PYY$_{3-36}$ injection in mice produces an acute anorexigenic effect followed by a delayed orexigenic effect not observed with other anorexigenic gut hormones

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Receptors within the ARC integrate appetite-regulating signals by altering neuropeptide release within two distinct neuronal populations (18, 26, 37). One population of neurons coexpresses the orexigenic peptides agouti-related peptide (AgRP) and NPY and the other population releases the proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript, which inhibit feeding (18, 26, 37).

It has been suggested that peripheral administration of PYY$_{3-36}$ may mediate its anorectic effects by increasing hypothalamic POMC levels and reducing NPY levels (8). However, PYY$_{3-36}$ retains its anorexigenic effect in mice that are either deficient in POMC (14) or lack the melanocortin-4 receptor (25). This suggested that the inhibition of NPY neurons by PYY$_{3-36}$ may be more important in mediating its anorexigenic effect than activation of POMC neurons, a proposal supported by electrophysiological evidence (3).

Peripheral administration of PYY$_{3-36}$ has been shown by several groups to reduce short-term food intake (8, 15, 25, 31, 36, 40). Furthermore, it was shown that repeated twice daily injection of PYY$_{3-36}$ decreases food intake and body weight in rats (8). In addition, sustained intravenous infusion of PYY$_{3-36}$ in rats, mimicking postprandial levels of the peptide, potently inhibited food intake (17). Chronic infusion of PYY$_{3-36}$ via osmotic minipump has also been shown to significantly reduce food intake and weight gain in both leptin-deficient ob/ob mice and diet-induced obese mice (4, 35).

However, a few groups have had difficulty replicating the chronic anorectic effects of repeated PYY$_{3-36}$ injections (12, 41). Challis et al. (14) found no change in cumulative food intake or body weight in mice treated with intraperitoneal PYY$_{3-36}$ injection daily for 1 wk. Moran et al. (32) recently found daily intramuscular administration of PYY$_{3-36}$ failed to produce a sustained reduction in food intake over successive days in rhesus monkeys. It remains unclear what causes the inconsistency in effect of chronic PYY$_{3-36}$ administration on food intake. One possibility is that insufficient acclimatization to handling and injection of animals results in a stress-induced reduction in appetite and a subsequent failure of anorexigenic peptides to further reduce food intake (2, 25).

We investigated the effects of 0, 6, 12, 18, 24, and 30 h fasting upon the anorexigenic effect of acute PYY$_{3-36}$ injection. PYY$_{3-36}$ caused an acute reduction in food intake followed by an increased food intake in the dark phase in nonfasted mice. To confirm this delayed orexigenic effect, we...
measured the effects of early light-phase PYY$_{3-36}$ injection on dark-phase food intake in ad libitum-fed mice fasted for 4 h postinjection. The 4-h fast was introduced to cover the period of anorexigenic activity of PYY$_{3-36}$ and ensure subsequent dark-phase food intake measurements were based on the effect of the peptide as opposed to prior differences in appetite in the initial 4 h following injection. To investigate the mechanism for this delayed orexigenic effect, we measured the effects of early light-phase injection of PYY$_{3-36}$ in ad libitum-fed mice on the levels of hypothalamic NPY, AgRP, and POMC and plasma PYY$_{3-36}$ and ghrelin at the beginning of the dark phase. Finally, we investigated if in this paradigm the delayed orexigenic effect was unique to the PYY$_{3-36}$ System or whether or not it occurs after peripheral injection of other anorectic substances, including glucagon-like peptide (GLP-1), oxyntomodulin (OXM), and lithium chloride (LiCl).

MATERIALS AND METHODS

Animals

Male C57Bl/6j mice (specific pathogen free; Harlan) weighing 20–30 g were maintained in individually ventilated cages under controlled conditions of temperature (21–23°C) and light (12:12-h light-dark cycle, lights on at 8:00 A.M.) with ad libitum access to food (RM1 diet; Special Diet Services, Witham, Essex, UK) and water. Animals were acclimatized with daily handling, body weight monitoring, and two sham injections during the week before the first study day. All animal procedures undertaken were approved under the United Kingdom Animals (Scientific Procedures) Act 1986 (Project License nos. 70/5516 and 70/6402). All animal groups were randomized by weight. All injections were delivered intraperitoneally. Ad libitum-fed animals were given a fresh quantity of chow at the time of injection.

Materials

All chemicals were purchased from Merck (Poole, Dorset, UK) and peptides from Phoenix Pharmaceuticals (Belmont, CA) except d-Allo Ile$^3$ PYY$_{3-36}$ which was purchased from Bachem (Liverpool, UK). The analog d-Allo Ile$^3$ PYY$_{3-36}$ was designed with an NH$_2$-terminal stereochemical change to protect against endogenous circulating pro- teases. Characterization of d-Allo Ile$^3$ PYY$_{3-36}$ showed it bound with similar affinity as PYY$_{3-36}$ to the Y2R (IC$_{50}$: PYY$_{3-36}$ 0.12 nM, d-Allo Ile$^3$ PYY$_{3-36}$ 0.15 nM) (data not shown).

