0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(12):6054–6061

doi: 10.1210/en.2007-0898

Combination Therapy with Amylin and Peptide YY[3–36] in Obese Rodents: Anorexigenic Synergy and Weight Loss Additivity

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Circulating levels of the pancreatic β-cell peptide hormone amylin and the gut peptide PYY[3–36] increase after nutrient ingestion. Both have been implicated as short-term signals of meal termination with anorexigenic and weight-reducing effects. However, their combined effects are unknown. We report that the combination of amylin and PYY[3–36] elicited greater anorexigenic and weight-reducing effects than either peptide alone. In high-fat-fed rats, a single ip injection of amylin (10 μg/kg) plus PYY[3–36] (1000 μg/kg) reduced food intake for 24 h ($P < 0.05$ *vs.* vehicle), whereas the anorexigenic effects of either PYY[3–36] or amylin alone began to diminish 6 h after injection. These anorexigenic effects were dissociable from changes in locomotor activity. Subcutaneous infusion of amylin plus PYY[3–36] for 14 d suppressed food intake and body weight to a greater extent than either agent alone in both rat and mouse diet-induced obesity (DIO) models ($P < 0.05$). In DIO-prone rats, 24-h metabolic rate was maintained despite weight loss, and amylin plus PYY[3–36] (but not monotherapy) increased 24-h fat oxidation ($P < 0.05$ *vs.* vehicle). Finally, a 4 × 3 factorial design was used to formally describe the interaction between amylin and PYY[3–36]. DIO-prone rats were treated with amylin (0, 4, 20, and 100 μg/kg·d) and PYY[3–36] (0, 200, 400 μg/kg·d) alone and in combination for 14 d. Statistical analyses revealed that food intake suppression with amylin plus PYY[3–36] treatment was synergistic, whereas body weight reduction was additive. Collectively, these observations highlight the importance of studying peptide hormones in combination and suggest that integrated neurohormonal approaches may hold promise as treatments for obesity. (*Endocrinology* 148: 6054–6061, 2007)

THE INCREASING PREVALENCE of obesity in recent years has led to an expanded interest in the mechanisms regulating appetite control and long-term energy homeostasis. The central nervous system receives, integrates, and responds to many signals from the periphery and is recognized as the key regulator of balance between food intake and energy expenditure (EE). Among the signals implicated in the inhibition of food intake are the neurohormones amylin and peptide YY (PYY).

Amylin is a 37-amino-acid peptide hormone produced in pancreatic β-cells and cosecreted with insulin in response to nutrient ingestion. Plasma amylin concentrations rise rapidly in response to meals, peak within 60 min, and remain elevated for up to 4 h (1). Once in the periphery, amylin binds with high affinity to receptors in the central nervous system, particularly within the area postrema (AP) (2–4). Peripheral administration of amylin to lean or diet-induced obese (DIO) rats decreased food intake and weight (2, 5–7). In DIO rats, pair-feeding studies revealed that whereas food intake reduction was the predominant mode of action for overall weight loss, weight loss in amylin-treated rats was entirely attributable to a reduction in fat mass, with relative preservation of lean mass. In contrast, pair-fed control animals experienced reductions in both fat and lean body mass. Importantly, amylin-induced weight loss was not associated with counterregulatory decreases in EE (7). Several of these findings in rodents have been mirrored in various clinical studies. Pramlintide, a synthetic analog of human amylin, has been shown to increase satiety, resulting in decreased 24-h food intake and weight loss in obese subjects (8–11). Randomized, double-blind, phase 2 studies in obese subjects have demonstrated placebo-corrected weight loss of 3.1% over 16 wk (12) and up to 6–7% over 1 yr with pramlintide monotherapy (13).

PYY is a 36-amino-acid peptide hormone that is secreted from intestinal L-cells after a meal and cleaved by dipeptidyl peptidase-IV (DPP-IV) to generate PYY[3–36], the major circulating form (14). After nutrient ingestion, plasma PYY[3–36] levels increase within 15 min, reach a peak at approximately 90 min, and remain elevated for up to 6 h (15). PYY[3–36] levels reflect meal size and content, with fat being the most potent stimulator of PYY secretion (16). PYY[3–36] has been suggested to bind to Y2 receptors expressed by neurons within the hypothalamic arcuate nucleus (17). Experiments in rodents have shown that PYY[3–36] inhibits food intake, reduces body weight gain, and increases utilization of fat stores for energy (17, 18). Obese humans may have lower levels of PYY than lean subjects, and PYY levels have been shown to be inversely correlated with body mass index (19). Infusion of PYY[3–36] into obese (19) and lean (19, 20) subjects reduced caloric intake, and a single sc injection of PYY[3–36] resulted in a trend toward increased hunger suppression and satiety quotient after a meal (21, 22). However,

First Published Online August 30, 2007

Abbreviations: AP, Area postrema; DIO, diet-induced obese; EE, energy expenditure; HSD, Harlan Sprague Dawley; PYY, peptide YY; RQ, respiratory quotient; RSM, response surface methodology.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

a phase 2 randomized, placebo-controlled study found that nasally administered PYY[3–36] did not significantly reduce weight, compared with placebo, after 12 wk of treatment (23).

The full potential of peptide hormone therapeutics for obesity treatment may not be realized if each neurohormone is evaluated only in isolation. It is conceivable that greater weight loss could be achieved with combinatorial regimens of neurohormones, especially with those that have complementary mechanisms of action and naturally occurring synergies. Furthermore, the metabolic counterregulatory adaptations (increased appetite, decline in metabolic rate, reduced thyroid hormone levels, and increased muscle efficiency) elicited by reduced caloric intake and body weight (24) may be easier to override with combination therapies. We hypothesized that combination therapy with amylin and PYY[3–36] would elicit additive, and possibly synergistic, anorexigenic and weight-lowering effects. We report our findings on the interactive effects of amylin and PYY[3–36] in multiple rodent models.

Materials and Methods

Animals, housing, diet, and drug

All studies were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, Inc., in accordance with Animal Welfare Act guidelines. Animals were housed individually in standard caging at 22 C in a 12-h light (lights on at 0500 h), 12-h dark (lights off at 1700 h) cycle. Studies in rats were conducted using either male Harlan Sprague Dawley (HSD) rats (24-h feeding, locomotor activity) or male DIO-prone rats (chronic drug infusion). HSD rats were obtained from Harlan (Indianapolis, IN) and maintained on standard chow (7012; Harlan Teklad, Madison, WI). For 24-h feeding studies, HSD rats were fed a moderately high-fat diet (32% kcal from fat, D12266B; Research Diets, New Brunswick, NJ) for 10 d before study initiation. DIO-prone rats were adult male Levin rats obtained from Charles River Laboratories (Wilmington, MA). These rats were originally developed from a line of Crl:CD(SD)BR rats that are prone to becoming obese on a diet relatively high in fat and energy (25). DIO-prone rats were fattened *ad libitum* on a moderately high-fat diet (32% kcal from fat, D12266B; Research Diets) for 6 wk before and throughout drug treatment. DIO mice were adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) fattened on a high-fat diet (58% kcal from fat, D12331; Research Diets) for 4 wk before and during drug treatment. All studies used rat amylin and human PYY[3–36] obtained from Peptisynthia (Torrance, CA).

