeated measures two-way ANOVA. In the Sham group, at 4 hr, PYY_{3-36} significantly reduced food intake by 25% ± 10%, and only a trend toward reduction of food intake, without significance, was observed by 6 hr (Figure 2B). In contrast, the BSDV group responded to PYY_{3-36} for an extended period of time, such that even by 6 hr, PYY_{3-36} treated animals ate ~30% less than saline-treated BSDV and PYY_{3-36}-treated Sham mice (Figure 2B). In fact, at 8 hr post injection, a significant reduction of food intake was measured in the PYY_{3-36}-treated BSDV mice as compared to saline-treated Sham controls (Figure 2B), suggesting that BSDV mice actually exhibit prolonged sensitivity to the anorexigenic actions of a single bolus dose of PYY_{3-36}.

Dose-dependent activation of AP and intermediate NTS neurons with nocturnal peripheral administration of PYY_{3-36}

In order to begin to address the mechanisms by which PYY_{3-36} inhibits food intake, it was important to characterize the minimum dose of peripheral PYY_{3-36} required to inhibit food intake reliably and reproducibly. We previously showed that PYY_{3-36} dose-dependently and transiently reduces food intake at high doses (3 and 10 μg/100 g) both in fasted and in nocturnal free-feeding paradigms in acclimated mice (Halatchev et al., 2004). However, at the lowest dose, 0.3 μg/100 g, PYY_{3-36} neither reliably nor significantly reduced food intake at any time point in either feeding paradigm. Here, using a 16 hr fast-induced refeeding paradigm, acclimated wt mice were injected i.p. with increasing doses of PYY_{3-36} (0.3, 1.5, and 5 μg/100 g), and their cumulative food intake was measured hourly for 4 hr. PYY_{3-36} dose-dependently reduced food intake for the duration of the experiment (Figure 3A). As shown previously, although a trend for reduction in food intake at 0.3 μg/100 g of PYY_{3-36} was observed, statistical significance was not reached at that dose compared to saline treatment (Figure 3A). However, PYY_{3-36} significantly and reliably reduced food intake at the 1.5 μg/100 g dose (Figure 3A) at 1, 2, and 3 hr. The highest dose of PYY_{3-36} (5 μg/100 g) tested significantly reduced food intake for the duration of the experiment, 4 hr (Figure 3A), as it did in the nocturnal feeding paradigms in Sham, BSDV (Figure 2B), Lep^{ob}/Lep^{ob} (Figure 1A), and $Lepr^{db}/Lepr^{db}$ mice (Figure 1B).

We next wanted to identify specific sites in the brain that are activated by peripherally administered PYY_{3-36}. We decided to focus on two brainstem nuclei in particular, the AP and NTS, both known to be involved in mediating the effects of gut-released peptides like CCK and ghrelin (Date et al., 2002; Fan et al., 2004). Nocturnal i.p. injection (1900 hr) of increasing doses of PYY_{3-36} (0.3, 1.5, and 5 μg/100 g) caused a significant dose-dependent activation of c-Fos IR in neurons in AP and intermediate NTS (Figures 3C, 3F, 3I, 3K, and 3L), compared to saline injections (Figures 3B, 3E, 3H, 3K, and 3L). The minimum peripheral dose of PYY_{3-36} to reliably and reproducibly inhibit food intake, 1.5 μg/100 g (Figure 3A), was also the minimal dose to show a 2-fold statistically significant activation of c-Fos in the intermediate NTS, compared to a saline i.p. injection (Figure 3K). While increasing doses of i.p. PYY_{3-36} consistently induced c-Fos IR in a very discrete subnucleus of the NTS, the positive control, CCK-8 (10 μg/kg), dramatically induced widespread c-Fos expression in neurons throughout the entire NTS and AP (Figures 3D, 3G, 3J, 3K, and 3L) to levels