Study 1: Effect of Variable Fast Times on the Anorexigenic Effect of Peripherally Administered PYY$_{3-36}$ in Mice

This study was designed to evaluate the anorexigenic effects of PYY$_{3-36}$ in mice fasted for increasing periods of time. Mice received injections of PYY$_{3-36}$ (23 nmol/kg) or saline at 8:00 A.M. after a specific fast time of 0, 6, 12, 18, 24, or 30 h (n = 8/group). This dose of PYY$_{3-36}$ has been previously shown to reduce appetite (8). Food intake was measured at 1, 2, 4, 6, and 24 h postinjection.

Study 2: Effects of Early Light-Phase Injection of PYY$_{3-36}$ on Food Intake in Mice

This study was carried out to confirm the delayed orexigenic effect seen in the PYY$_{3-36}$-injected animals from study 1. In study 1, animals fasted for 0 or 6 h and then injected with PYY$_{3-36}$ demonstrated an increase in food intake during the 6- to 24-h period postinjection. However, the reductions in food intake of PYY$_{3-36}$-injected animals in the 0- and 6-h fast groups during the first 0–4 h postinjection could have stimulated the delayed orexigenic effect of PYY$_{3-36}$. To negate this possible effect, a protocol was used that introduced a 4-h fast, postinjection of saline or PYY$_{3-36}$. This 4-h fast was used to cover the period of anorexigenic activity of PYY$_{3-36}$ and ensure subsequent dark-phase food intake measurements were based upon the effect of the peptide as opposed to prior differences in food intake in the initial 4-h postinjection of PYY$_{3-36}$. In addition to the 4-h fast experiments, a separate group of animals fasted overnight was administered saline or PYY$_{3-36}$, and food was returned postinjection to confirm the anorexigenic effect of PYY$_{3-36}$.

Forty-eight mice were split into two groups (n = 24), one of which was fasted overnight. The group of nonfasted mice was injected at 8:00 A.M. with either PYY$_{3-36}$ (23 nmol/kg) or saline (n = 12/group), then fasted for 4 h, and normal chow was returned. Food intake was measured 6, 8, 12, and 24 h post the initial injection (i.e., 2, 4, 8, and 20 h post the 4-h fast). A second group of fasted animals was injected intraperitoneally at 8:00 A.M. with either PYY$_{3-36}$ (23 nmol/kg) or saline (n = 12/group). Normal chow was returned immediately postinjection, and food intake was measured at 1, 2, 4, and 24 h postinjection.

Study 3: Time Course Effects of Early Light-Phase Injection of PYY$_{3-36}$ on Food Intake, Ambulatory Activity, and Oxygen Consumption in Mice

To further investigate the significantly increased food intake seen during the dark phase of PYY$_{3-36}$-injected animals in studies 1 and 2, a study was carried out to measure the time course of the effects of PYY$_{3-36}$ on food intake, activity, and oxygen consumption (VO$_2$) using the comprehensive laboratory animal monitoring system (CLAMS) as previously described (38). Ad libitum-fed animals (ground food, RM1 diet; Special Diet Services) were acclimatized in the metabolic cages for 2 days before they received an intraperitoneal injection of either saline or PYY$_{3-36}$ (23 nmol/kg) (n = 8/group) at approximately 8:00 A.M. and then fasted for 4 h. Food intake was measured every 30 min for 24 h postinjection. During CLAMS monitoring, metabolic parameters [VO$_2$ and carbon dioxide production (VCO$_2$)] were measured by indirect calorimetry. Exhaust air from each chamber was sampled at 30-min intervals for a period of 1 min. Sample air was sequentially passed through O$_2$ and CO$_2$ sensors (Columbus Instruments) for determination of O$_2$ and CO$_2$ content. To compare animals of differing sizes, the O$_2$ consumption and CO$_2$ production values were normalized with respect to body weight, and O$_2$ consumption was corrected to an effective mass value of 0.75. Ambulatory activity of each individually housed animal was measured simultaneously using the optical beam technique (Opto M3; Columbus Instruments). Consecutive photo-beam breaks were scored as an ambulatory movement. Activity counts (XAMB) were recorded every minute for 24 h and were used to determine horizontal activity.

Study 4: Effect of Early Light-Phase Injection of Various Doses of PYY$_{3-36}$ on Food Intake in Ad Libitum-Fed Mice

This study was carried to determine if the delayed orexigenic effects following PYY$_{3-36}$ administration were dependent on the dose of peripherally injected PYY$_{3-36}$. Thirty-six ad libitum-fed C57Bl/6j mice were injected at 8:00 A.M. with either saline or PYY$_{3-36}$ (7, 23, or 69 nmol/kg) (n = 9/group) and fasted for 4 h. Food intake was measured at 12 and 24 h post the initial injection. The PYY$_{3-36}$ 23 nmol/kg dose was chosen since it had shown a delayed orexigenic effect in study 2. The threefold lower and higher doses were chosen to establish a dose response for the effects of early light-phase injection of PYY$_{3-36}$ on food intake.

