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ORIGINAL ARTICLE

Pulmonary delivery of peptide YY for food intake suppression and reduced body weight gain in rats

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Aims: Peptide YY (PYY) is an endogenous anorectic gut-secreted peptide that has been shown to suppress appetite in animals and humans, when given by injection. This study tested if needle-free pulmonary delivery of PYY enables food intake suppression and reduced body weight gain in rats. The PYY pharmacokinetics and effects on brain neuropeptide levels were also examined.

Methods: Rats received single or once-daily 7-day pulmonary administration of saline or PYYs. Food intake and body weight gain were monitored to study the effects of different doses (0.08–0.90 mg/kg) of PYY3-36, PYY1-36 and PYY13-36. Plasma PYY pharmacokinetics were determined via enzyme-linked immunosorbent assay. Changes in orexigenic neuropeptide Y (NPY) and c-Fos protein levels in the hypothalamus arcuate nucleus (ARC) were measured by immunofluorescence microscopy.

Results: PYY3-36 caused dose-dependent and 4- to 6-h food intake suppression following pulmonary delivery. At 0.80 mg/kg, the effect was significant with 35.1 ± 5.7 and 19.7 ± 4.2% suppression at 4 and 6 h, respectively. Repeated administration for 7 days reduced cumulative body weight gain by 39.4 ± 11.0%. PYY1-36, but not PYY13-36, was equipotent to PYY3-36 in food intake suppression. The plasma PYY concentration reached its peak at 10 min following pulmonary delivery with 12–14% of bioavailability. Increased c-Fos and reduced NPY expressions were observed in the hypothalamus ARC, consistent with the magnitude of food intake suppression by each of the PYYs.

Conclusions: Pulmonary delivery of PYY enabled significant 4- to 6-h food intake suppression via 12–14% of lung absorption and hypothalamic ARC interaction, leading to reduced body weight gain in rats.

Keywords: animal pharmacology, appetite control, antiobesity drug, experimental pharmacology, obesity therapy, pharmacokinetics

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Introduction

Peptide tyrosine-tyrosine (PYY1-36) is an endogenous 36-amino acid peptide found in the endocrine L cells of the intestinal mucosa, particularly lining the ileum and colon [1–3]. It is secreted into the blood circulation post-prandially, primarily in proportion to calorie intake, while its blood level remains low under the fasting state [1–3]. Upon secretion, a substantial portion of PYY1-36 is cleaved by dipeptidyl peptidase IV (DPPIV), yielding its N-terminus truncated, 34-amino acid peptide, PYY3-36 [1–3]. This PYY3-36 is considered to be one of the gut-secreted satiety (anorectic) signals that accesses the appetite regulatory centre of the brain, while also acting locally to delay gastric emptying and slow intestinal transit [1–3]. Many studies have now shown that exogenous administration of PYY3-36 caused significant suppression of food intake and appetite in both animals and humans [4–11]. Hence, the peptide is believed to be a potential therapeutic molecule in the treatment and/or management of obesity, a critical global epidemic sweeping the developed and parts of the developing world [3].

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Meal intake in humans is primarily controlled by the appetite regulatory centre of the brain, namely the hypothalamus and brainstem, although local peripheral actions by the adrenals, pancreas and gastrointestinal tract may also contribute to this control [12]. In this brain appetite regulatory centre, PYYs appear to act on the presynaptic neuropeptide Y (NPY) family receptors and alter the expression of orexigenic NPY for their effects on appetite and meal intake [1–3]. While PYY1-36 binds to all known NPY receptor subtypes, PYY3-36 exhibits the highest affinity to the Y2-receptor and less to the Y1- and Y5-receptors, the latter leading to reduced expression of orexigenic NPY [1–3,13]. It is likely therefore that the suppression of appetite and meal intake by PYY3-36 involves this Y2-receptor subtype that is abundantly expressed in the hypothalamus, specifically within the arcuate nucleus (ARC) [1–3,14]. Indeed, intra-ARC injection of PYY3-36 failed to suppress food intake in Y2-receptor knockout mice, and food intake suppression of PYY3-36 in wild-type mice was diminished by a Y2-receptor-specific antagonist, BIIE0246 [4,15]. Even so, a role of the vagus nerve and thus, the brainstem has been proposed for this PYY's effect on appetite, based on the Y2-receptor expression in the nodose ganglion and vagal afferents. However, evidence has been inconsistent and inconclusive, even in animals [16–20].

Regarding its therapeutic use in obesity, parenteral injections have been a logical choice for PYY, as the peptide is a

macromolecule with a molecular weight of 4 kDa and likely susceptible to enzymatic degradation, both of which would preclude adequate epithelial absorption, for example, from the intestinal mucosa following oral administration [21]. As a result, the effective routes of PYY administration have been limited to injections, such as intravenous, intraperitoneal or subcutaneous injection and/or infusion, in order to cause significant suppression of food intake or appetite in animals and humans [4–11,22]. Clearly, however, a needle-free and parenteral administration would be most desirable, given the likely need for repeated administration on a daily basis to achieve effective appetite control and body weight management. Indeed, attempts have been made to formulate PYY3-36 in a nasal spray with an absorption modulator; however, in a clinical trial, its preprandial administration failed to cause significant body weight loss [23]. It is therefore possible that the lung offers a better needle-free portal for PYY's systemic delivery and appetite and meal intake reduction by inhalation, especially without the need for absorption modulators, a successful scenario achieved in inhaled insulin for postprandial glycaemic control in diabetes [24].

Hence, this study tested the hypothesis that needle-free pulmonary delivery of PYY is effective in producing food intake suppression via systemic lung absorption and hypothalamic interaction, leading to reduced body weight gain in rats. PYY3-36 was tested with single and repeated pulmonary administration, as it is the 'active' form of circulating PYY for satiety. The endogenously secreted peptide, PYY1-36, and its 24-amino acid, smaller fragment peptide retaining the Y2-receptor-related activities, PYY13-36 [25–27], were also assessed to compare the molar potencies of food intake suppression following pulmonary delivery. The plasma PYY pharmacokinetics and bioavailability for pulmonary delivery of PYY3-36 were then determined to characterize its lung absorption and increased systemic levels. Finally, changes in neuronal activation of c-Fos protein and the expression of orexigenic NPY by pulmonary delivery of PYYs were measured in the hypothalamus ARC using immunofluorescence microscopy. This study was designed to show proof-of-concept for needle-free pulmonary delivery of PYY to cause effective appetite suppression and may lead to the future development of new treatment options for the management of obesity.