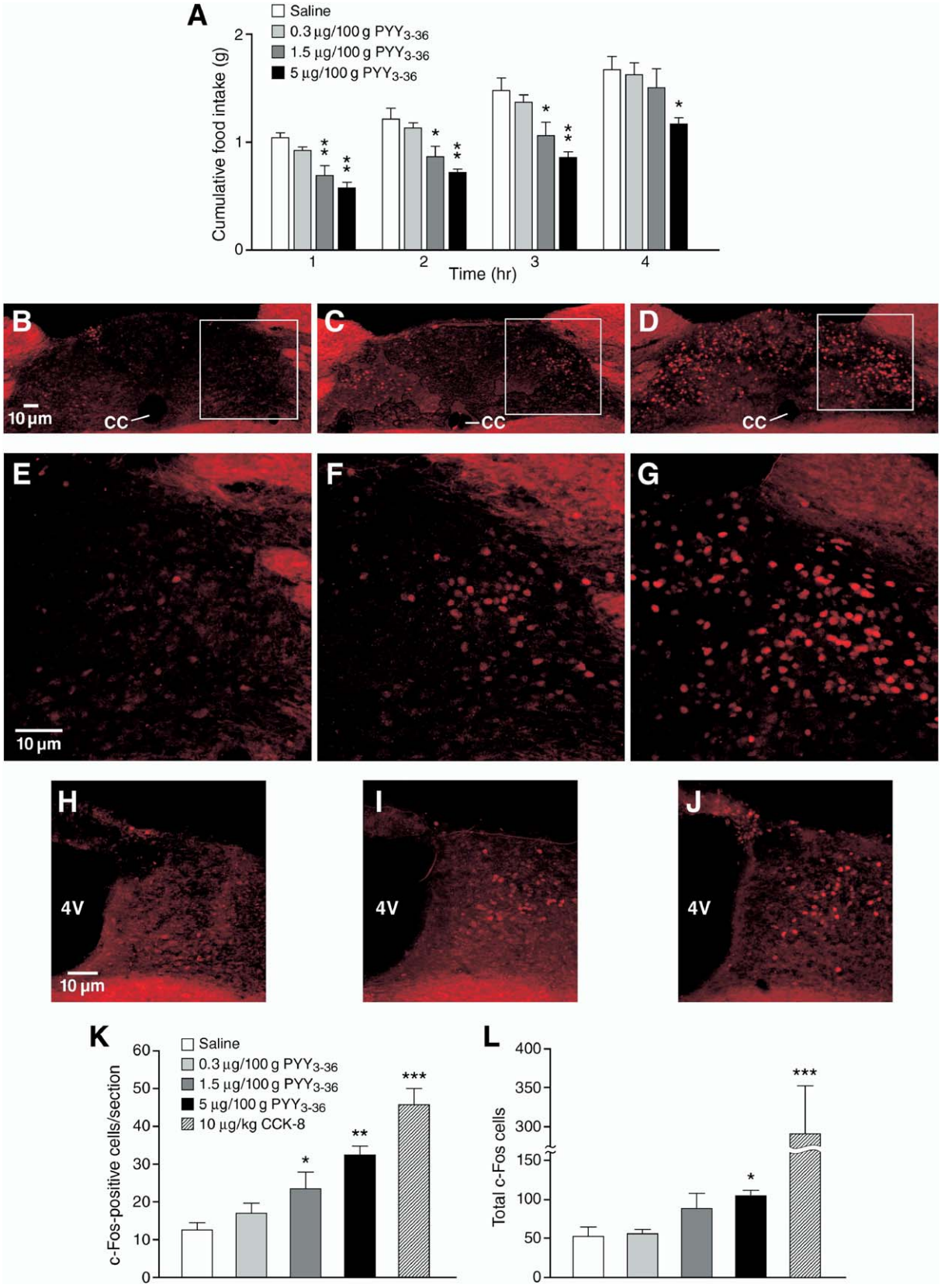

Figure 3. Peripheral PYY_{3-36} dose-dependently inhibits food intake and activates c-Fos expression in AP and intermediate NTS neurons

A) Dose-dependent inhibition of food intake at 1, 2, 3, and 4 hr after i.p. injections of saline or $PYY_{3–36}$ at doses of 0.3, 1.5, and 5 µg/100 g in wt mice acclimated for 5 days (n = 5).

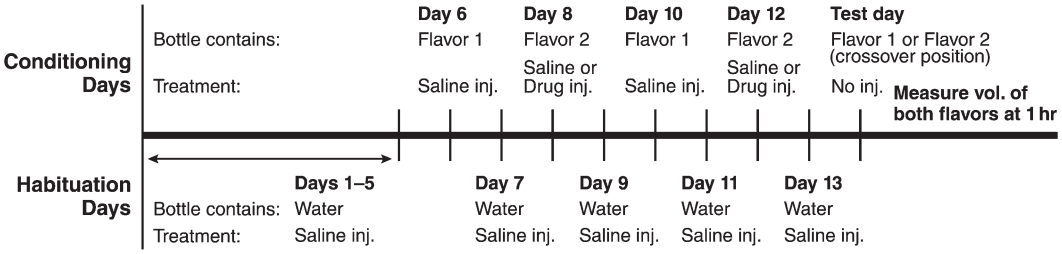

Figure 4. Schematic timeline of the conditioned taste aversion protocol

All male age-matched mice were individually housed and received 1 hr of water or flavor in two separate bottles (equidistant from the food) daily for 13 days. Each animal was weighed and i.p. injected daily with either saline (habituation days, days 1–7, 9–11, and 13, or saline/peptide/LiCl days, 8 and 12). On the test day, mice received both flavors in a crossover position, and intake from each bottle was measured after 1 hr.

much greater than even the highest dose of $PYY_{3–36}$ (Figures 3C, 3F, 3I, 3K, and 3L).

Peripheral administration of $PYY_{3–36}$ causes conditioned taste aversion in mice

A growing number of reports and observations have failed to identify neuronal and/or hormonal systems required for the anorexigenic actions of $PYY_{3–36}$. However, previous reports have demonstrated a link between $PYY_{1–36}$ and emesis in dogs (Harding and McDonald, 1989; Perry et al., 1994). Additionally, in this report, we have shown that there is a dose-dependent, statistically significant activation of c-Fos IR, after peripheral administration of $PYY_{3–36}$, in intermediate NTS and AP, nuclei known to mediate the response to aversive stimuli (Schafe et al., 1995). This led us to the hypothesis that peripherally administered $PYY_{3–36}$ may be mediating its anorexic effects through visceral illness. We chose a two-bottle, two-flavor conditioned taste aversion assay (Figure 4) to test whether $PYY_{3–36}$, at doses that reliably and reproducibly reduce food intake (1.5 and 5 μg/100 g), causes conditioned taste aversion in mice. Both peripheral doses of $PYY_{3–36}$ (1.5 and 5 μg/100 g) significantly reduced drinking of the flavor associated with peptide administration ($PYY_{3–36}$-paired flavor) compared to the flavor associated with the saline treatment (saline-paired flavor) (Figure 5A). Saline-treated animals (saline pairing to both flavors) showed no preference for either bottle (Figure 5A). Intriguingly, the minimum peripheral dose of $PYY_{3–36}$ to reliably and reproducibly inhibit food intake, 1.5 μg/100 g (Figure 3A), and significantly activate c-Fos IR in intermediate NTS (Figure 3K) also caused a significant reduction in consumption of the $PYY_{3–36}$-paired flavor, compared to the saline-paired flavor (Figure 5A). Mice that received 1.5 μg/100 g $PYY_{3–36}$ drank 31% ± 17%, and mice that received 5 μg/100 g $PYY_{3–36}$ drank 50% ± 15% less of the $PYY_{3–36}$-paired versus the saline-paired flavor (Figure 5A) at 1 hr. The largest reduction of stimulus-paired flavor consumption was seen in the LiCl group (positive control), in which the difference between LiCl-paired and saline-paired flavor consumption was 96% ± 2% (Figure 5A) at 1 hr.