Study 5: Effects of Early Light-Phase Injection of PYY$_{3-36}$ on NPY, AgRP, and POMC Hypothalamic mRNA Levels and Plasma Hormone Concentrations in Ad Libitum-Fed Mice at the Beginning of the Dark Phase

Intraperitoneal injection of PYY$_{3-36}$ in ad libitum mice at 8:00 A.M., followed by a 4-h fast, resulted in an increase in food intake during the subsequent dark phase in studies 2, 3, and 4. To investigate a possible mechanism for this delayed orexigenic effect, the hypothalamic mRNA levels of NPY, POMC, and AgRP and plasma ghrelin and PYY$_{3-36}$ were measured at the start of the dark phase (8:00 P.M.)
in ad libitum-fed mice injected with saline or PYY3-36 at 8:00 A.M. Thirty-six ad libitum-fed mice were injected intraperitoneally at 8:00 A.M. with either saline or PYY3-36 (7, 23, or 69 nmol/kg) \((n = 9/\text{group})\) and then fasted for 4 h after which time fresh chow was returned. After measuring food intake at 8:00 P.M., cardiac puncture and hypothalamic extraction were performed. Hypothalamic levels of AgRP, NPY, and POMC were measured by ribonuclease protection assay (RPA), and plasma ghrelin and PYY3-36 were measured by radioimmunoassay (RIA) as detailed below.

**Hypothalamic extraction.** Mice were killed by carbon dioxide inhalation, and brains were rapidly removed. A block of tissue encompassing the hypothalamus was subsequently cut from the brains and placed in an Eppendorf tube in liquid nitrogen. Hypothalami were stored at \(-70^\circ\text{C}\) until RNA extraction.

**RPA.** Total RNA was extracted from the hypothalami using Tri-Reagent (Helena Biosciences, Sunderland, UK) following the manufacturer’s protocol. Hypothalamic AgRP, NPY, and POMC (all 5 \(\mu\)g) mRNA were quantified by RPA (RPA III kit; Ambion, Austin, TX) using in-house probes \((10)\). AgRP corresponded to nucleotides 17–353 (accession no. XM226404), NPY corresponded to nucleotides 81–538 (accession no. NM_012614), and POMC corresponded to nucleotides 185–674 (accession no. NM_139326). Rat cytophilin (Ambion) was used as an internal control. cDNAs corresponding to the above probes were made by PCR and cloned into pBluescript. Linearized cDNAs were transcribed using T3 polymerase (Promega, Madison, WI) to produce antisense riboprobes labeled with \[^{32}\text{P}\]CTP (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK). RNA was hybridized overnight at \(42^\circ\text{C}\) and separated on a 5% polyacrylamide gel. The dried gel was exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) overnight, and protected RNA hybrids were quantified using ImageQuant software (Molecular Dynamics). For each neuropeptide, the ratio of the optical density of the band of neuropeptide mRNA to that of cytophilin was calculated and expressed in relative units \((8, 28, 29)\).

**Plasma ghrelin.** All samples were assayed in duplicate and in a single assay to eliminate the effects of interassay variation. Ghrelin-like immunoreactivity was measured with a specific and sensitive RIA as previously described \((33)\). Briefly, the antisera (SC-10368) was obtained from Santa Cruz biotechnology and used at a final dilution of 1:10,000. The dose of D-Allo Ile3 PYY3-36 was chosen since it significantly reduced food intake in \(\text{CTA in mice (30)}\) and was used to investigate if it produced a similar anorexic effect. Ad libitum-fed mice were injected with either saline or D-Allo Ile3 PYY3-36 (23 nmol/kg) \((n = 8/\text{group})\) at 8:00 A.M. then fasted for 4 h. Fresh chow was returned, and food intake was measured at 6, 8, 12, and 24 h postinjection. An additional test study in fasted animals was carried out simultaneously to confirm the anorectic effects of the peptide during the 4-h fast; mice were fasted overnight and injected at 8:00 A.M. with either D-Allo Ile3 PYY3-36 (23 nmol/kg) or saline \((n = 8/\text{group})\). Food intake was measured at 1, 2, 4, and 24 h postinjection. The dose of D-Allo Ile3 PYY3-36 was chosen since it significantly reduced food intake in study 6.

**Plasma PYY.** All samples were assayed in duplicate and in one assay to eliminate the effects of interassay variation. PYY-like immunoreactivity was measured with a specific and sensitive RIA as previously described \((6)\). Briefly, the antiseraum (Y21) was produced by immunizing rabbits with the C-terminal leucine residue. This study was designed to test the potency of D-Allo Ile3 PYY3-36 vs. PYY3-36 on food intake in fasted mice. C57BL/6 mice were fasted overnight and injected intraperitoneally at 8:00 A.M. with saline, PYY3-36 (7 or 23 nmol/kg), or D-Allo Ile3 PYY3-36 (7 or 23 nmol/kg) \((n = 10/\text{group})\). Food intake was measured at 1, 2, 4, and 24 h postinjection.