Materials and Methods

Materials

Human PYY1-36 (PYY1-36; molecular weight of 4310 Da) and PYY3-36 (molecular weight of 4050 Da) were purchased from American Peptide Company (Sunnyvale, CA, USA), whereas rat PYY13-36 (PYY13-36; molecular weight of 3014 Da) was from Bachem Americas (Torrance, CA, USA). These were received as lyophilized powders, certified to contain 82.8, 82.2 and 80.7% of the peptide, respectively, and freshly reconstituted with saline to prepare their dosing solutions prior to each experiment. Isoflurane (USP) and sodium pentobarbital (Nembutal®; 50 mg/ml), respectively, were obtained from Webster Veterinary Supply (Sterling, MA, USA) and Ovation Pharmaceuticals (Deerfield, IL, USA). The total human PYY enzyme-linked immunosorbent assay (ELISA) kit was from Millipore Corporation (Billerica, MA, USA). Paraformaldehyde (EM grade) and Tissue-Tek® OCT embedding medium were from Ted Pella (Redding, CA, USA). The rabbit anti-rat c-Fos and NPY antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Bachem, respectively, while the Cy3- and Cy2-labelled goat anti-rabbit immunoglobulin G (IgG) antibodies and normal goat serum were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Other reagents such as phosphate-buffered saline (PBS), Triton-X 100, bovine serum albumin and Sudan Black B were from Sigma-Aldrich (St. Louis, MO, USA).

Animals

This research adhered to the National Institutes of Health (NIH) policy, Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised in 1985). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU). Male Sprague-Dawley rats (specific pathogen free) weighing 250–275 g were purchased from Hilltop Lab Animals (Scottdale, PA, USA). Each animal was housed individually in a metabolic cage (Harvard Apparatus, Holliston, MA, USA) and acclimatized for 7 days at the VCU animal care facility under controlled conditions with respect to temperature (20–23 °C), relative humidity (40–70%) and light–dark cycling (12–12 h; the light cycle between 06:00 and 18:00 hours). Throughout the experiments, animals had free access to preweighed standard rat diet (7012; Harlan Teklad, Boston, MA, USA) and tap water, so that their intake was determined and monitored by weight difference in addition to daily body weight gain. As suggested elsewhere [28], the 7-day acclimatization was found to be necessary to yield physiological values of daily food and water intake and body weight gain; these were indeed reproducible 30.4 ± 0.6, 30.5 ± 0.3 and 5.8 ± 0.1 g, respectively, across the animals used in this study.

Pulmonary Administration of PYYs in Rats

As rats are nocturnal in nature [29], PYYs or saline was administered at ~1 h before the dark cycle commenced, that is, ~17:00 hours, in all experiments, in order to monitor their dark-phase free food intake following administration. Each of the PYY (PYY1-36, PYY3-36 or PYY13-36) solutions, or saline, was administered into the rat lung via either intratracheal or orotracheal instillation under short-term (<5 min) anaesthesia with isoflurane. After 7-day acclimatization, each rat was anaesthetized in a small chamber box with 4% (v/v) isoflurane generated from a vaporizer (Ohmeda Tech 4 Surgivet®; Smith Medical North America, Waukesha, WI, USA). For the intratracheal instillation, the animal was placed in a supine position on a surgical board inclined at 30°. The tracheal region was surgically exposed, and 0.06 ml of PYY solution or saline was directly instilled into the lung using an insulin syringe (1 ml; Becton Dickinson, Franklin Lakes, NJ, USA) by projecting its needle tip just

before the bifurcation in the airway lumen. Immediately, the incision was sealed with the Nexaband® surgical glue (Webster Veterinary Supply). Meanwhile, the orotracheal instillation was carried out to the animals placed on the surgical board inclined at 60°. Using a small animal fibre-optic laryngoscope (LS-1; Penn-Century, Philadelphia, PA, USA), the tracheal lumen was visually confirmed through the oral cavity and then, 0.1 ml of PYY solution or saline was directly instilled into the lung using a custom-made dosing device, a 1-ml microsyringe attached to 8.5-cm PEEK™ extension tubing (Upchurch Scientific, Oak Harbor, WA, USA); the tubing was inserted 3 cm down within the tracheal lumen to position its tip just before the bifurcation. In both cases, animals recovered from the anaesthesia within 5 min post-administration, followed by monitoring of food and water intake and body weight gain in the animal care facility. Our in-house satellite validation using a marker solute, 4.4 kDa fluorescein isothiocyanate-labelled dextran, had ensured consistent 95.7 ± 4.0% of the lung lobar delivery by the two lung-dosing techniques.

Subcutaneous Injection of PYY in Rats

This route of administration was tested to compare the food intake suppression of PYY3-36 with pulmonary administration. Rats were first subjected to a sham operation of the intratracheal instillation under the isoflurane anaesthesia, as described above, that is, without solution instillation. Subsequently, 0.3 ml of PYY3-36 solution was subcutaneously injected into the fold of the neck skin. Following full recovery from the anaesthesia, food and water intake and body weight gain were monitored at the animal care facility.

Food Intake Suppression Following Single Pulmonary or Subcutaneous PYY Administration

Rats were divided into seven groups to assess the effects of different doses, PYYs and routes of administration on the food and water intake and the body weight gain. The groups were composed of intratracheal instillation of (i) saline (n = 12); (ii–iv) PYY3-36 at 0.08, 0.40 and 0.80 mg/kg (n = 7–8); (v) PYY1-36 at 0.90 mg/kg (n = 6); (vi) PYY13-36 at 0.60 mg/kg (n = 7) and (vii) subcutaneous injection of PYY3-36 at 0.80 mg/kg in the sham-operated animals (n = 5). Cumulative food intake was assessed at 2, 4, 6 and 24 h following administration, determined by weight difference from the preweighed diet placed at the time of administration. Water intake and body weight gain were also monitored daily by weight difference.