To directly compare the effect of the treatment (saline versus different peptide concentrations and LiCl) on the relative consumption of the stimulus-paired flavor, we also expressed the results in ratios, in which the amount of stimulus-paired flavor is divided by the total volume (see Experimental Procedures) consumed by an animal (Figure 5B). $PYY_{3–36}$ dose-dependently and significantly reduced the ratios, compared to saline control (Figure 5B). Compared to the saline control ratio of 0.52 ± 0.05 (n = 17), $PYY_{3–36}$ had a ratio of 0.42 ± 0.05 (ns; n = 17) at 1.5 μg/100 g $PYY_{3–36}$ dose and 0.33 ± 0.06 ($p < 0.0115$; n = 17) at 5 μg/100 g $PYY_{3–36}$ dose (Figure 5B). The drastic reduction of stimulus-paired flavor consumed by the LiCl-treated group translated to a ratio of 0.05 ± 0.03 ($p < 0.0001$; n = 9) (Figure 5B).

Discussion

Two decades after the original discovery of PYY by Tatemoto in 1980, it was demonstrated that peripheral administration of the Y2 receptor-preferring $PYY_{3–36}$ form of the peptide reduced food intake in mice, rats, and humans (Batterham et al., 2002). In their study, Batterham et al. (2002) also reported that $PYY_{3–36}$ decreases the tonic inhibitory drive from NPY neurons onto POMC neurons in the ARC by activating inhibitory Y2 autoreceptors on NPY neurons or terminals. Furthermore, they demonstrated that peripheral administration of $PYY_{3–36}$ caused activation of a small percentage (~12%) of POMC neurons in ARC, assessed by expression of c-Fos, a marker of neuronal activation. From these observations, the effects of $PYY_{3–36}$ on food intake were proposed to be mediated through the central

B–J) c-Fos expression in AP and NTS neurons. (**B**) Ninety minutes after i.p. saline injection, (**C**) 5 μg/100g $PYY_{3–36}$ injection, and (**D**) 10 μg/kg CCK-8 injection. Peripheral $PYY_{3–36}$ injection (5 μg/100g) specifically induces c-Fos expression in intermediate NTS at the level of AP (**F**) or 4V (**I**), as compared to the diffuse activation by CCK-8 (10 μg/kg) at the level of AP (**G**) or 4V (**J**), and low c-Fos expression with i.p. saline-injected controls at the level of AP (**E**) or 4V (**H**).

K) Quantization of c-Fos IR in the intermediate NTS neurons after i.p. administration of saline (n = 5) or $PYY_{3–36}$ at increasing concentrations (0.3 μg/100 g, n = 4; 1.5 μg/100 g, n = 4; and 5 μg/100 g, n = 5; or CCK-8 at 10 μg/kg, n = 4).

L) Quantization of c-Fos IR in the AP neurons after i.p. administration of saline (n = 5) or $PYY_{3–36}$ at increasing concentrations (0.3 μg/100g, n = 4; 1.5 μg/100 g, n = 4; and 5 μg/100 g, n = 5; or CCK-8 at 10 μg/kg, n = 4). Data is expressed as mean ± SEM. Statistics by one-way ANOVA with Dunnett's test post-hoc, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

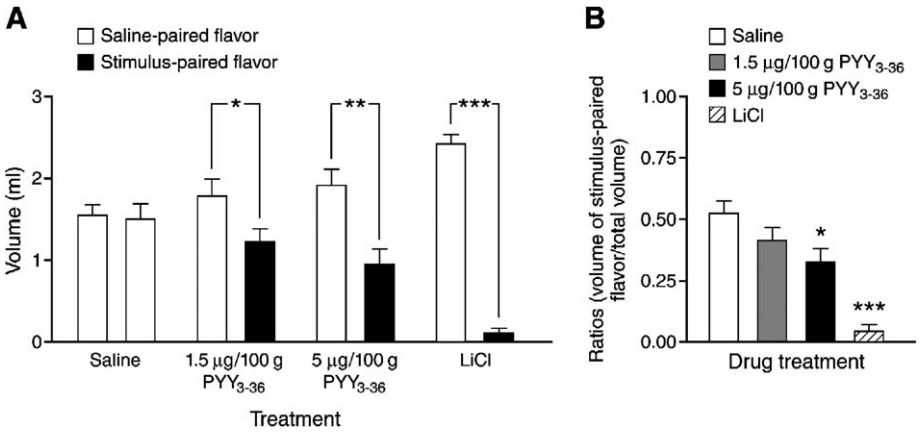

Figure 5. Peripheral administration of $PYY_{3–36}$ at doses that reliably inhibit food intake causes conditioned taste aversion in wt mice

A) Individual flavor consumption (saline-paired flavor versus stimulus-paired flavor) after stimulus-paired flavor is associated with i.p. LiCl (2% BW of 0.15 M LiCl) or $PYY_{3–36}$ (1.5 and 5 µg/100 g) treatment.

B) Ratios of volume of stimulus-paired flavor consumed versus total volume consumed across treatment groups (saline, 1.5 and 5 µg/100 g, n = 17; LiCl, n = 9). Data is expressed as mean ± SEM, statistics by Student's (two-tailed) t test for (**A**) and one-way ANOVA with Dunnett's test post-hoc for (**B**), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

melanocortin system. However, since this report, a number of published observations have raised serious doubts about the role of the central melanocortin system in the anorexigenic effects of peripherally administered $PYY_{3–36}$. Mice lacking the POMC gene (Challis et al., 2004) or the melanocortin-4 receptors (Halatchev et al., 2004) are fully responsive to the peptide. Likewise, lean and obese agouti mice are also fully responsive to peripheral injection of $PYY_{3–36}$ (Martin et al., 2004). Nevertheless, $PYY_{3–36}$ is still considered by some to be a potentially promising therapeutic agent, since it appears to reduce food intake in lean and obese humans (Batterham et al., 2002; Batterham et al., 2003) as well as in normal and obese mice (Pittner et al., 2004). In two studies from the same laboratory, human subjects were infused with the peptide intravenously and showed 33% reduction of calories consumed in a meal. Additional studies will be necessary to validate these observations in the human as well as to determine if there is any clinical efficacy in the treatment of obesity. Nevertheless, elucidation of mechanisms by which this postprandially-released gut peptide reduces food intake remains highly relevant.