**Study 8: Effects of Early Light-Phase Injection of GLP-1, OXM, and LiCl on Food Intake in Mice**

This study investigated if the delayed orexigenic effect induced by PYY3-36 and D-Allo Ile3 PYY3-36 observed in studies 2 and 7, respectively, also occurred with other anorexigenic agents. Ad libitum-fed C57BL/6 mice were given intraperitoneal injections of either saline, GLP-1 (1,000 nmol/kg), OXM (1,400 nmol/kg), or LiCl (100 \(\mu\)l of 3 M solution) \((n = 8/\text{group})\) at 8:00 A.M. and then fasted for 4 h. After the 4-h fast, fresh chow was returned, and food intake measured at 6, 8, 12, and 24 h postinjection. To confirm the expected anorectic effects of GLP-1, OXM, and LiCl, a second group of mice was fasted overnight and injected intraperitoneally at 8:00 A.M. with saline, GLP-1 (1,000 nmol/kg), OXM (1,400 nmol/kg), or LiCl (100 \(\mu\)l of 3 M solution) \((n = 8/\text{group})\) at 8:00 A.M. and then fasted for 4 h. The doses of GLP-1 and OXM used have previously been shown to significantly alter food intake following intraperitoneal injection \((20)\). It has been suggested that the anorexigenic effects of PYY3-36 may be due to a conditioned taste aversion (CTA) response \((24)\). LiCl at the dose used in this study is known to cause CTA in mice \((30)\) and was used to investigate if it produced a similar delayed orexigenic effect to PYY3-36.

**Statistical Analysis**

Statistical advice was provided by J. Eliahou of the Statistical Advisory Service, Imperial College London. Food intake data \((g)\) is expressed as means ± SE and analyzed by unpaired Student’s \(t\)-test (GraphPad Prism, GraphPad Software, San Diego, CA) unless otherwise.
wise stated. Data parameters, including $\dot{V}O_2$, $\dot{V}CO_2$, and ambulatory activity generated by the CLAMS metabolic cages, were analyzed by the general estimating equation and the Mann-Whitney $U$-test, using commercial software (Stata 9.1; Statacorp, College Station, TX). In all cases, values of $P < 0.05$ were considered statistically significant.

RESULTS

Study 1: Effect of Variable Fast Times on the Anorexigenic Effect of Peripherally Administered PYY$_{3-36}$ in Mice

Nonfasted mice injected with PYY$_{3-36}$ showed a significant reduction in food intake between 2 and 4 h compared with the saline group [0-h fasted group; 2–4 h food intake: saline: 0.13 ± 0.02 g, PYY$_{3-36}$: 0.06 ± 0.01 g ($P < 0.05$ vs. saline)] (Fig. 1A). The first significant decrease in food intake in animals fasted for 6 h and then injected with PYY$_{3-36}$ occurs at the earlier time period of 1–2 h compared with nonfasted mice [6-h fasted group; 1–2 h food intake: saline: 1.7 ± 0.02 g, PYY$_{3-36}$: 0.05 ± 0.01 g ($P < 0.001$ vs. saline)] (Fig. 1B). Mice fasted for 12, 18, 24, and 30 h and injected with PYY$_{3-36}$ showed a significant decrease in food intake in the 0- to 1-h and 1- to 2-h period compared with saline-injected animals (Fig. 1, C–F).

A trend toward increased food intake is seen in all PYY$_{3-36}$-injected groups at 6–24 h following injection, and this was statistically significant in the 0- and 6-h fasted groups [0-h

Fig. 1. Effect of variable fast times on the anorexigenic effects of peptide YY (PYY$_{3-36}$). The effect of ip administration of PYY$_{3-36}$ on food intake in mice fasted for 0 (A), 6 (B), 12 (C), 18 (D), 24 (E), and 30 (F) h preinjection. Food intake was measured at 1, 2, 4, 6, and 24 h postinjection; PYY$_{3-36}$ dose 23 nmol/kg; $n = 8$ animals/group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. saline. Results are means ± SE.

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fasted group; 6–24 h food intake: saline: 3.1 ± 0.2 g, PYY3-36: 3.8 ± 0.1 g (P < 0.05 vs. saline); 6-h fasted group; 6–24 h food intake: saline: 3.8 ± 0.1 g, PYY3-36: 4.3 ± 0.09 g (P < 0.01 vs. saline) (Fig. 1, A and B).

Study 2: Effects of Early Light-Phase Injection of PYY3-36 on Food Intake in Mice

Mice injected at 8:00 A.M. with PYY3-36 and then fasted for 4 h ate significantly more during the 12- to 24-h period postinjection compared with saline controls [12–24 h food intake: saline: 2.8 ± 0.1 g, PYY3-36: 3.26 ± 0.1 g (P < 0.01 vs. saline)] (Fig. 2A). Mice fasted overnight and injected with PYY3-36 at 8:00 A.M. confirmed the anorexigenic effect of intraperitoneal PYY3-36 compared with saline-injected animals [0–4 h food intake: saline: 1.76 ± 0.03 g, PYY3-36: 1.1 ± 0.08 g (P < 0.001 vs. saline)] (Fig. 2B).

Study 3: Time Course Effects of Early Light-Phase Injection of PYY3-36 on Food Intake, Ambulatory Activity, and V̇O₂ in Mice

PYY3-36 injection followed by a 4-h fast resulted in a significantly increased nighttime food intake compared with saline controls [12–24 h food intake: PYY3-36: 3.72 ± 0.1 g; saline: 3.2 ± 0.2 g (P < 0.05 vs. saline)] (Fig. 3). There was no difference in food intake between PYY3-36- and saline-injected animals in the 4- to 12-h period postinjection (data not shown). Intraperitoneal administration of PYY3-36 followed by a 4-h fast had no significant effect on V̇O₂, V̇CO₂, or XAMB when compared with saline-injected controls (data not shown).