Twenty-four-hour food intake monitoring

HSD rats were habituated to the testing chamber, food hopper, and diet for 10 d before the study. Testing chambers (10.5 × 19 × 8 in., BioDAQ Food Intake Monitor; Research Diets) were equipped with a tunnel containing a food hopper at the end. Rats received daily ip injections of vehicle (10% dimethylsulfoxide in sterile water) before lights off on d 8, 9, and 10 of habituation. After habituation, rats (initial body weight was ~441 g at time 0) were divided into treatment groups (seven to eight rats per group) of equal food consumption. On test day, rats received a single ip injection of vehicle or peptide (10 μg/kg amylin and/or 1000 μg/kg PYY[3–36]) 15 min before lights off and were placed immediately back into the BioDAQ Food Intake Monitor cages. These doses were chosen because previous data showed near-maximal anorexigenic effects in this model (data not shown). Food hoppers were weighed automatically every 5 sec throughout the experiment. Cumulative food intake (in grams) was monitored for 24 h.

Locomotor activity

To examine the acute effects of amylin and PYY[3–36] on locomotor activity, HSD rats (six rats per group) received a single ip injection of vehicle or peptide (10 μg/kg amylin and/or 1000 μg/kg PYY[3–36]) just before lights out. Immediately after injection, rats were placed into individual cages within SmartFrame Cage Racks (Kinder Scientific, San Diego, CA) with *ad libitum* access to food and water and were undisturbed for 24 h. This cage system measures locomotor activity via photo beam breaks. Total zone distance (meters) was recorded for 12-h intervals. MotorMonitor software (Kinder Scientific) was used to analyze the data.

DIO mouse and rat: single-dose combinations

For chronic infusion studies, each drug (or vehicle) was delivered by a separate surgically implanted sc osmotic minipump (Durect Corp., Cupertino, CA) containing either drug or vehicle (50% dimethylsulfoxide in sterile water). DIO mice (11–14 mice per group) were weight-matched into treatment groups (initial body weight was ~27 g on d 0) and implanted with minipumps containing vehicle, 300 μg/kg·d amylin, 1000 μg/kg·d PYY[3–36], or amylin plus PYY[3–36]. Similarly, DIO-prone rats (five rats per group) were weight-matched into treatment groups (initial body weight was ~500 g on d 0) and implanted with minipumps containing vehicle, 100 μg/kg·d amylin, 200 μg/kg·d PYY[3–36], or amylin plus PYY[3–36]. Thus, both studies had a total of four treatment groups: vehicle plus vehicle, vehicle plus amylin, vehicle plus PYY[3–36], or PYY[3–36] plus amylin. Because the weight-loss efficacy of amylin and PYY[3–36] varies somewhat across DIO mice and DIO-prone rats (unpublished observations), a higher dose range was tested in mice. Body weight [expressed as percent change (vehicle-corrected)] and cumulative food intake [expressed as percent change [vehicle-corrected)] were measured weekly for 2 wk. At termination, plasma from DIO-prone rats that were fasted for approximately 3 h (postabsorptive; ~0800 h) before plasma collection were assayed for amylin and PYY[3–36] by ELISA (Millipore, Billerica, MA). Amylin and PYY[3–36] concentrations are expressed as mean ± SE.

Indirect calorimetry

On d 4 of treatment, DIO-prone rats (five rats per group) were placed in an indirect calorimeter (Oxymax Equal Flow System; Columbus Instruments, Columbus, OH). Rats were habituated in the Oxymax cages for 24 h before testing. Measurements were taken over a 24-h period during which the animals had *ad libitum* access to food and water. Respiratory quotient (RQ) and EE were calculated and averaged across the 24-h measurement session.

DIO-prone rat: response surface methodology (RSM)

In an independent group of animals, DIO-prone rats were weight-matched (initial body weight was ~500 g on d 0) into one of the 12 groups (five rats per group) resulting from a full factorial design using four doses of amylin (0, 4, 20, and 100 μg/kg·d) and three doses of PYY[3–36] (0, 200, and 400 μg/kg·d) for a total of 60 rats. Body weight and cumulative food intake were measured weekly for 2 wk.

Statistical analyses

Treatment differences for locomotor activity, RQ, EE, 24-h feeding, and vehicle-corrected changes from baseline in food intake and body weight were analyzed using one-way ANOVA followed by contrasts. Due to the small sample size, multiple comparisons were not performed. Statistical analyses were done in SAS version 8.2 (SAS Institute, Inc., Cary, NC) or Prism 4 for Windows (GraphPad Software, San Diego, CA). Graphs were generated using Prism 4 for Windows. Data are presented as mean ± SE.

For RSM, a first-order plus interaction model was fit to the observed percent vehicle-corrected body weight. The model contained effects for the intercept, linear effect of amylin, linear effect of PYY[3–36], and linear interaction effect for the amylin plus PYY[3–36] combination. To improve the fit of the statistical model, the doses of amylin were expressed as the base 10 logarithm, where 1 was added to avoid problems with the zero dose [*i.e.* dose amylin = $\log_{10}$(dose amylin + 1)]. A reduced second-order model was fit to the observed percent vehicle-corrected food intake to improve the fit. This model contained effects for the intercept, linear, and quadratic effects of amylin, linear effect of PYY[3–36], and

linear interaction effect for the amylin plus PYY[3–36] combination. The doses of amylin were again expressed using the base 10 logarithm modified for the zero dose. Lack of fit for the model was tested by the usual F test derived by partitioning the error sum of squares into pure error and lack of fit. Using this lack of fit test, other quadratic and higher-order effects were not necessary and were excluded from the model.

Using these models, a response surface was created to predict weight loss and food intake over the entire dose region. A formal test for synergy or antagonism was performed by comparing the *P* value for the interaction term of each model to 0.05. A significant interaction indicates that the slope of the response surface changes as the combination doses are increased. The sign of the interaction term coefficient is indicative of the type of departure from additivity; a negative coefficient represents synergistic reductions in body weight or food intake. *P* values ≥ 0.05 suggest additivity. For first-order models, the *P* value from the interaction term has been shown (26) to be the correct *P* value for testing departures from additivity using Berenbaum's interaction index derived from isobolograms (27), a widely accepted method of rigorously evaluating combinatorial relationships.