Reduced Body Weight Gain in Response to Repeated Pulmonary Administration of PYY

A total of 20 rats were divided into three groups with n = 6–7. At ~1 h before the dark cycle, that is, ~17:00 hours, a group of animals received once-daily orotracheal instillations of (i) saline (n = 7) or (ii–iii) PYY3-36 at 0.08 (n = 6) or 0.80 mg/kg (n = 7) for 7 days. Cumulative food and water intake and body weight gain were monitored daily (every 24 h) for 8 days following the first instillation. In addition, the 4-h dark-phase food intake was also determined on days 1, 4 and 7, in order to assess the continuity and consistency of the food intake suppression.

Plasma PYY3-36 Pharmacokinetics Following Pulmonary Administration

Rats were anaesthetized with an intraperitoneal injection of Nembutal at 50 mg/kg. PYY3-36 was then administered to the lungs at 0.08 or 0.80 mg/kg by intratracheal instillation (n = 4 at each dose), as described above. An aliquot (0.1 ml) of the blood was withdrawn from the jugular vein at 0, 5, 10, 20, 30, 45, 60, 90 and 120 min following instillation. The plasma samples were obtained via centrifugation and then analysed with the PYY ELISA kit, in accordance with the manufacturer's protocol using the matrix-matched calibration standards. This kit had been shown to have no specificity to rat PYY. Our in-house validation yielded ≤11.5 and ≤12.3% of the assay accuracy and precision, respectively, and 5.0 ng/ml in rat plasma as the lower limit of quantification. A separate group of animals (n = 4) received an intravenous bolus injection of PYY3-36 at 0.08 mg/kg, followed by blood sampling for 120 min and PYY determination via the validated ELISA.

The plasma concentration–time profile of PYY3-36 following intratracheal instillation or intravenous injection in each animal was analysed to derive the following pharmacokinetic parameters. The peak concentration (C_{max}) and the time required to reach C_{max} (T_{max}) were determined visually. The terminal half-life ($t_{1/2}$) was computed from the slope (β) of the semi-logarithmic plasma profile at ≥60 min ($t_{1/2} = 0.693/\beta$) [30]. The area under the plasma concentration-time curve (AUC0-inf) was calculated by the trapezoidal method for 120 min plus Clast/β, where Clast was the plasma concentration at 120 min [30]. The percent absolute bioavailability (%F) for intratracheal instillation was calculated from its dose-normalized AUC0-inf, relative to that for intravenous injection [30].

Immunofluorescence c-Fos and NPY Detection in the Hypothalamus ARC

Changes in c-Fos protein and orexigenic NPY levels in the hypothalamus ARC in response to PYY administration were assessed via immunofluorescence microscopy at 4 h following single intratracheal instillation. Under the Nembutal anaesthesia, the brain was fixed via 20-min transcardial perfusion of 4% (w/v) paraformaldehyde at 25 ml/min, after which the midbrain section including the hypothalamus was dissected, referenced to the optic chiasm and cerebellum as landmarks [31]. This tissue block was further fixed for 1 h under vacuum and cryoprotected for 48 h with 30% (w/v) sucrose. They were embedded in Tissue-Tek OCT, snap frozen under dry ice and liquid N2 and stored at −70 °C prior to sectioning. Frozen 30 µm thick coronal sections were prepared using a cryostat (Microm HM 550; Thermo Fisher Scientific, Waltham, MA, USA); they were air-dried and stored at −20 °C prior to immunofluorescent detection.

The sections were selected to include the ARC of the hypothalamus [31]. They were first processed for the removal of the

embedding medium, followed by dehydration in 70–100% (v/v) serially graded ethanol baths. The sections were then rehydrated and blocked from non-specific binding via 1 h incubation with PBS containing 0.5% (v/v) Triton-X, 4% (w/v) bovine serum albumin, 5% (w/v) dry non-fat milk and 10% (v/v) normal goat serum. They were incubated overnight at 4 °C with rabbit anti-rat c-Fos or NPY antibody diluted to 1 : 100 or 1 : 200, respectively, and then for 1 h with Cy3- or Cy2-labelled goat anti-rabbit IgG antibody diluted to 1 : 200. Sudan Black B counter stain was applied for morphological identification of ARC and quenching of autofluorescence signals. The immunofluorescence images were captured under the BX40 fluorescence microscope (×400 magnification; Olympus, Center Valley, PA, USA) at respective excitation/emission wavelengths of 510/590 nm and 450/515 nm for c-Fos and NPY, using a CCD camera (Retiga-2000R; QImaging, Surrey, BC, Canada). For c-Fos, they were analysed with Image-Pro® Plus 5.1 (Media Cybernetics, Bethesda, MD, USA) to be quantified as the c-Fos-positive cell counts within the representative rectangular 488 × 720 pixel image areas of the bilateral ARC regions. In contrast, the NPY immunofluorescence was visually assessed and thus, remained semi-quantitative, as its quantification was not practical with pixel values.

Statistical Analysis

Results of the food and water intake, body weight gain and c-Fos-positive cell counts were expressed as group mean ± s.e. Statistical analyses of group comparisons were carried out using Prism® 4 (GraphPad Software, San Diego, CA, USA) or JMP® 8 (SAS Institute, Cary, NC, USA). One- or two-way analysis of variance (ANOVA) was first used to identify the statistical difference between groups, where $p < 0.05$ was considered significant. *Post hoc* analysis of the multiple comparison testing was performed by Tukey's or Dunnett's method, the latter being specifically used to compare each of the multiple test groups against the control group, but not among each other. The p-values were classified into $p < 0.05$ (significant) and $p < 0.01$ (highly significant). No comparisons reached $p < 0.001$ (extremely significant).