Study 4: Effect of Early Light-Phase Injection of Various Doses of PYY3-36 on Food Intake in Ad Libitum-Fed Mice

An increase in dark-phase food intake was observed in animals administered the 23 and 69 nmol/kg doses of PYY3-36 [12–24 h food intake: saline: 2.57 ± 0.14 g, 23 nmol/kg PYY3-36: 2.85 ± 0.1 g, 69 nmol/kg PYY3-36: 2.87 ± 0.08 g (both P < 0.05 vs. saline)] (Fig. 4). There was a trend toward an increase in nighttime food intake in the 7 nmol/kg PYY3-36 group compared with saline controls, but this did not reach statistical significance {12–24 h food intake: saline: 2.57 ± 0.14 g, 7 nmol/kg PYY3-36: 2.7 ± 0.15 g [P = not significant (NS)] (Fig. 4).

Study 5: Effects of Early Light-Phase Injection of PYY3-36 on NPY, AgRP, and POMC Hypothalamic mRNA Levels and Plasma Hormone Concentrations in Ad Libitum-Fed Mice at the Beginning of the Dark Phase

There was a dose-dependent trend toward a decrease in POMC mRNA levels and an increase in hypothalamic NPY and AgRP levels at the beginning of the dark phase in animals injected with PYY3-36 in the early light phase and fasted for 4 h, but this did not reach statistical significance [POMC mRNA: PYY3-36 7 nmol: 87.7 ± 31.7% of saline mRNA, PYY3-36 23 nmol/kg: 77.2 ± 35.4% of saline mRNA, PYY3-36 69 nmol: 65.5 ± 19.4% of saline mRNA (P = NS for all groups); NPY mRNA: PYY3-36 7 nmol: 105.6 ± 13.5% of saline mRNA, PYY3-36 23 nmol/kg: 122.2 ± 19.9% of saline mRNA, PYY3-36 69 nmol: 114.6 ± 14.2% of saline mRNA (P = NS for all groups); AgRP mRNA: PYY3-36 7 nmol: 106.6 ± 21.1% of saline mRNA, PYY3-36 23 nmol/kg: 105.9 ± 19.9% of saline mRNA, PYY3-36 69 nmol: 125.0 ± 18.5% of saline mRNA (P = NS for all groups)]. There was a nonsignificant trend toward an increase in the AgRP-to-POMC ratio between saline- and PYY3-36-injected animals (data not shown).

There was a nonsignificant trend toward reduced plasma PYY3-36 at the beginning of the dark phase in animals injected with PYY3-36 in the early light phase and fasted for 4 h [plasma PYY3-36: saline: 86.1 ± 13.5 pmol/l, PYY3-36 7 nmol: 66.9 ± 8.2 pmol/l, PYY3-36 23 nmol/kg: 50.5 ± 8.9, PYY3-36 69 nmol: 58.3 ± 10.4 (P = NS for all groups)]. There was a trend toward an increase in plasma ghrelin in mice injected with both the 7 and 69 nmol/kg doses of PYY3-36, and a significant increase in plasma ghrelin was seen in animals injected with 23 nmol/kg PYY3-36 [plasma ghrelin: saline-injected animals: 896 ± 62.4 pmol/l, PYY3-36 7 nmol/kg: 1,154 ± 76.1 pmol/l (P = NS), PYY3-36 23 nmol/kg: 1,291 ± 137.1 pmol/l (P < 0.05 vs. saline), PYY3-36 69 nmol/kg: 1,150.7 ± 126.7 pmol/l (P = NS)] (Fig. 5B).
PYY3-36 INDUCES A DELAYED OREXIGENIC EFFECT

In agreement with previous studies, we confirm the anorexigenic effects of acute administration of PYY3-36 (15, 25, 32, 36, 40). Furthermore, data from study 1, in which animals were either ad libitum-fed or fasted for a variable time, show that peripheral injection of PYY3-36 is capable of significantly reducing food intake in both fed and fasted states. An early light-phase injection of PYY3-36 appears to act more rapidly and potently to significantly reduce food intake in mice that have been fasted for longer periods of time. This may be due to the reduced appetite of ad libitum-fed compared with fasted animals, minimizing the differences in food intake between PYY3-36 and saline injections in fed animals. It has been suggested that PYY3-36 may mediate its anorexigenic effects by reducing NPY release via the autoinhibitory Y2R (8). Because hypothalamic NPY levels are lower in ad libitum-fed compared with fasted animals (13), PYY3-36 would be expected to have a reduced anorexigenic effect in ad libitum-fed animals.