A variety of peripheral peptides that relay information about energy availability have synergistic effects on key circuits in the brain involved in regulation of energy intake and expenditure. For example, leptin, the adipostatic hormone released from adipose tissue, has synergistic actions with CCK (Barrachina et al., 1997; Emond et al., 1999; Matson et al., 1997). However, animal models lacking the key signal relaying long-term energy availability, like rodents lacking leptin signaling, show desensitized responses to bolus injections of some satiety factors. CCK, for example, shows a decreased ability to induce satiety in a number of obese rodent models, like $MC4R^{-/-}$ mice and obese Zucker rats (Halatchev et al., 2004; McLaughlin and Baile, 1980; Niederau et al., 1997). In contrast, bolus peripheral injections of $PYY_{3–36}$ reduce food intake to the same amount and time duration in $MC4R^{-/-}$, (Halatchev et al., 2004), Agouti (Martin et al., 2004), and $POMC^{-/-}$ mice (Challis et al., 2004) as they do in wt mice. Therefore, we wanted to test the efficacy of $PYY_{3–36}$ to reduce food intake in mice lacking the leptin gene Lep^{ob}/Lep^{ob} and in mice with a mutation in the long form of the leptin receptor $Lepr^{db}/Lepr^{db}$. $PYY_{3–36}$ reduced food intake, using a nocturnal feeding paradigm, in Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice (Figure 1). This experiment shows that intact leptin signaling is not essential for the anorexigenic actions of $PYY_{3–36}$. Furthermore, the lack of any potent desensitization to $PYY_{3–36}$ in animals lacking leptin signaling argues that $PYY_{3–36}$, in contrast to CCK, may not act via a leptin-regulated satiety pathway.

We next wanted to determine if the effects of $PYY_{3–36}$ on food reduction require an intact vagus nerves, like the gut-derived factors CCK and ghrelin (Date et al., 2002; Smith et al., 1985). We chose a nocturnal nonfasted feeding paradigm to assay the effects of peripherally administered $PYY_{3–36}$ on vagotomized mice because we and others have observed that fasted vagotomized animals consume less food than sham-operated controls, presumably due to stress or gastric distention (Moran et al., 1997; Reidelberger, 1992). Our previous observation demonstrated that stress could easily mask the anorexigenic effects of $PYY_{3–36}$ (Halatchev et al., 2004).

$PYY_{3–36}$ (5 µg/100 g) reduced food intake to the same extent in vagotomized mice and Sham controls (Figure 2B). However, in contrast to Sham mice, $PYY_{3–36}$ had a prolonged duration of effect in vagotomized mice at that dose, reducing food intake for up to 8 hr (Figure 2B). This observation was quite striking; instead of attenuating the effects of $PYY_{3–36}$ on food intake, total (afferent and efferent) subdiaphragmatic vagotomy, in fact, prolonged them. This may reflect that even though an intact vagus nerve is not required for the short-term anorexigenic actions of peripheral $PYY_{3–36}$, unlike CCK and ghrelin, intact vagal tone could modulate the duration of action of $PYY_{3–36}$. Moreover, it has been shown in the literature that intact efferent vagal parasympathetic tone modulates basal and food-induced release of PYY (Zhang et al., 1993), as well as some of the ability of the peptide to modulate gastrointestinal (GI) transit time and glandular secretion (Chen et al., 1996; Masuda et al., 1994). In contrast to the results reported here, a recent study has demonstrated blockade of $PYY_{3–36}$-mediated inhibition of food intake in the rat by bilateral subdiaphragmatic vagotomy (BSDV) (Koda et al., 2005). Additional work will be required to determine if the different findings result from species differences between rat and mouse or from methodological differences. For example, regulation of growth hormone release by ghrelin appears to be dependent on the vagus in rats (Date et al., 2002) but not in humans (Takeno et al., 2004).

Even though dose response curves for $PYY_{3–36}$ can be found

in the literature showing the magnitude and duration of food reduction by $PYY_{3–36}$, the minimum dose required to produce significant and reliable food inhibition by i.p. administration has not been reported in mice. Here we show that the minimum dose of $PYY_{3–36}$ needed to reliably and reproducibly cause a short-term inhibition of food in mice is 1.5 μg/100 g (Figure 3A), with varying duration of action from 1 to 3 hr (data not shown). This dose was then used in both the neuroanatomical and behavioral tests performed in the rest of this study.

Certain sites in the brain have been implicated in mediating the effects of gut peptides and other hormones in the regulation of satiety and long-term energy homeostasis. The hypothalamus has been proposed to be a primary site of action of the long-term adipostatic factor leptin (Elias et al., 1999). In the brainstem, the NTS is the primary site for reception of satiety signals from the vagus, provided by both neural and hormonal signals from the gut, such as gastric distension and CCK (Jean, 1991). Other gut-derived factors, like amylin, appear to be vagus independent and act directly on brainstem structures like the AP to inhibit feeding (Lutz et al., 1995; Lutz et al., 2001; Riediger et al., 2002). It was thus, perhaps, surprising that a gut-released peptide, like $PYY_{3–36}$, would mediate its short-term anorexigenic effects directly via the ARC of the hypothalamus. Additional work will be required to determine the mechanism and relevance of activation of small numbers of arcuate POMC neurons by $PYY_{3–36}$. However, in this report, we looked at the ability of peripherally administered $PYY_{3–36}$ to activate neurons throughout the entire NTS and other brainstem nuclei known to be part of the gut-brain axis. Specifically, we focused on the AP, a circumventricular organ, and the NTS, two sites known to be involved in satiety and emesis. In a previous report, focusing on a different region of the NTS expressing POMC, we did not observe activation of neurons in the NTS by $PYY_{3–36}$ (Halatchev et al., 2004). However, here, after examining the entire NTS, we observed a dose-dependent activation of AP and NTS neurons with increasing concentrations of $PYY_{3–36}$, as assayed by c-Fos IR, a marker of neuronal activation (Figure 3). In contrast to CCK, which dramatically activated neurons throughout the AP and NTS, $PYY_{3–36}$ activated a specific subnucleus of the NTS, the intermediate nucleus of the NTS (Figure 3). Even though both peptides, CCK and $PYY_{3–36}$, are secreted in response to fatty acids in the GI lumen and reduce food intake by similar amounts and over similar time courses, the strikingly different pattern of c-Fos activation in NTS and AP likely reflects inherent differences in their mechanisms of action. A functional example of this is the fact that CCK requires MC4-R signaling for the inhibition of food intake while $PYY_{3–36}$ does not (Fan et al., 2004; Halatchev et al., 2004).