Results

Food Intake Suppression Following Single Pulmonary Administration of PYY3-36

Figure 1 shows (a) the cumulative food intake at 2, 4, 6 and 24 h following single intratracheal instillation of saline or PYY3-36 at 0.08, 0.40 and 0.80 mg/kg and (b) its comparison with subcutaneous injection at 0.80 mg/kg. Following pulmonary delivery, the highest dose at 0.80 mg/kg caused significant 35.1 ± 5.7 and 19.7 ± 4.2% suppression of the cumulative food intake at 4 and 6 h, respectively, compared to the saline control ($p < 0.01$ and $p < 0.05$, Dunnett's test). However, the food intake suppression was not significant at 2 and 24 h or at lower doses (0.08 and 0.40 mg/kg) at any of the time points (figure 1a). These data show that PYY's effect of food intake suppression was short-lived (i.e. transient) and dose-related

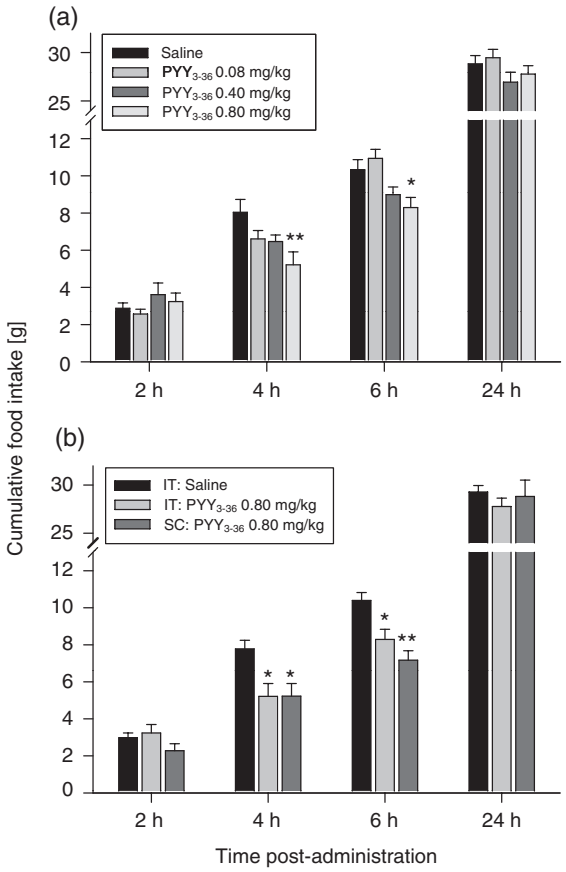

Figure 1. Cumulative free food intake at 2, 4, 6 and 24 h in rats (a) following single intratracheal (IT) instillation of saline (n = 12) and PYY3-36 at 0.08 (n = 8), 0.40 (n = 7) and 0.80 mg/kg (n = 7) and (b) following single IT instillation of saline (n = 12) and PYY3-36 at 0.80 mg/kg (n = 7) and single subcutaneous injection (SC) of PYY3-36 at 0.80 mg/kg (n = 5). Data represent mean ± s.e. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the corresponding values in the saline control animals, derived from the one-way ANOVA followed by Dunnett's multiple comparison test. PYY, peptide YY.

following pulmonary delivery. Correspondingly, the food intake during the 6- to 24-h period was consistent across the groups ($p > 0.05$, ANOVA; data not shown), also suggesting that appetite rebound or postponed food intake initiation was unlikely the reason for the PYY's effect by this pulmonary delivery. Meanwhile, subcutaneous injection of PYY3-36 at 0.80 mg/kg caused significant food intake suppression, also at 4 and 6 h ($p < 0.05$ and $p < 0.01$, Dunnett's test), which was mirrored to that by pulmonary delivery at the same dose (figure 1b). This supported the notion that, when compared at the same dose of 0.80 mg/kg, pulmonary delivery of PYY3-36 was equipotent to subcutaneous injection. Neither the daily water intake nor the body weight gain was affected by this single administration of PYY3-36 ($p > 0.05$, ANOVA; data not shown).

Food Intake Suppression Following Single Pulmonary Administration of Different PYYs

Figure 2 shows the cumulative food intake at 2, 4, 6 and 24 h following single intratracheal instillation of PYY1-36 at

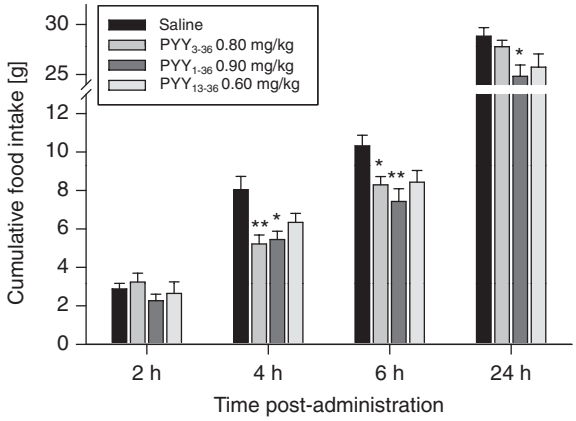

Figure 2. Cumulative free food intake at 2, 4, 6 and 24 h in rats following single intratracheal instillation of saline (n = 12), PYY3-36 at 0.80 mg/kg (n = 7), PYY1-36 at 0.90 mg/kg (n = 6) and PYY13-36 at 0.60 mg/kg (n = 7). The PYY doses are all equimolar at 0.2 µmol/kg. Data represent mean ± s.e. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the corresponding values in the saline control animals, derived from the one-way ANOVA followed by Dunnett's multiple comparison test. PYY, peptide YY.

0.90 mg/kg and PYY13-36 at 0.60 mg/kg, compared to that for saline (control) and PYY3-36 at 0.80 mg/kg (the latter comparator data were already shown in figure 1). To assess their molar potencies with this pulmonary delivery, the doses of PYY1-36 and PYY13-36 were chosen to be 0.2 µmol/kg, as significant food intake suppression was seen in figure 1 at its equimolar dose of PYY3-36, that is, 0.80 mg/kg. PYY1-36, the endogenously secreted form of PYY, exhibited significant 32.2 ± 5.3 and 28.0 ± 6.4% suppression of the cumulative food intake at 4 and 6 h, respectively, compared to the saline control ($p < 0.05$ and $p < 0.01$, Dunnett's test). This food intake suppression for PYY1-36 was consistent with that for PYY3-36 (35.1 ± 5.7 and 19.7 ± 4.2%, respectively), and thus, PYY1-36 and PYY3-36 were equipotent for the 4- and 6-h effects following pulmonary administration. Notably, however, the cumulative food intake for PYY1-36 at 24 h remained significantly lower than that for the saline control by 13.9 ± 3.9% ($p < 0.05$, Dunnett's test). This may have implied the sustained effect of this endogenously secreted gut peptide with pulmonary administration, which was not the case for PYY3-36. In contrast, at this 0.2 µmol/kg dose, the smaller Y2-receptor-active fragment peptide, PYY13-36 (0.60 mg/kg) caused marginal (18–21%) and statistically insignificant food intake suppression at 4 and 6 h, compared to the saline control ($p > 0.05$, Dunnett's test; figure 2). It was likely therefore that this smaller peptide was not as potent and thus beneficial as PYY3-36 and PYY1-36 with respect to the food intake suppression by pulmonary delivery, despite its faster lung absorption expected from a smaller molecular size (i.e. 3 kDa).