Results

Acute administration of amylin and PYY[3–36] in high-fat-fed HSD rats: 24-h food intake and locomotor activity

To determine whether amylin and PYY[3–36] have complementary effects on suppression of 24-h food intake, nondeprived high-fat-fed HSD rats were given a single administration of vehicle, amylin (10 μg/kg), and/or PYY[3–36] (1000 μg/kg) (Fig. 1A). As expected, food consumption increased progressively throughout the dark cycle in all treatment groups, whereas the rate of food intake tapered off during the light cycle. Cumulative food intake was suppressed by individual administration of amylin or PYY[3–36] (both $P < 0.05$ *vs.* vehicle at 1, 2, 3, and 5 h). The combination exerted longer-lasting effects, with food intake reduction still evident 24 h after administration ($P < 0.05$ *vs.* vehicle at all time points except 11 h).

To ascertain whether or not the decreases in food intake could be attributed to changes in activity, a separate group of HSD rats received the same doses of amylin, PYY[3–36], or amylin plus PYY[3–36], and locomotor activity was monitored for 24 h. There were no significant differences compared with vehicle, suggesting that locomotor activity was unchanged with treatment (Fig. 1B).

Chronic administration of amylin and PYY[3–36] in DIO mice and DIO-prone rats

The observation that acute administration of amylin plus PYY[3–36] was anorexigenic at 24 h after administration led us to test the durability and potential weight-reducing effects of the amylin plus PYY[3–36] combination using continuous drug administration. After 2 wk of therapy in DIO mice, cumulative food intake (vehicle corrected) was reduced by 3.5 ± 1.5%, 9.7 ± 0.9% ($P < 0.05$), and 11.4 ± 1.9% ($P < 0.05$) in amylin, PYY[3–36] and combination-treated groups, respectively (Fig. 2A). Similarly, body weight (vehicle corrected) was reduced in all treatment groups: amylin −3.1 ± 0.7% ($P < 0.05$), PYY[3–36] −6.1 ± 0.9% ($P < 0.05$), and combination −9.2 ± 0.9% ($P < 0.05$) (Fig. 2B). Combination therapy also significantly reduced body weight compared with either monotherapy at 2 wk ($P < 0.05$).

The anorexigenic and weight-lowering effects of amylin plus PYY[3–36] were also more effective than monotherapy in DIO-prone rats. Although amylin and PYY[3–36] individually reduced food intake (−26.8 ± 2.3 and −13.0 ± 2.5%, $P < 0.05$ *vs.* vehicle; Fig. 3A) and body weight (−6.8 ± 0.7 and −3.2 ± 0.6%, $P < 0.05$ *vs.* vehicle; Fig. 3B) at 2 wk, combination therapy reduced food intake by 40.5 ± 3.0% ($P < 0.05$ *vs.* vehicle) and body weight by 9.8 ± 0.6% ($P < 0.05$ *vs.* vehicle). Reductions with amylin plus PYY[3–36] were significantly greater than those achieved with either monotherapy ($P < 0.05$). In addition, the effect of combination therapy on food intake and body weight was similar to the sum of the monotherapies, suggesting that the two peptides combine additively at these doses.

Plasma PYY[3–36] levels were elevated to a similar extent in PYY[3–36]-treated (3013 ± 825 pM) and amylin plus PYY[3–36]-treated DIO-prone rats (2119 ± 275 pM) compared with vehicle-treated (14.2 ± 2.0 pM) and amylin-treated rats (12.6 ± 1.2 pM). Likewise, plasma amylin levels were elevated in amylin-treated (891 ± 79 pM) and amylin plus PYY[3–36]-treated rats (582 ± 36 pM) compared with vehicle-treated (21 ± 7.0 pM) and PYY[3–36]-treated rats (12.6 ± 0.2 pM).

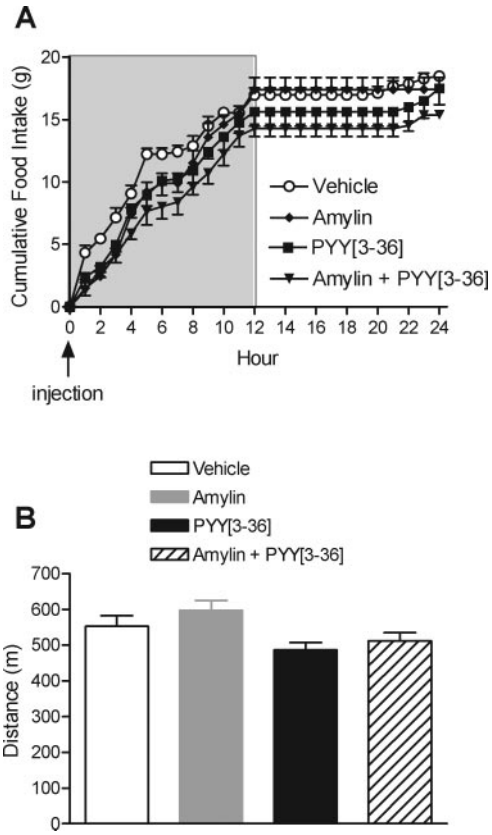

FIG. 1. Effects of acute amylin (10 μg/kg) and PYY[3–36] (1000 μg/kg) monotherapy and combination therapy on 24-h food intake and locomotor activity in HSD rats. A, Vehicle, amylin, PYY[3–36], or amylin plus PYY[3–36] were injected ip at the start of the experiment, 15 min before lights out (0–12 h, designated by *shaded rectangle*). Cumulative food intake data were collected over 24 h in specialized feeding chambers and are displayed as mean ± SE. B, Vehicle, amylin, PYY[3–36], or amylin plus PYY[3–36] were injected ip at the start of the experiment just before lights out, and locomotor activity was measured over 12 h. Data are displayed as mean ± SE.