Aversive stimuli are well established to activate specific neuronal populations within AP and intermediate NTS, such as those induced by i.p. injection of LiCl (Schafe et al., 1995). In fact, we observed a very similar pattern of activation of c-Fos in the same regions of the intermediate NTS, as observed immediately after i.p. LiCl and/or in response to a flavor that has been associated with an aversive stimulus (Houpt et al., 1997; Schafe et al., 1995), such as LiCl. Furthermore, Schafe et al. (1995) have demonstrated that the acquisition of the aversive memory to a substance, required for conditioned taste aversion, seems to be dependent on an intact forebrain-hindbrain relay through nerve tracts above the superior colliculus (Schafe et al., 1995). Here we show that a single bolus i.p. injection of $PYY_{3–36}$ produces c-Fos IR in the same brainstem nuclei as LiCl and reduces food intake. Thus, it is consistent with these findings that, like LiCl, $PYY_{3–36}$ may, at least in part, reduce food intake by causing a short-lived aversive response. Additional data will be required, however, to determine if $PYY_{3–36}$ treatment activates the same subgroup of intermediate NTS neurons as other aversive substances like LiCl.

A number of gut-released peptides have been shown to mediate part of their effects on food intake through induction of an aversive response. At high doses, CCK causes taste aversion and activates neurons in AP and NTS (Deutsch and Hardy, 1977; Ervin et al., 1995). However, unlike other well-characterized substances that cause an aversive response, like LiCl, the association produced by high doses of peripheral CCK is dependent on the vagus nerve (Verbalis et al., 1986). Here we wanted to examine whether the dose-dependent inhibition of food intake and c-Fos activation by $PYY_{3–36}$, in the AP and intermediate NTS, might be due to the production of a short-term aversive response in mice. At doses that reliably and reproducibly inhibit short-term food intake, $PYY_{3–36}$ caused conditioned taste aversion to a flavor paired with $PYY_{3–36}$ treatment (Figure 5). These data raise the possibility that the primary mechanism by which $PYY_{3–36}$ inhibits food intake in mice is through a short-term aversive response.

Experimental Procedures

Animals

Transgenic mice (C57BL/6J background) with enhanced green fluorescent protein under the control of the POMC promoter (EGFP-POMC) were derived from animals described previously (Cowley et al., 2001). All transgenic animals were raised in group housing with their siblings and maintained at 23°C ± 1°C on a 12 hr light, 12 hr dark cycle (0700–1900 hr light). Mice were allowed ad libitum access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO; ~4.5% fat by weight). $Lepr^{db}/Lepr^{db}$ and wt experimental animals of the C57BL/6J strain were purchased to be age, sex, and weight matched (The Jackson Laboratory, Bar Harbor, ME). Lep^{ob}/Lep^{ob} mice, male and female, were purchased from the same source to be age and weight matched. BSDV male mice (C57BL/6J strain) were purchased with both the anterior and posterior trunks of the vagus nerves cut below the level of the diaphragm (Charles River Laboratories, Wilmington, MA). To create Sham-operated animals, C57BL/6J mice from the same source underwent a comparable surgery without transection of the vagus nerves. Upon arrival, Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$, BSDV, Sham, and wt mice were allowed to acclimate for 1 week under the conditions stated above. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of Oregon Health and Science University.

Source of reagents/peptides

All experiments were performed with human $PYY_{3–36}$ (Bachem, Torrance, CA; lot 0562660), CCK-8 (Sigma-Aldrich, St. Louis, MO; lot 062K11181), and LiCl (Sigma-Aldrich, St. Louis, MO; lot 50K0198). Peptides were certified by the manufacturer and came with HPLC data showing a single peak with the correct molecular weight from mass spectrogram and a purity grater than 97%. Peptides were dissolved in sterile isotonic saline and injected in a total volume of 500 μl/injection. Fresh human $PYY_{3–36}$ and CCK-8 dilutions were prepared on the day of the injection from frozen stock solutions. LiCl was dissolved in sterile millipore-filtered water (0.66 g in 100 ml of water).

Feeding protocols

16 hr fast refeeding in acclimated wt mice

Age-matched wt male mice (8 weeks) were used for the feeding study as described previously (Halatchev et al., 2004). In short, animals were individ-

ually housed for a week. During the second week, animals were acclimated to the procedure by i.p. saline injections daily, with food in a petri dish and mock food measurements every hour for 4 hr. Mice were fasted for 16 hr the night before the experiment (1900–1100 hr). Animals were injected with either saline or $PYY_{3–36}$ i.p. at a dose of 0.3, 1.5, and 5 μg/100 g, and cumulative food intake was measured hourly for 4 hr after injections by placing two pellets of chow in petri dishes on the floor of the cage. To minimize error attributable to loss of food particles or bedding in petri dishes, petri dishes and bedding were screened.

Nighttime feeding protocol in acclimated Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$ mice

Age-matched Lep^{ob}/Lep^{ob} (8- to 9-week-old, half male and half female) and $Lepr^{db}/Lepr^{db}$ (10-week-old female mice) were used for the nocturnal study. As described previously (Halatchev et al., 2004), mice were individually housed for a week. In the following week, mice were acclimated to the procedure by injecting 500 μl sterile isotonic saline i.p. immediately before lights out (1900 hr). Two pellets of food were then placed in a petri dish and weighed every 2 hr for 6 hr under a red light. Animals were acclimated to the procedure until their food intake stabilized for at least 4 consecutive days. On the 2 experimental days, separated by 48 hr of rest, animals were injected i.p. in a crossover manner with either saline or 5 μg/100 g $PYY_{3–36}$ and their food intake measured every 2 hr for 6 hr. On nonexperimental days, animals were injected i.p. with saline and food measured to establish any deviations from baseline. Measurement error was minimized by careful screening for food particles under red light.