It is possible that the acute reduction in food intake in nonfasted animals injected with PYY3-36 during the initial 4-h postinjection seen in study 1 could have resulted in the delayed orexigenic effects of PYY3-36 seen during the dark phase in these animals. To negate this possible effect, a 4-h fast was introduced postinjection. This fast was designed to cover the period of anorexigenic activity of PYY3-36. Using this protocol, early light-phase injections of PYY3-36 caused a significant increase in food intake during the subsequent dark phase, suggesting that this delayed orexigenic effect is not a compensatory increase in feeding due to a prior anorexigenic effect. This delayed orexigenic effect following peripheral injection of PYY3-36 could be contributing to the inconsistency in reproducing the anorexigenic effects following chronic administration of PYY3-36 (12, 14, 32, 41), especially if administered insufficiently frequently.

**DISCUSSION**

Study 6: Comparison of the Anorexigenic Effect of Early Light-phase Injection of d-Allo Ile3 PYY3-36 vs. PYY3-36 on Food Intake in Fasted Mice

Both PYY3-36 and d-Allo Ile3 PYY3-36 significantly reduced food intake in fasted mice at the 23 nmol/kg dose [0–4 h food intake: saline 1.6 ± 0.09 g, PYY3-36 (23 nmol/kg): 1.17 ± 0.09 g (P < 0.001 vs. saline); d-Allo Ile3 PYY3-36 (23 nmol/kg): 0.99 ± 0.07 g (P < 0.001 vs. saline)] (Fig. 6). The 7 nmol/kg dose of PYY3-36 did not significantly reduce food intake compared with saline; however, intraperitoneal injection of the 7 nmol of d-Allo Ile3 PYY3-36 did cause a significant reduction in food intake [0–4 h food intake: saline 1.6 ± 0.09 g, d-Allo Ile3 PYY3-36 (7 nmol/kg): 1.17 ± 0.06 g (P < 0.01)] (Fig. 6).

Study 7: Effects of Early Light-phase Injection of d-Allo Ile3 PYY3-36 on Food Intake in Ad Libitum-fed Mice

Peripheral injection of the analog d-Allo Ile3 PYY3-36 followed by a 4-h fast resulted in a significantly increased food intake at 12–24 h postinjection [12–24 h food intake: saline: 2.76 ± 0.14 g, d-Allo PYY3-36: 3.24 ± 0.16 g (P < 0.01 vs. saline)] (Fig. 7A). Animals fasted overnight and injected with d-Allo Ile3 PYY3-36 showed a significant reduction in food intake during the 4-h postinjection [0–4 h food intake: saline: 1.55 ± 0.05 g, d-Allo PYY3-36: 1.01 ± 0.09 g (P < 0.001 vs. saline)] (Fig. 7B).

Study 8: Effects of Early Light-phase Injection of GLP-1, OXM, and LiCl on Food Intake in Mice

Intraperitoneal injection of GLP-1, OXM, and LiCl significantly reduced food intake in animals fasted overnight [0–2 h food intake: saline: 1.22 ± 0.06 g, GLP-1: 0.88 ± 0.07 g (P < 0.01 vs. saline); saline: 1.5 ± 0.06 g, OXM: 1.33 ± 0.07 g (P < 0.05 vs. saline); saline: 1.24 ± 0.1 g, LiCl: 0.8 ± 0.5 g (P < 0.001 vs. saline)] (Fig. 8, B, D, and F, respectively). However, no nighttime increase in food intake was seen in ad libitum-fed animals injected with GLP-1, OXM, or LiCl and then subjected to a 4-h fast [12–24 h food intake: saline: 3.48 ± 0.08 g, GLP-1: 3.48 ± 0.06 g (P = NS); saline: 3.85 ± 0.07, OXM: 3.72 ± 0.08 (P = NS); saline: 3.2 ± 1.4 g, LiCl: 3.0 ± 0.1 g (P = NS)] (Fig. 8, A, C, and E, respectively).

**Fig. 4.** Dose-dependent effects of peripheral injection of PYY3-36 at the beginning of the light phase on food intake in mice. Ad libitum-fed mice were injected ip at 8:00 A.M. with either saline or PYY3-36 (7, 23, or 69 nmol/kg) and fasted for 4 h. Food intake was measured 8 and 24 h postinjection; n = 9/group. *P < 0.05 vs. saline. Results are means ± SE.

**Fig. 3.** The effect of ip injection of PYY3-36 or saline on cumulative food intake in mice. Ad libitum-fed mice acclimatized for 2 days in the comprehensive laboratory animal monitoring system (CLAMS) were injected at 8:00 A.M. with either saline or PYY3-36, followed by a 4-h fast, after which food was returned. Food intake was recorded at 30-min intervals for 24 h following the 4-h fast; PYY3-36 dose 23 nmol/kg; n = 8/group. Error bars indicate 95% confidence intervals. The hatched lines indicate the duration of the 4-h fast postinjection at time (t) = 0. Open bars, light phase; filled bars, dark phase.

**Study 1:** Effects of Early Light-phase Injection of PYY3-36 on Food Intake in Mice

Peripheral injection of PYY3-36 at time (t) = 0 significantly reduced food intake during the 4 h postinjection [0–4 h food intake: saline: 1.55 ± 0.05 g (P < 0.001 vs. saline); PYY3-36 dose 23 nmol/kg; n = 8/group. Error bars indicate 95% confidence intervals. The hatched lines indicate the duration of the 4-h fast postinjection at time (t) = 0. Open bars, light phase; filled bars, dark phase.