Reduced Body Weight Gain in Response to Repeated Pulmonary Administration of PYY3-36

Figure 3 shows the profiles of cumulative food intake and body weight gain in response to once-daily orotracheal instillations

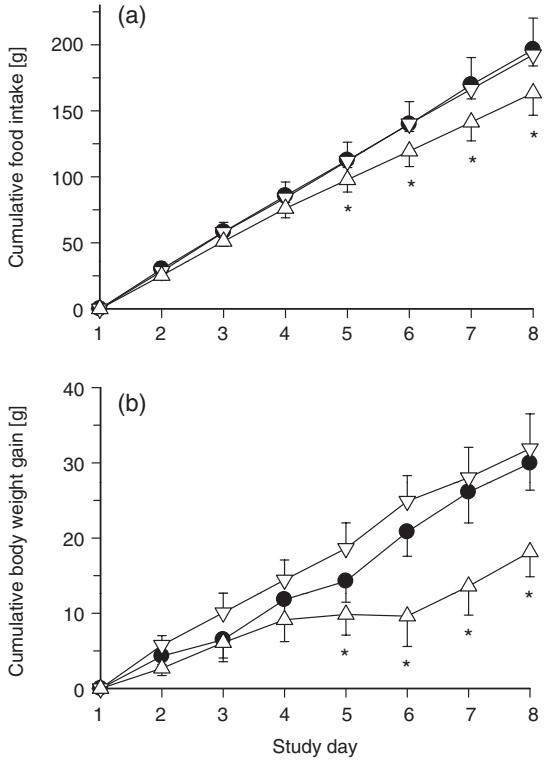

Figure 3. Cumulative (a) food intake and (b) body weight gain in response to once-daily 7-day orotracheal instillations of saline (●; n = 7) and PYY3-36 at 0.08 (▽; n = 6) and 0.80 mg/kg (△; n = 7) in freely feeding rats. Orotracheal instillations were carried out daily just before the dark cycle on day 1 through day 7. Data represent mean ± s.e. * indicates $p < 0.05$, compared to the corresponding value in the saline control animals, derived from the two-way ANOVA followed by Tukey's multiple comparison test. PYY, peptide YY.

of saline or PYY3-36 at 0.08 and 0.80 mg/kg for 7 days. The cumulative food intake for PYY3-36 at 0.80 mg/kg became significantly lower than that for the saline control on day 5 and thereafter ($p < 0.05$, Tukey's test; figure 3a), resulting in 16.7 ± 8.6% suppression by day 8. During this period, percent food intake suppression at 4 h post-instillation on days 1, 4 and 7 remained significant ($p < 0.01$, Dunnett's test) and consistent at 30.8 ± 6.3, 32.4 ± 9.8 and 35.7 ± 11.0%, respectively. Accordingly, the cumulative body weight gain for PYY3-36 at 0.80 mg/kg became significantly lower than that for the control on day 5 and thereafter ($p < 0.05$, Tukey's test; figure 3b), resulting in 39.4 ± 11.0% reduction by day 8. Meanwhile, the lower 0.08 mg/kg dose failed to reduce either the cumulative food intake or the body weight gain, showing the profiles overlaid to those for the saline control (figure 3). The daily water intake was again unaffected by this repeated daily administration of PYY3-36 throughout the experimental periods.

Plasma PYY3-36 Pharmacokinetics Following Pulmonary Administration

Figure 4 shows the plasma concentration–time profiles of immunoreactive PYY following intratracheal instillation of

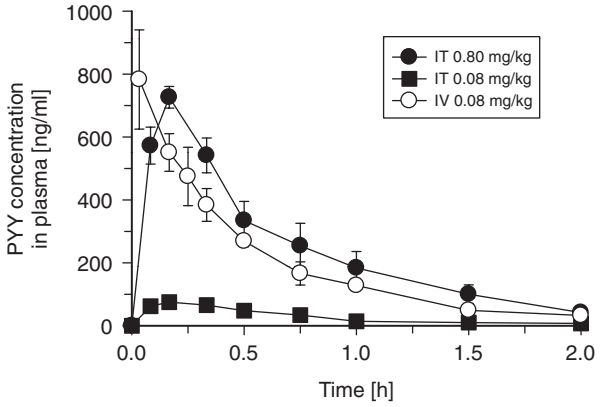

Figure 4. Immunoreactive peptide YY (PYY) concentration in plasma versus time profiles in rats following intratracheal instillation of PYY3-36 at 0.08 and 0.80 mg/kg and bolus intravenous injection of PYY3-36 at 0.08 mg/kg. Data represent mean ± s.e. from four rats (n = 4).

PYY3-36 at 0.08 and 0.80 mg/kg and intravenous bolus injection of PYY3-36 at 0.08 mg/kg. Following pulmonary administration, the plasma PYY concentrations were elevated quite rapidly with a T_{max} of 10 min, reaching the supraphysiological level at both these two doses. However, the profiles also declined rapidly with the terminal $t_{1/2}$ values of 25.5 ± 9.0 and 32.6 ± 4.0 min, respectively. As these $t_{1/2}$ values for pulmonary administration were consistent with 26.1 ± 1.9 min for intravenous injection, it was likely that the systemic elimination was still the slowest kinetic process for PYY3-36 following pulmonary delivery, while its lung absorption was fast, given the short T_{max} of 10 min. Even so, the percent absolute bioavailability (%F) remained low at 14.1 ± 1.1 and 12.2 ± 3.5% at these two doses, which suggested substantial loss of the peptide within the lung via processes other than absorption.