Nighttime feeding protocol in acclimated Sham and BSDV mice

Age-matched Sham and BSDV male mice (8 weeks) were used for the nocturnal vagotomy feeding experiment. Mice underwent surgery a week before arrival and were allowed to recover their body weight and food intake to presurgical levels for a week in group housing. Mice were individually housed for a week, and their body weight was monitored and compared across surgical groups and to age-matched sex-matched nonsurgical mice. Only mice that recovered completely from surgery were used for the experiment. Sham and BSDV mice were injected with 500 μl of sterile isotonic saline i.p. immediately before lights out (1900 hr), and two preweighed pellets of Purina chow in petri dishes were measured every 2 hr for 8 hr using red light. Acclimatization to the procedure was established by stabilization of food intake over 4 consecutive days. On experimental days, sham and BSDV mice were injected i.p. in a crossover manner with either saline or 5 μg/100 g $PYY_{3–36}$ and their food intake measured every 2 hr for 8 hr.

CCK-8, a peptide demonstrated to require the vagus nerve to mediate its effects (Ritter and Ladenheim, 1985), was used to confirm that the BSDV mice were vagotomized and that the Sham-vagotomized animals were not. Animals received 2 days of isotonic saline injections i.p. after $PYY_{3–36}$ administration to confirm that baseline food consumption was not altered due to experimental manipulations. On the third and fifth days, animals were injected i.p. in a crossover manner with either saline or CCK-8 (10 μg/kg) and their food intake measured at 2 hr. Each animal was used as its own control to determine if its vagus nerves were cut. Only Sham animals that responded to CCK-8 by reducing their food intake and only BSDV mice that did not show a reduction of food intake with CCK-8 were used for analysis of the effect of vagotomy on the actions of $PYY_{3–36}$. For consistency, each individual animal's response to CCK-8 was tested several times.

c-Fos immunohistochemistry

POMC-EGFP mice (23–27 g) were handled and injected with 250 μl sterile saline i.p. at 1900 hr for 5 days prior to the experiment to minimize background c-Fos immunoreactivity caused by stress. During the acclimatization period, food was withdrawn from animals 2 hr prior to injections (for 3.5 hr) to minimize neuronal activation due to feeding (Fan et al., 2004). Animals received an i.p. injection of $PYY_{3–36}$ (0.3, 1.5, and 5 μg/100 g), CCK-8 (10 μg/kg), or sterile saline 90 min (at lights out, 1900 hr) before being deeply anaesthetized and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Sections from perfused brains (25 μM) were cut and stored free-floating in 0.01 M PBS containing 0.03% sodium azide. The sections were incubated for 1 hr at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS + 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos (Ab-5), human (rabbit) polyclonal antibody (Oncogene Research Products, La Jolla, CA; Lot D21570) and diluted 1:80,000 in blocking reagent for 24 hr at 4°C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes Inc., Eugene, OR) for 1 hr at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations, the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda Corp., Foster City, CA), and viewed under a fluorescence microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). The number of c-Fos immunoreactive cells was counted in sections representing certain nuclei.

Conditioned taste aversion

Age-matched (7-week-old) male wt (C57BL/6J) mice were individually housed for 1 week with ad libitum access to food and water. After 1 week of acclimatization to single housing, water was withdrawn right before lights out (day 0). To acclimate mice to the i.p. injections, weighing, and timing of water presentation, animals were presented with two water bottles (equidistant from the food, and the original water bottle presented during the first week of individual housing) at the same time (1000 hr–1100 hr) for 1 hr for 5 consecutive days (day 1–day 5). At the end of the hour, water bottles and mice were weighed, and each animal was injected i.p. with a volume of sterile isotonic saline, equal to 2% of its body weight (BW). Individual water consumption from each bottle was measured for each mouse every day to ensure mice knew that they had a choice between two bottles and drank equally from both. On day 6, each mouse was given 1 hr access to a novel flavor (either 0.15% saccharin with 0.05% cherry Kool-Aid or 0.15% saccharin with 0.05% grape Kool-Aid) instead of water in both bottles. Immediately following the 1 hr access to a novel flavor, each mouse was weighed and received i.p. injection of 2% BW sterile isotonic saline. On the following day (day 7), mice were given access to water for 1 hr, weighed, and injected i.p. with 2% BW saline. On the second Kool-Aid day (day 8), each mouse received a novel access to the other flavor (mice that received cherry on day 6 received grape on day 8, and mice that received grape on day 6 received cherry on day 8) in both bottles, and, immediately following 1 hr access to the second novel flavor, each mouse was weighed and injected i.p. with either 2% BW of saline, 0.15M LiCl, or $PYY_{3–36}$ at doses of 1.5 or 5 μg/100 g. Animals received another day with 1 hr water access (day 9) followed by weighing and 2% BW i.p. saline injection. On the third day of Kool-Aid presentation (day 10), mice were allowed access to 1 hr of original Kool-Aid flavor in both bottles (Kool-Aid flavor from day 6) immediately followed by weighing and 2% BW i.p. saline injection for all animals. After a day with 1 hr water access, weighing, and 2% BW i.p. saline injections (day 11), mice received a fourth day of Kool-Aid access to second flavor (same as day 8) for 1 hr in both bottles followed by weighing and i.p. injection with either 2% BW of saline, 0.15 M LiCl, or $PYY_{3–36}$ at doses of 1.5 or 5 μg/100 g (day 12). Animals were allowed to recover for 48 hr by another day of 1 hr access to water, weighing, and 2% BW i.p. saline injections (day 13). On test day, each mouse was presented with both Kool-Aid flavors in random fashion, alternating bottle positions (e.g., left bottle grape versus right bottle grape), simultaneously, and the respective individual bottle/flavor consumption was measured for 1 hr. See Figure 4 for schematic representation of the conditioned taste aversion (CTA) protocol.

The CTA data was represented as raw data (Figure 5A) of the volume consumption by each animal (for the saline group, volume consumed from either right or left bottle and, for stimulus-paired groups, volume consumed of the saline-paired flavor [day 6 and day 10] versus $PYY_{3–36}$-paired or LiCl-paired flavor [day 8 and day 12]). Additionally, ratios of volume consumed (Figure 5B) were calculated as (volume bottle 1 or 2 consumed)/(total volume consumed) for saline controls or (volume of saline-paired flavor consumed)/(volume of stimulus-paired flavor consumed + volume of saline-paired flavor consumed) for experimental treatments. Even though individual C57BL/6J mice showed some flavor preference (for either grape or cherry), on average no preference was seen. On the test day, the saline-treated group of mice showed a significant preference for the cherry flavor due to two mice preferring the cherry flavor over the grape. However, when a metaanalysis was performed, the flavor preference did not affect other individual animals' preference for flavor in the other treatment groups.