**Summary:**

- Both PYY3-36 and d-Allo Ile3 PYY3-36 significantly reduced food intake in fasted mice.
- In fasted mice, the 23 nmol/kg dose of PYY3-36 reduced food intake significantly compared with saline.
- In ad libitum-fed mice, PYY3-36 did not significantly reduce food intake compared with saline.
- Both PYY3-36 and d-Allo Ile3 PYY3-36 caused a significant reduction in food intake in fasted mice at the 23 nmol/kg dose compared with saline.
- Peripheral injection of PYY3-36 is capable of significantly reducing food intake in both fed and fasted states.
- Early light-phase injection of PYY3-36 appears to act more rapidly and potently to significantly reduce food intake in mice that have been fasted for longer periods of time.

**Fig. 5:** Cumulative food intake in mice. Ad libitum-fed animals injected with GLP-1, OXM, or LiCl and then subjected to a 4-h fast [12-24 h food intake: saline: 3.48 ± 0.08 g, GLP-1: 3.48 ± 0.06 g (P = NS); saline: 3.85 ± 0.07, OXM: 3.72 ± 0.08 (P = NS); saline: 3.2 ± 1.4 g, LiCl: 3.0 ± 0.1 g (P = NS)] (Fig. 8, A, C, and E, respectively).
Feeding data obtained from analysis in metabolic cages also indicate that animals injected with PYY3-36 and fasted for 4 h have a significantly increased feeding during the dark phase compared with their saline-injected counterparts. This manifested as a gradual increase in feeding over the course of the dark phase opposed to a sharp rise at a particular time point. Changes in food intake affect both hypothalamic neuropeptide expression and plasma ghrelin and PYY3-36 levels (39). Therefore, if measurements of hypothalamic ARC neuropeptides and plasma PYY3-36 and ghrelin were made during the dark phase, it would not be possible to exclude changes in food intake causing secondary changes in these measurements as opposed to a direct effect of early light phase injection of PYY3-36. As such, it was decided to measure the plasma levels of gut hormones and hypothalamic neuropeptide mRNA concentrations at the start of the dark phase, before any incremental differences in food intake could manifest. Here we show that early light-phase administration of PYY3-36 followed by a 4-h fast resulted in a nonsignificant trend toward increased levels of hypothalamic NPY and AgRP mRNA and a decrease in POMC mRNA 12 h postinjection, at the beginning of the dark phase. Furthermore, in mice injected with 23 nmol/kg of PYY3-36 at 8:00 A.M., 12 h later at the beginning of the dark phase, plasma levels of ghrelin were increased significantly, and there was a trend toward decreased plasma PYY3-36 levels. These changes in ARC neuropeptide mRNA and plasma levels of ghrelin and PYY3-36 seen 12 h postinjection of PYY3-36 correlate with the effects on food intake and may be responsible for the delayed orexigenic effect we have observed.

In addition to the hypothalamus, regions of the brain stem, in particular the area postrema, have also been implicated in mediating the anorexigenic effects of peripherally administered PYY3-36 (1, 27). Furthermore, large concentrations of the Y2R are expressed within the brain stem (43). Our data show that early light-phase injection of PYY3-36 results in a significant increase in plasma ghrelin levels and trends toward an increase in hypothalamic orexigenic neuropeptides at the start of the dark phase, which may be responsible for the delayed orexigenic effect. However, it cannot be excluded that the brain stem is involved in mediating the delayed orexigenic effect we have observed following acute peripheral injection of PYY3-36. Interestingly, mice with area postrema ablations have been...
shown to have slightly enhanced feeding suppression after peripheral injection of PYY$_{3-36}$ suggesting that certain areas of the brain stem are not required to transmit the anorexigenic effect of PYY$_{3-36}$ (19). Further work is required to investigate the central nervous system pathways of PYY$_{3-36}$ action.

To determine if the delayed orexigenic effect observed following PYY$_{3-36}$ injection was unique to the PYY$_{3-36}$ molecule, we investigated the effects of a PYY$_{3-36}$ analog, D-Allo Ile$^3$ PYY$_{3-36}$, on food intake. The only structural difference between this particular analog and PYY$_{3-36}$ is a stereochemical reorientation of side chains on two of the chiral atoms on the NH$_2$-terminal leucine residue. The 7 nmol/kg dose of D-Allo Ile$^3$ PYY$_{3-36}$ had similar anorectic effects to the 23 nmol/kg dose of PYY$_{3-36}$, suggesting a threefold greater potency of D-Allo Ile$^3$ PYY$_{3-36}$ compared with PYY$_{3-36}$. The degradation of PYY$_{3-36}$ by dipeptidyl peptidase IV occurs via the NH$_2$ terminal, limiting its half-life (21). It is possible that the altered NH$_2$-terminal configuration seen in D-Allo Ile$^3$ PYY$_{3-36}$ imparts added protection against circulating proteases, resulting in a longer half-life and enhanced anorexigenic effects of D-Allo Ile$^3$ PYY$_{3-36}$ compared with PYY$_{3-36}$. Interestingly, intraperitoneal injection of D-Allo Ile$^3$ PYY$_{3-36}$ in the early light phase followed by a 4-h fast caused a similar delayed orexigenic effect in dark-phase food intake to that of PYY$_{3-36}$. This suggests that the delayed orexigenic effect following PYY$_{3-36}$ injection is not unique but also occurs with analogs, such as D-Allo Ile$^3$ PYY$_{3-36}$, by stimulating the same physiological pathways as PYY$_{3-36}$.