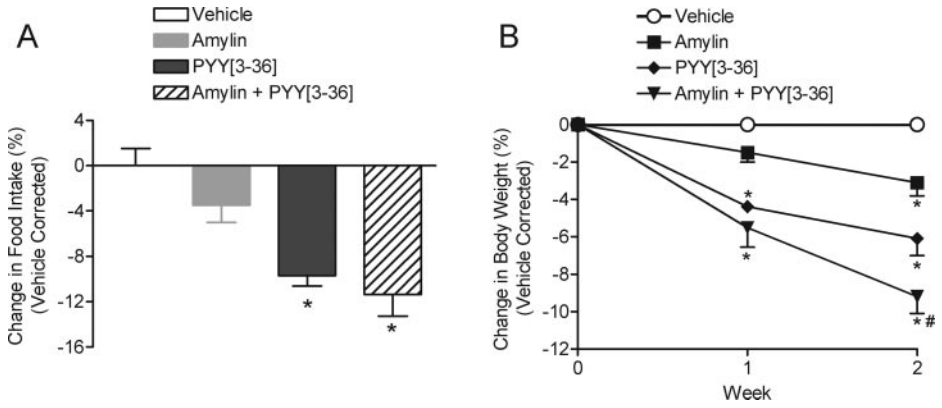

FIG. 2. Effects of chronic amylin (300 μg/kg·d) and PYY[3–36] (1000 μg/kg·d) monotherapy and combination therapy on food intake and body weight in DIO mice. A, Change in cumulative food intake (vehicle-corrected; mean ± SE) after 2 wk of treatment; B, change in body weight (vehicle-corrected; mean ± SE) after 1 and 2 wk of treatment. *, $P < 0.05$ *vs.* vehicle; #, $P < 0.05$ *vs.* monotherapies.

Indirect calorimetry in DIO rats treated with amylin and PYY[3–36] combination therapy

Indirect calorimetry was performed in DIO-prone rats after the first week of treatment to test whether weight changes observed with monotherapy and/or combination therapy were associated with increased fatty acid oxidation (RQ) and/or changes in EE (Table 1). RQ was significantly reduced compared with vehicle in rats treated with combination therapy ($P < 0.05$) but not in rats treated with amylin or PYY[3–36] alone. EE was unchanged across treatment groups.

Response surface analysis of amylin and PYY[3–36] combination therapy

Although these findings suggest mathematical additivity between amylin and PYY[3–36], the inherent limitation of single-dose studies precludes a broad claim for pharmacological additivity. To formally evaluate mathematical additivity over a wide range of doses, RSM was used. RSM is an efficient method of exploring responses for combination drug therapy and has been widely applied in the development of efficacious mixtures of anesthetics (28) as well as in the identification of polytherapies for HIV (29), hypertension (30), and cancer (31). *P* values generated using RSM indicate additivity if $P \geq 0.05$ and synergy if $P < 0.05$. In addition to formally testing for antagonism or synergy, RSM can be used to predict the response over an entire combination dose region. Using this predictive model, optimal dose combinations for a specific response can be easily identified. DIO-prone rats were treated for 2 wk with a range of doses of amylin (0, 4, 20, and 100 μg/kg·d) and PYY[3–36] (0, 200, and 400 μg/kg·d). At wk 2, RSM analysis of cumulative food intake (Fig. 4) showed a statistically significant dose response for both amylin ($P = 0.011$) and PYY[3–36] ($P = 0.0004$), and cumulative food intake was synergistically reduced ($P = 0.022$) across treatment combinations by up to 54.7 ± 1.1%. RSM analysis of body weight (Fig. 5) showed a statistically significant linear dose response for both amylin ($P < 0.0001$) and PYY[3–36] ($P = 0.0003$). Body weight was additively reduced ($P = 0.27$) across treatment combinations by up to 15.6 ± 0.9%. There was no statistically significant lack of fit for either model ($P = 0.69$ for food intake and $P = 0.44$ for body weight).

Discussion

The aforementioned studies demonstrated that the combination of amylin and PYY[3–36] 1) elicited a greater reduction in 24-h food intake after a single acute injection than either monotherapy without significantly changing locomotor activity, 2) reduced food intake and body weight in two species, and 3) synergistically reduced food intake and additively reduced body weight in DIO-prone rats. These findings provide important new insights into the interactions of amylin and PYY[3–36] and demonstrate that their combined effects may be useful in the treatment of obesity.

Several groups have described the anorexigenic effects of a single peripheral injection of either amylin (32) or PYY[3–36] (33) on 24-h food intake. Similar to the results of the current study, the anorexigenic effects of individually administered amylin and PYY[3–36] were most evident during the first few hours after injection. Food intake rebounded after the initial hypophagia, most likely due to the relatively

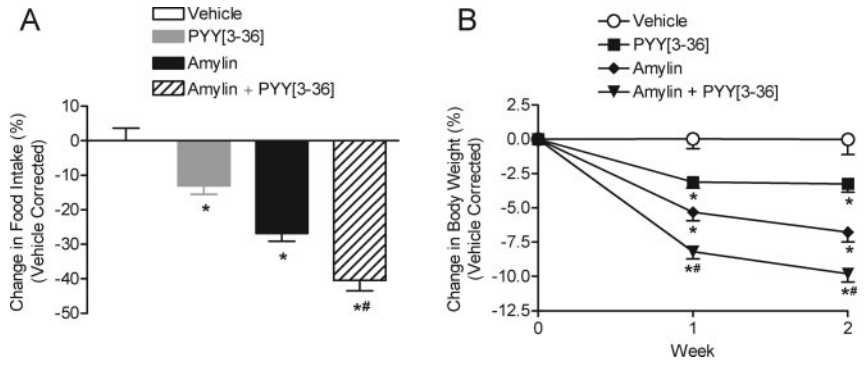

FIG. 3. Effects of single-dose chronic amylin (100 μg/kg·d) and PYY[3–36] (200 μg/kg·d) monotherapy and combination therapy on food intake and body weight in DIO-prone rats. A, Change in cumulative food intake (vehicle-corrected; mean ± SE) after 2 wk of treatment; B, change in body weight (vehicle-corrected; mean ± SE) after 1 and 2 wk of treatment. *, $P < 0.05$ *vs.* vehicle; #, $P < 0.05$ *vs.* monotherapies.

TABLE 1. Changes in RQ and EE after the first week of treatment in DIO-prone rats

Group	RQ	EE (kcal/h·kg)
Vehicle	0.85 ± 0.02	6.32 ± 0.28
PYY[3–36] (200 μg/kg·d)	0.86 ± 0.01	6.34 ± 0.13
Amylin (100 μg/kg·d)	0.83 ± 0.01	6.45 ± 0.25
Amylin + PYY[3–36]	0.80 ± 0.01[a]	6.09 ± 0.25

Data were averaged across 24 h (mean ± SE).
[a] $P < 0.05$ *vs.* vehicle control.

short half-lives of these peptides and/or to compensatory responses related to energy balance (34). For example, decreased food intake can result in a net reduction in both gastric distention and secretion of nutrient-stimulated satiety signals (35), which could in turn prevent the full manifestation of the anorexigenic properties of an individually administered peptide. Pharmacologically replacing these neurohormonal signals through the use of peptide combinations represents one potential approach to overcoming such compensatory mechanisms and may have contributed to the more robust, sustained reduction in food intake. For example, in the current study, the combination of amylin plus PYY[3–36] maintained an anorexigenic effect for the entire 24-h study period. The increased effect observed with amylin plus PYY[3–36] is consistent with an emerging literature regarding the acute synergy of pancreatic, intestinal, and adipose signals (33, 36–41).