Statistics

Statistical analyses were performed using PRISM (GraphPad Software, Inc., San Diego, CA). Data is expressed as mean ± SEM. For Figure 1, a repeated measures two-way ANOVA was used to determine the effect of time on drug treatment followed by an unpaired Student's (two-tailed) t test to determine significance at each time point. Significance was determined using

an unpaired Student's (two-tailed) t test Figures 2A and 5A. For Figure 2B, a repeated measures two-way ANOVA was used to assay the effects of time across the different treatment paradigms (surgery and drug) followed by one-way ANOVA with Bonferroni's tests posthoc to determine significance across individual treatments. One-way ANOVA with Dunnett's test posthoc was used to determine significance across treatments in Figures 3 and 5B. Significance was taken as $p < 0.05$.

Acknowledgments

We wish to thank Kate L.J. Ellacott, Ruth E. Thomas, and Christina Gremel for helpful discussion. POMC-EGFP mice were a kind gift from Dr. Malcolm Low (Oregon Health and Science University). Protocols for conditioned taste aversion were provided by Dr. Randy Seeley (University of Cincinnati). This work was supported by National Institutes of Health Grant DK070332 (to R.D.C.) and F31-NS048773-01 (to I.G.H). OHSU and R.D.C. have a financial interest in Orexigen, Inc.

Received: December 24, 2004
Revised: February 23, 2005
Accepted: February 25, 2005
Published: March 15, 2005

References

Adams, S.H., Won, W.B., Schonhoff, S.E., Leiter, A.B., and Paterniti, J.R., Jr. (2004). Effects of peptide YY[3–36] on short-term food intake in mice are not affected by prevailing plasma ghrelin levels. Endocrinology *145*, 4967–4975.

Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology *89*, 1070–1077.

Barrachina, M.D., Martinez, V., Wang, L., Wei, J.Y., and Tache, Y. (1997). Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. Proc. Natl. Acad. Sci. USA *94*, 10455–10460.

Batterham, R.L., Cowley, M.A., Small, C.J., Herzog, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A., et al. (2002). Gut hormone PYY(3–36) physiologically inhibits food intake. Nature *418*, 650–654.

Batterham, R.L., Cohen, M.A., Ellis, S.M., Le Roux, C.W., Withers, D.J., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2003). Inhibition of food intake in obese subjects by peptide YY3–36. N. Engl. J. Med. *349*, 941–948.

Bottcher, G., Alumets, J., Hakanson, R., and Sundler, F. (1986). Co-existence of glicentin and peptide YY in colorectal L-cells in cat and man. An electron microscopic study. Regul. Pept. *13*, 283–291.

Bottcher, G., Sjoberg, J., Ekman, R., Hakanson, R., and Sundler, F. (1993). Peptide YY in the mammalian pancreas: immunocytochemical localization and immunochemical characterization. Regul. Pept. *43*, 115–130.

Challis, B.G., Coll, A.P., Yeo, G.S., Pinnock, S.B., Dickson, S.L., Thresher, R.R., Dixon, J., Zahn, D., Rochford, J.J., White, A., et al. (2004). Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3–36). Proc. Natl. Acad. Sci. USA *101*, 4695–4700.

Chelikani, P.K., Haver, A.C., and Reidelberger, R.D. (2005). Intravenous infusion of peptide YY(3–36) potently inhibits food intake in rats. Endocrinology *146*, 879–888.

Chen, C.H., Rogers, R.C., and Stephens, R.L., Jr. (1996). Intracisternal injection of peptide YY inhibits gastric emptying in rats. Regul. Pept. *61*, 95–98.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480–484.

Cox, J.E., and Randich, A. (2004). Enhancement of feeding suppression by PYY(3–36) in rats with area postrema ablations. Peptides *25*, 985–989.

Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Niijima, A., Matsuo, H., Kangawa, K., and Nakazato, M. (2002). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology *123*, 1120–1128.

Deutsch, J.A., and Hardy, W.T. (1977). Cholecystokinin produces bait shyness in rats. Nature *266*, 196.

Elias, C.F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R.S., Bjorbaek, C., Flier, J.S., Saper, C.B., and Elmquist, J.K. (1999). Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron *23*, 775–786.

Ellacott, K.L., and Cone, R.D. (2004). The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. Recent Prog. Horm. Res. *59*, 395–408.

Emond, M., Schwartz, G.J., Ladenheim, E.E., and Moran, T.H. (1999). Central leptin modulates behavioral and neural responsivity to CCK. Am. J. Physiol. *276*, R1545–R1549.

Ervin, G.N., Mosher, J.T., Birkemo, L.S., and Johnson, M.F. (1995). Multiple, small doses of cholecystokinin octapeptide are more efficacious at inducing taste aversion conditioning than single, large doses. Peptides *16*, 539–545.

Fan, W., Ellacott, K.L., Halatchev, I.G., Takahashi, K., Yu, P., and Cone, R.D. (2004). Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. Nat. Neurosci. *7*, 335–336.

Grandt, D., Schimiczek, M., Beglinger, C., Layer, P., Goebell, H., Eysselein, V.E., and Reeve, J.R., Jr. (1994). Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1–36 and PYY 3–36. Regul. Pept. *51*, 151–159.

Halatchev, I.G., Ellacott, K.L., Fan, W., and Cone, R.D. (2004). Peptide YY3–36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology *145*, 2585–2590.

Harding, R.K., and McDonald, T.J. (1989). Identification and characterization of the emetic effects of peptide YY. Peptides *10*, 21–24.

Houpt, T.A., Berlin, R., and Smith, G.P. (1997). Subdiaphragmatic vagotomy does not attenuate c-Fos induction in the nucleus of the solitary tract after conditioned taste aversion expression. Brain Res. *747*, 85–91.

Jean, A. (1991). The nucleus tractus solitarius: neuroanatomic, neurochemical and functional aspects. Arch. Int. Physiol. Biochim. Biophys. *99*, A3–52.