It has been reported that PYY$_{3-36}$ may cause an aversive response in mice that might be responsible for its anorectic actions (24). LiCl was used to compare the effects of PYY$_{3-36}$ with those of an agent known to induce CTA. Although LiCl caused a decrease in food intake, its peripheral injection did not produce a delayed orexigenic effect in nighttime feeding as occurs with PYY$_{3-36}$. These data suggest that stimulation of CTA pathways is not likely to be responsible for the delayed orexigenic effect observed following light-phase PYY$_{3-36}$ injection.

It is important to consider the physiological relevance of the delayed orexigenic effect we have observed and how the data presented align with previous studies of PYY$_{3-36}$ on food intake. Previously, our group and others have shown that PYY$_{3-36}$ potently decreases food intake, and this is a consistent and reproducible effect (8, 15, 25, 36, 40). In this manuscript, we also show that PYY$_{3-36}$ reduces food intake in fasted (Figs. 1, C–F, and 2B) and ad libitum-fed animals (Fig. 1A). However, in addition to its anorexigenic effects, we show that PYY$_{3-36}$ has a small delayed orexigenic effect that is independent of the acute reduction in food intake caused by the peptide. This delayed orexigenic effect of PYY$_{3-36}$ may be a physiological response arising from the activation of anorectic circuits immediately following PYY$_{3-36}$ administration. Our studies suggest that the predominant effect of PYY$_{3-36}$ is anorectic, but this may be limited by the delayed orexigenic effect.

We have previously shown that PYY$_{3-36}$ has an anorectic effect that may be mediated via an increase in POMC and a decrease in NPY and AgRP levels in the ARC (8). In this previous study, we measured hypothalamic POMC, NPY, and AgRP mRNA levels in ad libitum-fed rats 6 h after PYY$_{3-36}$ injection. In the current study, we quantified hypothalamic mRNA levels 12 h postinjection in fasted mice. In addition, a 4-h fast was incorporated in the current study to allow differentiation between the immediate anorectic effects of PYY$_{3-36}$ and its delayed orexigenic effect. These changes to the protocol, including the timing of the observations with relation to whether PYY$_{3-36}$ is anorexigenic (6 h postinjection) or orexigenic (12 h postinjection) and the different species used, may explain the different findings between the two studies.

The studies reported here only examine the effects of a single administration of PYY$_{3-36}$ on food intake, and hence an effect on body weight would not be expected. We have previously demonstrated that chronic administration of PYY$_{3-36}$ causes a reduction in body weight (8). Repeat studies in our laboratory confirm that chronic administration of PYY$_{3-36}$ causes a reduction in body weight, and this has also been confirmed by other groups (4, 16, 35). The significant delayed orexigenic effect following early light-phase injection of PYY$_{3-36}$ reported in this manuscript was repeated in four separate groups of animals using PYY$_{3-36}$ from two different manufacturing runs. In addition, the PYY$_{3-36}$ analog demonstrated a similar delayed orexigenic effect, whereas GLP-1,

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**Fig. 7.** Effects of early light-phase injection of d-Allo Ile$^3$ PYY$_{3-36}$ on food intake in mice. A: ad libitum-fed mice were injected ip at 8:00 A.M. with either d-Allo Ile$^3$ PYY$_{3-36}$ or saline and fasted for 4 h. Food intake was measured 6, 8, 12, and 24 h postinjection. B: effects of ip injection at 8:00 A.M. of PYY$_{3-36}$ or saline on food intake in mice fasted overnight. d-Allo Ile$^3$ PYY$_{3-36}$ dose 23 nmol/kg; n = 12/group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. saline. Results are means ± SE.
OXM, and LiCl failed to induce a similar effect. Thus the delayed orexigenic effect following PYY3-36 administration appears to be a reproducible and specific effect.

PYY knockout mice have recently been shown to have an obese phenotype (9, 11, 42), suggesting that endogenous PYY3-36 has a predominantly anorexigenic effect. In this manuscript, we show that PYY3-36 reduces food intake using the same protocol we have previously used (8). In addition to its anorexigenic effects, we also show that PYY3-36 has a small delayed orexigenic effect that is independent of the acute reduction in food intake caused by PYY3-36. The studies in this manuscript are consistent with a predominantly anorectic effect of PYY3-36 that may be limited by a smaller delayed orexigenic effect.

In summary, we have shown that PYY3-36 acutely reduces food intake in both fed and fasted states. In addition, we have also shown that both PYY3-36 and its analog d-Allo Ile3-PYY3-36 can cause a delayed dark-phase orexigenic effect in mice fasted for 4 h postinjection, which did not occur following administration of GLP-1, OXM, or LiCl.
REFERENCES