Although our initial finding of an additive effect of amylin and PYY[3–36] on short-term food intake in normal rats was encouraging, it was important to demonstrate the durability of the effect and determine whether or not the peptide combination could elicit meaningful weight loss in rodent models of obesity. Therefore, DIO mice and DIO-prone rats were treated with amylin and/or PYY[3–36] via continuous infusion for 14 d. In both DIO models, food intake and body weight were reduced to a greater extent by the combination relative to either monotherapy. Previous studies indicated that monotherapy with either amylin or PYY[3–36] was associated with a decrease in RQ and a maintenance of metabolic rate in the face of decreased caloric intake and body weight loss (7, 42). Adding to these previous findings, our experiments showed that metabolic rate was maintained not only during amylin or PYY[3–36] monotherapy but also when both peptides were given in combination. Additionally, RQ was reduced relative to vehicle in DIO-prone rats treated with amylin plus PYY[3–36], but not monotherapy, suggesting an increase in fat oxidation with this treatment regimen. Of note, relative to the rats, the mice showed a less robust anorexigenic response to the combination of amylin and PYY[3–36] with a similar percent reduction in body weight. Although indirect calorimetry was not captured in these animals, these results suggest that mice may be more sensitive than rats to the metabolic effects of the combination of amylin plus PYY[3–36].

Having established in two DIO models that a combinatorial regimen with amylin plus PYY[3–36] elicited greater weight loss than was achievable with either peptide alone (at the doses tested), our next set of experiments sought to formally describe the interaction between the two peptide hormones. Given the complexity of the neurohormonal systems contributing to the regulation of food intake and body weight, it is difficult to capture the extent/efficacy of drug interactions using single-dose methodology. Using RSM (3 × 4 factorial design), we demonstrated that amylin and PYY[3–36] interacted in a synergistic manner to reduce food intake in DIO-prone rats, whereas body weight was reduced in an additive manner. This discordance between synergistic reduction of food intake and additive weight-loss suggests that some counterregulation was evident even with combination therapy. For example, at the time point queried, EE was not maintained as well with combination therapy as it was with vehicle control. Additional counterregulatory adaptations that could also explain the discordance include reduced chronic activity levels, increased skeletal muscle work efficiency, increased fat absorption (reduced fecal energy loss), decreased sympathetic nervous system tone, and changes in the thyroid axis (24, 43). These mechanisms should be evaluated in future studies. However, the fact that combination therapy reduced weight to a greater extent than either PYY[3–36] or amylin alone indicates that combination ther-

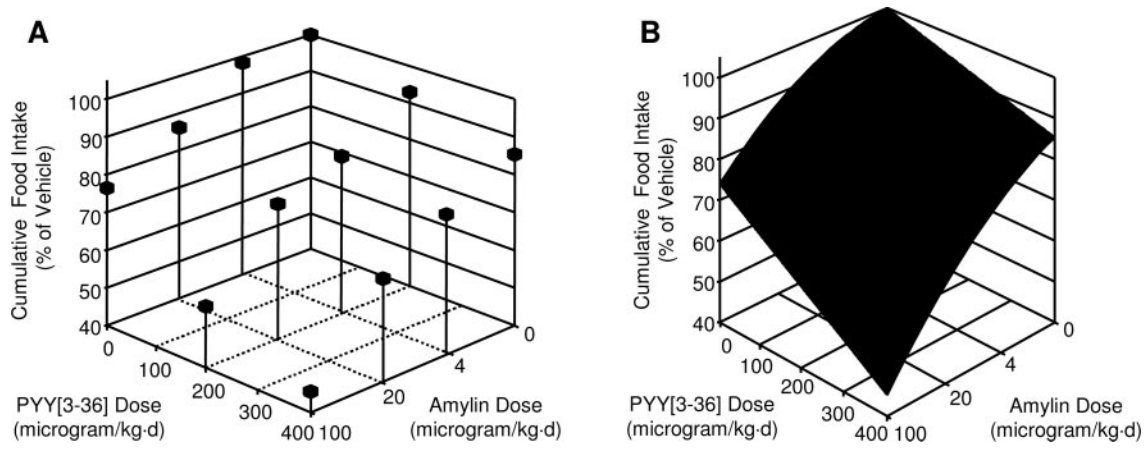

FIG. 4. Response surface analysis of the effects of amylin and PYY[3–36] on food intake in DIO-prone rats. A, Three-dimensional plot depicting mean changes in food intake (vehicle-corrected) for amylin (0, 4, 20, and 100 μg/kg·d) and PYY[3–36] (0, 200, and 400 μg/kg·d), alone and in combination; B, predicted response surface for mean change in food intake (vehicle corrected).

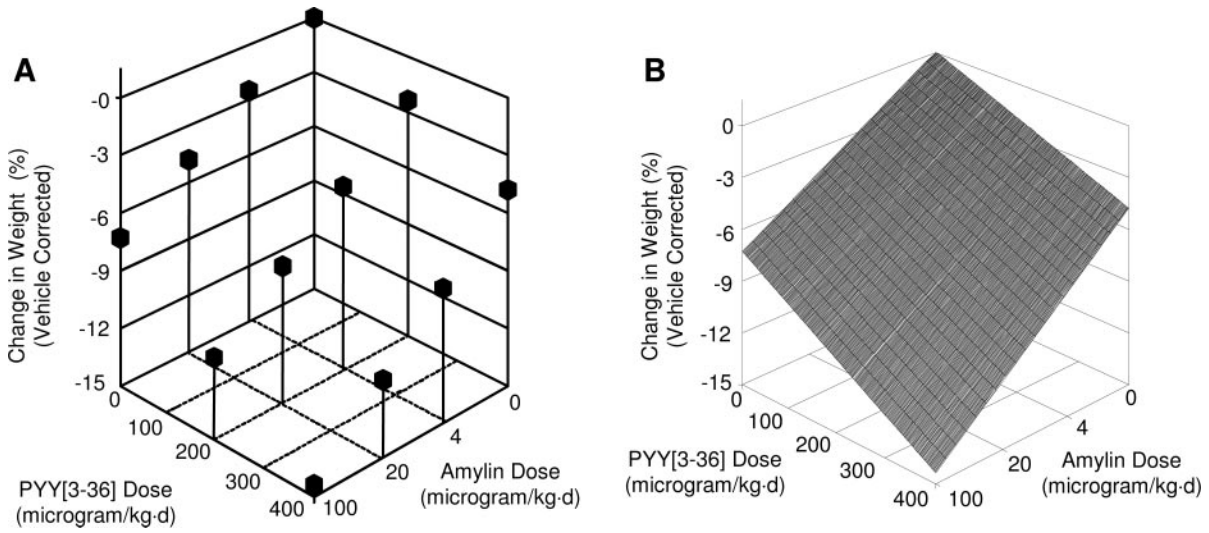

FIG. 5. Response surface analysis for the effects of amylin and PYY[3–36] on body weight in DIO-prone rats. A, Three-dimensional plot depicting mean changes in body weight (vehicle corrected) for amylin and PYY[3–36], alone and in combination; B, predicted response surface for mean change in body weight (vehicle corrected).

apies are a promising avenue for the development of neurohormones as potential antiobesity drugs.