Keire, D.A., Bowers, C.W., Solomon, T.E., and Reeve, J.R., Jr. (2002). Structure and receptor binding of PYY analogs. Peptides *23*, 305–321.

Koda, S., Date, Y., Murakami, N., Shimbara, T., Hanada, T., Toshinai, K., Niiji, A., Furuya, M., Inomata, N., Osuye, K., and Nakazato, M. (2005). The role of the vagal nerve in peripheral PYY3–36-induced feeding reduction in rats. Endocrinology *23*, 305–321. Published online February 17, 2005. 10.1210/en.2004-1266

Luckman, S.M. (1992). Fos-like immunoreactivity in the brainstems of the rat following peripheral administration of cholecystokinin. J. Neuroendocrinol. *4*, 149–152.

Lutz, T.A., Del Prete, E., and Scharrer, E. (1995). Subdiaphragmatic vagotomy does not influence the anorectic effect of amylin. Peptides *16*, 457–462.

Lutz, T.A., Mollet, A., Rushing, P.A., Riediger, T., and Scharrer, E. (2001). The anorectic effect of a chronic peripheral infusion of amylin is abolished in area postrema/nucleus of the solitary tract (AP/NTS) lesioned rats. Int. J. Obes. Relat. Metab. Disord. *25*, 1005–1011.

Martin, N.M., Small, C.J., Sajedi, A., Patterson, M., Ghatei, M.A., and Bloom, S.R. (2004). Pre-obese and obese agouti mice are sensitive to the anorectic effects of peptide YY(3–36) but resistant to ghrelin. Int. J. Obes. Relat. Metab. Disord. *28*, 886–893.

Masuda, M., Tomita, H., Okubo, K., and Miyasaka, K. (1994). Vagal efferent nerve-dependent inhibitory action of pancreatic polypeptide and peptide YY in conscious rats: comparison with somatostatin. J. Auton. Nerv. Syst. *50*, 131–138.

Matson, C.A., Wiater, M.F., Kuijper, J.L., and Weigle, D.S. (1997). Synergy between leptin and cholecystokinin (CCK) to control daily caloric intake. Peptides *18*, 1275–1278.

McLaughlin, C.L., and Baile, C.A. (1980). Decreased sensitivity of Zucker obese rats to the putative satiety agent cholecystokinin. Physiol. Behav. *25*, 543–548.

Miller, A.D., and Leslie, R.A. (1994). The area postrema and vomiting. Front. Neuroendocrinol. *15*, 301–320.

Moran, T.H., Baldessarini, A.R., Salorio, C.F., Lowery, T., and Schwartz, G.J. (1997). Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. Am. J. Physiol. *272*, R1245–R1251.

Moran, T.H., Smedh, U., Kinzig, K.P., Scott, K.A., Knipp, S., and Ladenheim, E.E. (2005). Peptide YY(3–36) inhibits gastric emptying and produces acute reductions in food intake in rhesus monkeys. Am. J. Physiol. Regul. Integr. Comp. Physiol. *288*, R384–R388.

Niederau, C., Meereis-Schwanke, K., Klonowski-Stumpe, H., and Herberg, L. (1997). CCK-resistance in Zucker obese versus lean rats. Regul. Pept. *70*, 97–104.

Perry, M.R., Rhee, J., and Smith, W.L. (1994). Plasma levels of peptide YY correlate with cisplatin-induced emesis in dogs. J. Pharm. Pharmacol. *46*, 553–557.

Pittner, R.A., Moore, C.X., Bhavsar, S.P., Gedulin, B.R., Smith, P.A., Jodka, C.M., Parkes, D.G., Paterniti, J.R., Srivastava, V.P., and Young, A.A. (2004). Effects of PYY[3–36] in rodent models of diabetes and obesity. Int. J. Obes. Relat. Metab. Disord. *28*, 963–971.

Reidelberger, R.D. (1992). Abdominal vagal mediation of the satiety effects of exogenous and endogenous cholecystokinin in rats. Am. J. Physiol. *263*, R1354–R1358.

Riediger, T., Schmid, H.A., Lutz, T.A., and Simon, E. (2002). Amylin and glucose co-activate area postrema neurons of the rat. Neurosci. Lett. *328*, 121–124.

Ritter, R.C., and Ladenheim, E.E. (1985). Capsaicin pretreatment attenuates suppression of food intake by cholecystokinin. Am. J. Physiol. *248*, R501–R504.

Schafe, G.E., Seeley, R.J., and Bernstein, I.L. (1995). Forebrain contribution to the induction of a cellular correlate of conditioned taste aversion in the nucleus of the solitary tract. J. Neurosci. *15*, 6789–6796.

Smith, G.P., Jerome, C., and Norgren, R. (1985). Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats. Am. J. Physiol. *249*, R638–R641.

Takeno, R., Okimura, Y., Iguchi, G., Kishimoto, M., Kudo, T., Takahashi, K., Takahashi, Y., Kaji, H., Ohno, M., Ikuta, H., et al. (2004). Intravenous administration of ghrelin stimulates growth hormone secretion in vagotomized patients as well as normal subjects. Eur. J. Endocrinol. *151*, 447–450.

Tschop, M., Castaneda, T.R., Joost, H.G., Thone-Reineke, C., Ortmann, S., Klaus, S., Hagan, M.M., Chandler, P.C., Oswald, K.D., Benoit, S.C., et al. (2004). Physiology: does gut hormone PYY3–36 decrease food intake in rodents? Nature *430*, 1 p following 165–discussion 162 p following 165.

Verbalis, J.G., McCann, M.J., McHale, C.M., and Stricker, E.M. (1986). Oxytocin secretion in response to cholecystokinin and food: differentiation of nausea from satiety. Science *232*, 1417–1419.

Wang, L., Barachina, M.D., Martinez, V., Wei, J.Y., and Tache, Y. (2000). Synergistic interaction between CCK and leptin to regulate food intake. Regul. Pept. *92*, 79–85.

Zhang, T., Uchida, T., Gomez, G., Lluis, F., Thompson, J.C., and Greeley, G.H., Jr. (1993). Neural regulation of peptide YY secretion. Regul. Pept. *48*, 321–328.