Hypothalamus c-Fos and NPY Expression Following Pulmonary PYY Administration

The immunofluorescence staining protocols were validated for the absence of detection via non-specific binding, as omission of the primary or secondary antibody resulted in negligible fluorescent signals (data not shown). Thus, figure 5 shows representative micrographs for c-Fos- and NPY-specific expressions in the hypothalamus ARC at 4 h following single intratracheal instillation of saline, PYY3-36 at 0.08 and 0.80 mg/kg, PYY1-36 at 0.90 mg/kg and PYY13-36 at 0.60 mg/kg. Each group was tested with three animals to ensure consistent expression, and as shown by the error bars for the c-Fos-positive cell counts in figure 6, the data were fairly reproducible for each group. Overall, the changes of both c-Fos and NPY expressions by each of the PYYs were consistent with the magnitude of the food intake suppression shown in figures 1 and 2. At 4 h, the c-Fos expression in the ARC appeared to be the highest for PYY3-36 at 0.80 mg/kg and PYY1-36 at 0.90 mg/kg, and their c-Fos-positive cell counts were indeed 5.0- and 4.6-fold higher than those in the saline control, respectively (figure 6); these corresponded to their greatest and equivalent food intake suppression by 30–35% (figures 1 and 2). In contrast, the c-Fos-positive cell counts were only moderately increased by

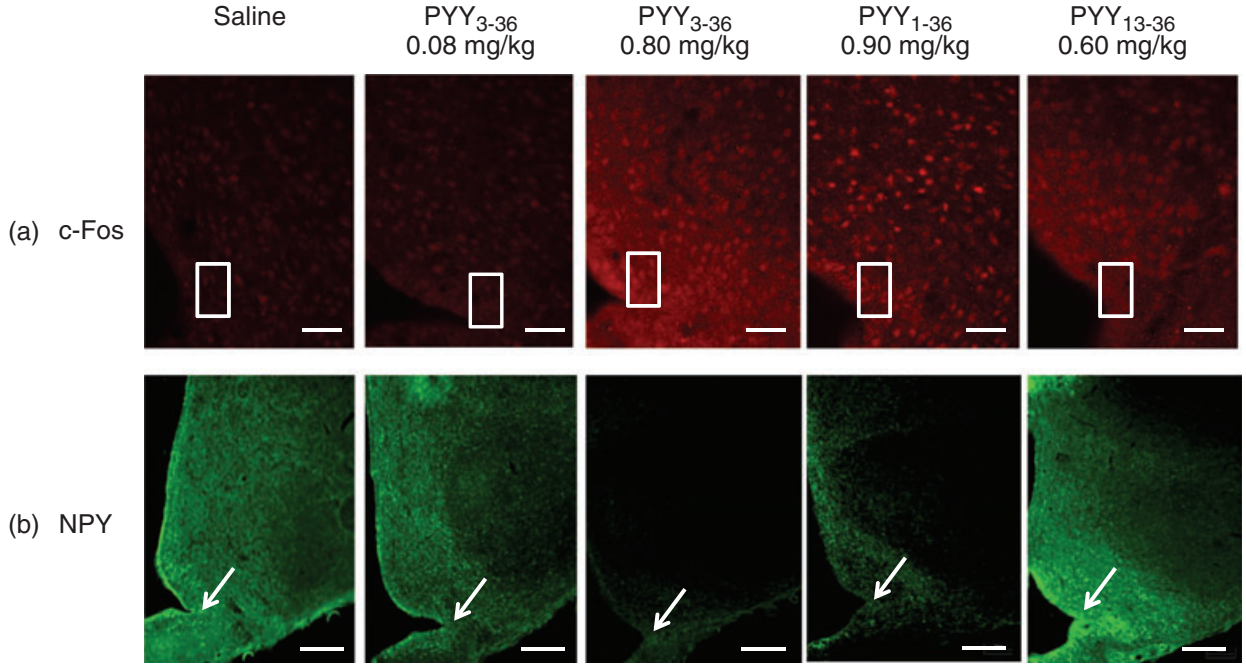

Figure 5. Representative immunofluorescence micrographs of the rat coronal brain sections (30 µm in thickness), showing (a) c-Fos and (b) neuropeptide Y (NPY) specific expression in the right hypothalamus. The brains were taken at 4 h following single intratracheal instillation of saline, PYY3-36 at 0.08 and 0.80 mg/kg, PYY1-36 at 0.90 mg/kg and PYY13-36 at 0.60 mg/kg. The scale bars represent 50 µm. The box in (a) c-Fos expression indicates the rectangular 488 × 720 pixel area in the arcuate nucleus (ARC) used for c-Fos-positive cell counts in figure 6; the arrow in (b) NPY expression indicates the approximate location of the ARC for visual comparison. PYY, peptide YY.

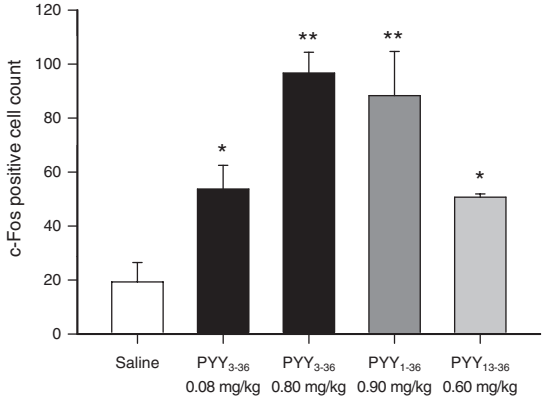

Figure 6. c-Fos-positive cell counts in the rectangular 488 × 720 pixel areas of the bilateral hypothalamus arcuate nucleus (ARC), obtained from each group of animals at 4 h following single intratracheal instillation of saline, PYY3-36 at 0.08 and 0.80 mg/kg, PYY1-36 at 0.90 mg/kg and PYY13-36 at 0.60 mg/kg. Data represent mean ± s.e. from three rats ($n = 3$). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the value of the saline control animals, derived from the one-way ANOVA followed by Dunnett's multiple comparison test. PYY, peptide YY.