With both monotherapy and combination therapy, it is important to consider the contribution of malaise or competing behaviors to the observed reduction in food intake and body weight. Typically, potential nonhomeostatic effects of compounds (*e.g.* conditioned taste aversion, locomotor effects, and pica) are evaluated in acute preclinical models. Because there are also clinical data regarding the tolerability and weight-reducing effects of the amylin analog pramlintide and the anorexigenic properties of PYY[3–36], it is now possible to draw parallels between these observations. Findings from several studies clearly suggest that acute amylin administration in rodents is not associated with malaise (conditioned taste aversion) or competing locomotor behaviors (44–47). Consistent with preclinical models, the effects of pramlintide on food intake and body weight were dissociable from the occurrence of nausea. Approximately 76% of patients who received pramlintide achieved a statistically significant reduction in body weight and did not report any malaise (11). Findings with PYY[3–36] in rodents are less clear, with some reports suggesting that PYY[3–36] supports taste aversion learning (48, 49), whereas other groups have not been able to confirm this observation (37, 50). PYY[3–36] administration decreased energy intake in lean and obese humans (after iv infusion) without incidence of nausea (17, 19), or with a range of nausea, fullness, and abdominal discomfort depending on the route of administration (iv, sc, or intranasal) (20, 23, 51, 52). In the present studies, the acute effects of a single-dose combination of amylin and PYY[3–36] on 24-h food intake, alone and in combination, were shown to be dissociable from any effects on locomotor activity in rats. With sustained administration, our plasma data (in rats) indicate that the effects on food intake and body weight were clearly achieved at pharmacological levels of each compound (~40- and ~200-fold above endogenous levels of amylin and PYY[3–36], respectively). Ongoing clinical research studies assessing the safety and tolerability of pramlintide and PYY[3–36] will hopefully define the therapeutic window for decreasing food intake and reducing body weight without unwanted side effects. The observation that additivity/synergy was achieved across a wide range of dose combinations in DIO rats suggests that it may be possible to select an optimally tolerated combination while retaining the desired effects on food intake and body weight.

Although the neural substrates mediating the observed anorexigenic synergy and weight loss additivity between amylin and PYY[3–36] were not measured in the present studies, potential neuroanatomical bases for convergence of their signaling pathways exist. Both amylin (53) and PYY[3–36] (54) bind receptors located within the AP in the caudal brainstem. Amylin's anorexigenic properties are attributable to its binding in the AP and subsequent activation of the nuclei tractus solitarii, lateral parabrachial nucleus, and the central nucleus of the amygdala (55, 56). PYY[3–36] mediates its effects on gastrointestinal motility through brainstem Y2 receptors (57, 58). However, unlike amylin, animals with lesions of the AP are still responsive to the anorexigenic effects of peripherally administered PYY[3–36] (59). The anorexigenic effects of PYY[3–36] are thought to be mediated through Y2 receptors located on neuropeptide Y neurons within the arcuate nucleus neurons of the hypothalamus (17). Interactions between amylin and PYY[3–36] may also take place via indirect projection pathways. For example, amylin-specific effects within the AP may influence upstream hypothalamic signaling. Amylin inhibited fasting-induced neuronal activation within the lateral hypothalamic area and reduced mRNA levels of orexin (56, 60), a food intake-stimulating peptide. Sustained administration of amylin (but not pair feeding) increased arcuate mRNA levels of proopiomelanocortin (7), a peptide linked to the anorexigenic actions of PYY[3–36] (61). However, because the acute anorexigenic effects of amylin are retained in agouti mice (7) and the acute effects of PYY[3–36] are retained in mice lacking proopiomelanocortin (62), intact melanocortinergic signaling may not be required for the expression of these effects. Studies

examining the interconnecting neuronal pathways regulating food intake and body weight will be useful in identifying other naturally occurring neurohormonal synergies.

Food intake and/or body weight have been successfully reduced after peripheral administration of other peptide/protein combinations. For example, PYY[3–36] and glucagon-like peptide-1[7–36] additively reduced food intake in both lean and obese mice as well as in lean human volunteers (33). Combination therapy with PYY[3–36] and the glucagon-like peptide-1 receptor agonist exendin-4 over a 2-wk time period resulted in synergistic reductions in food intake (37). More recently, we showed that conjunctive treatment with amylin and leptin resulted in synergistic reductions in both food intake and body weight in DIO-prone rats (40). Additionally, calorie restriction (by pair feeding to amylin) alone did not reduce weight to the same extent as amylin plus leptin treatment, suggesting that weight loss synergy was at least partially dissociable from amylin's anorexigenic effects (40).

Collectively, the results of these studies highlight the importance of studying neurohormones for potential interactions and suggest that combinatorial neurohormonal approaches may offer a promising, innovative approach to obesity pharmacotherapy.

Acknowledgments

We thank Julie Wilson, Chunli Lei, Julie Hoyt, Kimberly DeConzo, Calvin Vu, Jim Reynolds, and Luis Alvarado for their excellent technical assistance and Elaine Sherman for copyediting.

Received July 3, 2007. Accepted August 20, 2007.

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Disclosure Statement: J.R., T.C., C.J., H.M., J.A., C.M., C.W., and D.P. are employed by and own stock in Amylin Pharmaceuticals, Inc.

References

1. **Koda JE, Fineman M, Rink TJ, Dailey GE, Muchmore DB, Linarelli LG** 1992 Amylin concentrations and glucose control. Lancet 339:1179–1180
2. **Lutz TA, Mollet A, Rushing PA, Riediger T, 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
3. **Mollet A, Gilg S, Riediger T, Lutz TA** 2004 Infusion of the amylin antagonist AC 187 into the area postrema increases food intake in rats. Physiol Behav 81:149–155
4. **Lutz TA, Senn M, Althaus J, DelPrete E, Ehrensperger F, Scharrer E** 1998 Lesion of the area postrema nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. Peptides 19:309–317
5. **Rushing PA, Lutz TA, Seeley RJ, Woods SC** 2000 Amylin and insulin interact to reduce food intake in rats. Horm Metab Res 32:62–65
6. **Rushing PA, Hagan MM, Seeley RJ, Lutz TA, Woods SC** 2000 Amylin: a novel action in the brain to reduce body weight. Endocrinology 141:850–853
7. **Roth JD, Hughes H, Kendall E, Baron AD, Anderson CM** 2006 Anti-obesity effects of the β-cell hormone amylin in diet-induced obese rats: effects on food intake, body weight, composition, energy expenditure and gene expression. Endocrinology 147:5855–5864
8. **Chapman I, Parker B, Doran S, Feinle-Bisset C, Wishart J, Strobel S, Wang Y, Burns C, Lush C, Weyer C, Horowitz M** 2005 Effect of pramlintide on satiety and food intake in obese subjects and subjects with type 2 diabetes. Diabetologia 48:838–848
9. **Smith SR, Blundell JE, Burns C, Ellero C, Schroeder BE, Kesty NC, Chen KS, Halseth AE, Lush CW, Weyer C** 2007 Pramlintide treatment reduces 24-h caloric intake and meal sizes and improves control of eating in obese subjects: a 6-wk translational research study. Am J Physiol Endocrinol Metab 293:E620–E627
10. **Weyer C, Aronne L, Fujioka K, Aroda V, Edelman S, Chen K, Lush C, Wang Y, Burns C, Lutz K, McIntyre S, Kornstein J, Wintle M, Baron A** 2005 Safety, dose-tolerance, and weight-related effects of pramlintide in obese subjects with or without type 2 diabetes. Obes Rev 6(Suppl 1):21 (Abstract O050)
11. **Hollander P, Maggs DG, Ruggles JA, Fineman M, Shen L, Kolterman OG, Weyer C** 2004 Effect of pramlintide on weight in overweight and obese insulin-treated type 2 diabetes patients. Obes Res 12:661–668
12. **Wadden T, Klein S, Aronne L, Smith S, Halseth A, Kesty N, Burns C, Weyer C** 2006 Pramlintide treatment in obesity elicited progressive weight loss when used with a structured lifestyle intervention program: a randomized controlled trial. Obes Rev 7(Suppl 2):112–113 (Abstract PP0056)
13. **Smith S, Klein E, Burns C, Kesty N, Halseth A, Weyer C** 2007 Sustained weight loss following 1-y pramlintide treatment as an adjunct to lifestyle intervention in obesity. Diabetes 56(Suppl 1):A88 (Abstract 335-OR)
14. **Eberlein GA, Eysselein VE, Schaeffer M, Layer P, Grandt D, Goebell H, Niebel W, Davis M, Lee TD, Shively JE** 1989 A new molecular form of PYY: structural characterization of human PYY(3–36) and PYY(1–36). Peptides 10:797–803
15. **Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR** 1985 Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 89:1070–1077
16. **Lin HC, Chey WY** 2003 Cholecystokinin and peptide YY are released by fat in either proximal or distal small intestine in dogs. Regul Pept 114:131–135
17. **Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR** 2002 Gut hormone PYY(3–36) physiologically inhibits food intake. Nature 418:650–654
18. **Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S** 2003 Acute effects of PYY(3–36) on food intake and hypothalamic neuropeptide expression in the mouse. Biochem Biophys Res Commun 311:915–919
19. **Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghatei MA, Bloom SR** 2003 Inhibition of food intake in obese subjects by peptide $YY_{3–36}$. N Engl J Med 349:941–948
20. **Degen L, Oesch S, Casanova M, Graf S, Ketterer S, Drewe J, Beglinger C** 2005 Effect of peptide $YY_{3–36}$ on food intake in humans. Gastroenterology 129:1430–1436
21. **Lush C, Chen K, Hompesch M, Troupin B, LaCerte C, Burns C, Ellero C, Kornstein J, Vayser I, Wintle M, Blundell J, Baron A, Weyer C** 2005 A phase 1 study to evaluate the safety, tolerability, and pharmacokinetics of rising doses of AC162352 (synthetic human $PYY_{3–36}$) in lean and obese subjects. Obes Rev 6(Suppl 1):21 (Abstract O051)
22. **Troupin B, Lush C, Chen K, Hompesch M, LaCerte C, Burns C, Ellero C, Kornstein J, Vayser I, Wintle M, Blundell J, Baron A, Weyer C,** A phase 1 study to evaluate the safety, tolerability, and pharmacokinetics of rising doses of AC162352 (synthetic human $PYY_{3–36}$) in lean and obese subjects. Program of the 87th Annual Meeting of The Endocrine Society, San Francisco, CA, 2005, p 162 (Abstract OR58-1)
23. **Gantz I, Erondu N, Mallick M, Musser B, Krishna R, Tanaka EK, Snyder K, Stevens C, Stroh MA, Zhu H, Wagner JA, Macneil DJ, Heymsfield SB, Amatruda JM** 2007 Efficacy and safety of intranasal peptide $YY_{3–36}$ for weight reduction in obese adults. J Clin Endocrinol Metab 92:1754–1757
24. **Leibel RL, Rosenbaum M, Hirsch J** 1995 Changes in energy expenditure resulting from altered body weight. N Engl J Med 332:621–628
25. **Levin BE, Dunn-Meynell AA, Balkan B, Keesey RE** 1997 Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. Am J Physiol 273:R725–R730
26. **Carter WH, Gennings C, Staniswalis JG, Campbell ED, White KL** 1988 A statistical approach to the construction and analysis of isobolograms. J Am Coll Toxicol 7:963–973
27. **Berenbaum MC** 1981 Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 35:269–335
28. **Minto CF, Schnider TW, Short TG, Gregg KM, Gentilini A, Shafer SL** 2000 Response surface model for anesthetic drug interactions. Anesthesiology 92:1603–1616
29. **Patick AK, Boritzki TJ, Bloom LA** 1997 Activities of the human immunodeficiency virus type 1 (HIV-1) protease inhibitor nelfinavir mesylate in combination with reverse transcriptase and protease inhibitors against acute HIV-1 infection in vitro. Antimicrob Agents Chemother 41:2159–2164
30. **Pool JL, Cushman WC, Saini RK, Nwachuku CE, Battikha JP** 1997 Use of the factorial design and quadratic response surface models to evaluate the fosinopril and hydrochlorothiazide combination therapy in hypertension. Am J Hypertens 10:117–123
31. **Carter Jr WH, Wampler GL** 1986 Review of the application of response surface methodology in the combination therapy of cancer. Cancer Treat Rep 70:133–140
32. **Lutz TA, Del Prete E, Scharrer E** 1994 Reduction of food intake in rats by intraperitoneal injection of low doses of amylin. Physiol Behav 55:891–895
33. **Neary NM, Small CJ, Druce MR, Park AJ, Ellis SM, Semjonous NM, Dakin CL, Filipsson K, Wang F, Kent AS, Frost GS, Ghatei MA, Bloom SR** 2005 Peptide $YY_{3–36}$ and glucagon-like $peptide\text{-}1_{7–36}$ inhibit food intake additively. Endocrinology 146:5120–5127