2.8- and 2.6-fold, respectively, for PYY3-36 at 0.08 mg/kg and PYY13-36 at 0.60 mg/kg (figure 6), again in line with their lower and insignificant food intake suppression (figures 1 and 2, respectively). These results suggested quantitative association of the food intake suppression by PYYs administered to the lung with the neuronal activation of the increased c-Fos expression in the hypothalamus ARC. In contrast, it was observed that the NPY expression in the ARC was lowest for PYY3-36 at 0.80 mg/kg and PYY1-36 at 0.90 mg/kg, compared to PYY3-36 at 0.08 mg/kg, PYY13-36 at 0.60 mg/kg and importantly, the saline control (figure 5). This again conformed to the magnitude of the food intake suppression by each of these PYYs and doses (figures 1 and 2), which provided strong evidence that the reduced expression of orexigenic NPY in the hypothalamus ARC was involved in the mechanisms of the food intake suppression following pulmonary PYY administration, as is the case for its physiological control [1–3].

Discussion

The present study is the first to show that needle-free pulmonary delivery of PYY suppresses food intake and reduces body weight gain significantly in rats. Following pulmonary delivery, food intake suppression by PYY3-36 was shown to be time- and dose-related (figure 1). At 0.80 mg/kg, the food intake suppression was transient for 4–6 h, yet significant by 20–35%, which was equipotent to subcutaneous injection (figure 1). Its once-daily administration for 7 days enabled 39.4 ± 11.0% reduction of the cumulative body weight gain (figure 3), presumably by virtue of this transient (4–6 h) food intake suppression on a daily basis. Following PYY3-36 administration, the plasma PYY concentration reached the highest level in 10 min, suggesting kinetically fast lung absorption of this peptide, despite 12–14% of the systemic bioavailability (figure 4). Notably, the endogenously secreted form of PYY, PYY1-36, exhibited equipotent and possibly sustained suppression of food intake, compared to PYY3-36 (figure 2). In contrast, the fragment peptide, PYY13-36, appeared to be less potent (figure 2), despite its faster lung absorption expected from a smaller molecular size (i.e. 3 kDa). In the hypothalamus ARC, the reduced expression of orexigenic NPY was observed following pulmonary PYY administration, accompanied with the increased c-Fos expression of neuronal activation (figures 5 and 6), both of which conformed to the magnitude of the food intake suppression by each of the PYYs. Therefore, the food intake and body weight effects by pulmonary delivery of PYY shown in figures 1–3 can be attributed to their lung absorption and increased systemic level (figure 4) that then reduced orexigenic NPY in the hypothalamus ARC (figure 5).

It was quite essential in this study to first ensure near-physiological monitoring of food and water intake and body weight gain in rats, because pulmonary dosing operations of PYYs required anaesthesia with or without surgical incision. As indicated elsewhere [28], the acclimatization and habituation were so critical that individual housing in the metabolic cages for the 7-day period in the well-controlled facility was required prior to the experiment. In addition, anaesthetic duration and surgical incision were minimized for quick recovery from the dosing operation within 10 min. Even so, the daily food and water intake and body weight gain following saline administration in the control group were slightly lower than those during the acclimatization period (e.g. 28.8 ± 0.9 g vs. 30.4 ± 0.6 g of the daily food intake, respectively), which obliged this study to be carried out under near-physiological conditions. This limitation, however, appeared to arise from the isoflurane anaesthesia rather than the surgical incision, as the food intake profiles were perfectly mirrored between the intratracheal and orotracheal instillation of saline, the operations with and without the surgical incision, respectively (data not shown). Additionally, subcutaneous injection of PYY3-36 in the sham-operated, once-anaesthetized rats in this study was shown to cause food intake suppression (32.8 ± 8.7% at 4 h by 0.80 mg/kg; figure 1b) equivalent to that without the anaesthesia and surgical incision (30% at 4 h by 1.0 mg/kg) [22]. Finally, by virtue of relatively reproducible food intake data in our housing and monitoring system, the food intake suppression by PYYs was able to be assessed using 5–8 animals in each group, compared to over 10 animals typically used in the literature [4–8,10,11,22]. Hence, the present study was sufficiently sensitive and appropriately powered to assess the anorectic effects of PYYs in rats following pulmonary delivery.

The endogenously 'active' form of circulating PYY, PYY3-36, caused the transient 4- to 6-h food intake suppression in a dose-related fashion following pulmonary administration, achieving significant 20–35% suppression at 0.80 mg/kg (figure 1). Despite this success of the needle-free food intake suppression, the 0.80 mg/kg dose was considerably higher than the effective doses for intravenous or intraperitoneal injection in rats (0.01–0.1 mg/kg) [4,7,22]. This, by definition, implied low-to-marginal systemic bioavailability for this 4-kDa peptide following pulmonary delivery, as has been the case for many other peptides in this molecular weight range (<20%) [32].

Indeed, the bioavailability (%F) was found to be only 12–14%, presumably because of PYY's non-absorptive local degradation within the lung like other peptides [32]. Meanwhile, it is likely that the rapid systemic disappearance of PYY3-36 in 2 h (figure 4) resulted in only the transient (4–6 h) suppression of food intake, as shown in figure 1. Even so, the cumulative body weight gain was significantly reduced by 39.4% following once-daily instillations for 7 days (figure 3). In this regard, recent evidence supports PYY's effects on the body weight control via increased energy expenditure and altered fuel partitioning in favour of fat oxidation [33–35]. Indeed, when studied with diet-induced obese rats for over 2 weeks, certain modes of continuous PYY3-36 infusion enabled significant body weight loss [36,37]. Meanwhile, compensatory food intake increase or pharmacological tolerance was also suggested for PYY3-36 administered exogenously, which appears to have conflicted with PYY's effects on the reduced body weight gain; however, a bolus mode of injection was typically used, failing to show such body weight effects [38]. Hence, there may be an implication with the dosing route and/or regimen of PYY3-36, for example, dose, frequency and/or period, in order to attain effective body weight loss in a long term. Clearly, it is of interest to assess if pulmonary delivery of PYY causes such a body weight loss in obese animal models.