34. **Chelikani PK, Haver AC, Reidelberger RD** 2007 Intermittent intraperitoneal infusion of peptide YY(3–36) reduces daily food intake and adiposity in obese rats. Am J Physiol Regul Integr Comp Physiol 293:R39–R46
35. **Cummings DE, Overduin J** 2007 Gastrointestinal regulation of food intake. J Clin Invest 117:13–23
36. **Bhavsar S, Watkins J, Young A** 1998 Synergy between amylin and cholecystokinin for inhibition of food intake in mice. Physiol Behav 64:557–561
37. **Talsania T, Anini Y, Siu S, Drucker DJ, Brubaker PL** 2005 Peripheral exendin-4 and peptide $YY_{3–36}$ synergistically reduce food intake through different mechanisms in mice. Endocrinology 146:3748–3756
38. **Morton GJ, Blevins JE, Williams DL, Niswender KD, Gelling RW, Rhodes CJ, Baskin DG, Schwartz MW** 2005 Leptin action in the forebrain regulates the hindbrain response to satiety signals. J Clin Invest 115:703–710
39. **Williams DL, Baskin DG, Schwartz MW** 2006 Leptin regulation of the anorexic response to glucagon-like peptide-1 receptor stimulation. Diabetes 55: 3387–3393
40. **Roth J, Weyer C, Anderson C, Parkes D, Baron A,** 2006 Leptin responsivity restored in leptin-resistant diet-induced obese (DIO) rats: synergistic actions of amylin and leptin for reduction in body weight (BW) and fat. Proc American Diabetes Association 66th Scientific Sessions, Washington, DC, June 9–13, 2006 (Abstract 52-LB)
41. **Roth JD, Roland B, Cole R, Coffey T, Cronister C, Weyer C, Baron A, Parkes D** 2007 Responsiveness to leptin restored by amylin in diet-induced obese (DIO) rats: magnitude and mechanisms of synergy. Diabetes 56(Suppl 1):A72 (Abstract 277-OR)
42. **Adams SH, Lei C, Jodka CM, Nikoulina SE, Hoyt JA, Gedulin B, Mack CM, Kendall ES** 2006 PYY[3–36] administration decreases the respiratory quotient and reduces adiposity in diet-induced obese mice. J Nutr 136:195–201
43. **Wynne K, Stanley S, McGowan B, Bloom SR** 2005 Appetite control. J Endocrinol 184:291–318
44. **Chance WT, Balasubramaniam A, Chen X, Fischer JE** 1992 Tests of adipsia and conditioned taste aversion following the intrahypothalamic injection of amylin. Peptides 13:961–964
45. **Rushing PA, Seeley RJ, Air EL, Lutz TA, Woods SC** 2002 Acute 3rd-ventricular amylin infusion potently reduces food intake but does not produce aversive consequences. Peptides 23:985–988
46. **Naeve S, Parkes DG, Laugero KD** 2005 Amylin's inhibitory effect on food intake is not due to malaise in rats. Appetite 44:369
47. **Roan J, Wilson J, Parkes D, Mack C** 2005 Dissociation of acute food intake and locomotor activity effects in rats after peripheral treatment with rat amylin. Appetite 44:375
48. **Halatchev IG, Cone RD** 2005 Peripheral administration of PYY(3–36) produces conditioned taste aversion in mice. Cell Metab 1:159–168
49. **Chelikani PK, Haver AC, Reidelberger RD** 2006 Dose-dependent effects of peptide YY(3–36) on conditioned taste aversion in rats. Peptides 27:3193–3201
50. **Vrang N, Madsen AN, Tang-Christensen M, Hansen G, Larsen PJ** 2006 PYY(3–36) reduces food intake and body weight and improves insulin sensitivity in rodent models of diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 291:R367–R375
51. **Sloth B, Holst JJ, Flint A, Gregersen NT, Astrup A** 2007 Effects of $PYY_{1–36}$ and $PYY_{3–36}$ on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects. Am J Physiol Endocrinol Metab 292: E1062–E1068
52. **Sloth B, Davidsen L, Holst JJ, Flint A, Astrup A** 2007 The effect of subcutaneous injections of $PYY_{1–36}$ and $PYY_{3–36}$ on appetite, ad libitum energy intake and plasma free fatty acids concentration in obese males. Am J Physiol Endocrinol Metab 293:E604–E609
53. **Sexton PM, Paxinos G, Kenney MA, Wookey PJ, Beaumont K** 1994 In vitro autoradiographic localization of amylin binding sites in rat brain. Neuroscience 62:553–567
54. **Dumont Y, St-Pierre JA, Quirion R** 1996 Comparative autoradiographic distribution of neuropeptide Y Y1 receptors visualized with the Y1 receptor agonist. Neuroreport 7:901–904
55. **Rowland NE, Crews EC, Gentry RM** 1997 Comparison of Fos induced in rat brain by GLP-1 and amylin. Regul Pept 71:171–174
56. **Riediger T, Zuend D, Becskei C, Lutz TA** 2004 The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. Am J Physiol Regul Integr Comp Physiol 286:R114–R122
57. **Browning KN, Travagli RA** 2003 Neuropeptide Y and peptide YY inhibit excitatory synaptic transmission in the rat dorsal motor nucleus of the vagus. J Physiol 549:775–785
58. **Chen CH, Stephens Jr RL, Rogers RC** 1997 PYY and NPY: control of gastric motility via action on Y1 and Y2 receptors in the DVC. Neurogastroenterol Motil 9:109–116
59. **Cox JE, Randich A** 2004 Enhancement of feeding suppression by PYY(3–36) in rats with area postrema ablations. Peptides 25:985–989
60. **Barth SW, Riediger T, Lutz TA, Rechkemmer G** 2004 Peripheral amylin activates circumventricular organs expressing calcitonin receptor a/b subtypes and receptor-activity modifying proteins in the rat. Brain Res 997:97–102
61. **Renshaw D, Batterham RL** 2005 Peptide YY: a potential therapy for obesity. Curr Drug Targets 6:171–179
62. **Challis BG, Coll AP, Yeo GS, Pinnock SB, Dickson SL, Thresher RR, Dixon J, Zahn D, Rochford JJ, White A, Oliver RL, Millington G, Aparicio SA, Colledge WH, Russ AP, Carlton MB, O'Rahilly S** 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

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**