The literature has been so far inconsistent as to the effect and potency of the endogenously secreted form of PYY, PYY1-36, following exogenous administration, compared to PYY3-36. In humans, PYY1-36 caused no inhibitory effects on the appetite rating by subcutaneous injection, while PYY3-36 increased the satiety [11]. Similarly, in rats, PYY1-36 was an order of magnitude less potent than PYY3-36 in food intake suppression and gastric emptying inhibition by intravenous infusion [7,39]. In contrast, however, for subcutaneous infusion in rats, PYY1-36 and PYY3-36 exhibited equipotent food intake suppression, attributed to efficient PYY1-36 conversion to the 'active' PYY3-36 by DPPIV [9]. In the present study, pulmonary delivery of PYY1-36 was also shown to be equipotent to PYY3-36 in the 4- to 6-h food intake suppression (figure 2). As PYY1-36 itself is unlikely anorectic because of a lack of Y2-receptor selectivity [1–3,13], sufficient PYY1-36 conversion to PYY3-36 by DPPIV, not only in the systemic circulation but also in the lung, can be speculated. Indeed, physiological PYY1-36-to-PYY3-36 conversion appears to be capacity-limited by DPPIV and thus incomplete in the systemic circulation; PYY3-36 accounts for only 37–57% of the total PYY immunoreactivity in plasma [40]. Moreover, the enzyme has been shown to be more abundant in the lung than the serum or intestinal mucosa [41,42]. If so, it can be further speculated that the food intake suppression at 24 h by PYY1-36 administered to the lung (figure 3) was potentially associated with this conversion by DPPIV in the lung, in addition to that in the blood circulation, which somehow sustained PYY3-36 appearance in the circulation. Meanwhile, despite faster lung absorption expected from its smaller molecular size (3.0 kDa), the fragment peptide, PYY13-36 was shown to be only marginally effective following pulmonary delivery (figure 2). Unfortunately, this appeared to be consistent with less than a half potencies of PYY13-36 than those for the full-length peptide as to Y2-receptor-related activities, for example, NPY binding displacement in the rat brain and inhibition of catecholamine synthesis in the rat mesenteric bed [25–27]. It is therefore likely that this lower molar potency of PYY13-36 to the Y2-receptor could not be overcome by faster lung absorption, when administered at the equimolar dose of PYY1-36 and PYY3-36.

Suppression of appetite and food intake by PYY3-36 has been attributed, in most cases, to reduced expression of orexigenic NPY in the hypothalamus ARC via interactions with the Y2-receptor [1–3]. This was the reason why c-Fos and NPY expressions in the hypothalamus ARC were measured in this study, as evidence that PYYs administered to the lung reached the target brain location for the effects via systemic lung absorption. Indeed, neuronal c-Fos activation and reduced NPY expression were observed following pulmonary delivery (figures 5 and 6), in line with the magnitude of the food intake suppression by each of the PYYs and doses (figures 1 and 2). Even so, the literature showed elevated c-Fos expression in the brainstem and increased vagal afferent firing following peripheral injection of PYY3-36, leaving the possibility of vagus nerve involvement in the PYY's effects on appetite [16–20]. In this context, our satellite attempts of immunofluorescence microscopy had resulted in negligible c-Fos expression in the brainstem (e.g. area postrema) following pulmonary delivery of PYY3-36 at 0.80 mg/kg (data not shown). Hence, it is our conclusion that the mechanisms of the food intake suppression by pulmonary delivery of PYYs involved the hypothalamus ARC, but not the brainstem and vagal stimulation.

The effective lung dose of PYY3-36 at 0.80 mg/kg caused supraphysiological plasma PYY levels at 10.3–179.3 nM (41.8–726.3 ng/ml) for 2 h in rats (figure 4). This was nearly 1000-fold higher than their physiological PYY concentrations of 42–160 pM in response to mixed-nutrient meals [43], which raised a concern if the food intake suppression by pulmonary PYY delivery in figures 1–3 was merely as a result of the production of non-specific malaise (e.g. nausea). In fact, it was reported that PYY3-36 infusion at 3.8–14.6 μg/kg in rats caused dose-dependent conditioned taste aversion, that is, malaise, along with the food intake suppression [44]. Nevertheless, this seemed unlikely to be the case and otherwise, the lowest lung dose of PYY3-36 at 0.08 mg/kg should have caused malaise-induced food intake suppression, as its plasma PYY levels at 1.8–18.5 nM (7.2–75.0 ng/ml; figure 4) were still quite supraphysiological for 2 h (i.e. ~100-fold higher than the physiological level); yet, there was no food intake suppression at this dose, as shown in figure 1. In humans, however, this malaise or nausea appears to be the limitation of PYY's use for appetite suppression. Intravenous infusion of PYY3-36 at 0.4 and 0.8 pmol/kg/min caused nausea, vomiting and/or abdominal discomfort in 25 and 65% of the lean subjects, respectively, despite the significant anorectic effects [8]. Likewise, preprandial nasal delivery of PYY3-36 at 0.6 mg three times a day was discontinued in 59% of the obese subjects in a clinical study because of nausea and vomiting [23]. It was therefore clear that a significant challenge exists in pulmonary PYY delivery if appetite suppression and

body weight effects seen in this study with rodents can be obtained in humans without such malaise-induced adverse effects. However, such a translation seems to be quite hard between rodents and humans as a result of species differences of the effective dose required for the appetite suppression. Nevertheless, in favour of pulmonary delivery, the effective PYY3-36 doses appeared to be much lower in humans than in rodents. For intravenous infusion, an over ninefold lower dose of PYY3-36 (0.1 nmol/kg) was necessary for the effects of appetite suppression in humans, compared to rodents (0.9–9.0 nmol/kg) [4–9,22]. Similarly, for subcutaneous injection, a PYY3-36 dose of 0.2 nmol/kg was sufficiently effective in humans [11], while a 1000-fold higher dose of 0.2 μmol/kg (0.8 mg/kg) was necessary in rats, as shown in figure 1b in this study. Hence, it is quite critical to first identify the effective pulmonary dose for appetite suppression and body weight control in humans, once other concerns such as the effects in obese models, adverse effects, long-term safety and inhaled formulations are resolved in a preclinical level.

In conclusion, pulmonary delivery of PYY3-36 at 0.80 mg/kg (0.2 μmol/kg) was shown to cause significant 4- to 6-h food intake suppression in rats (figure 1), leading to the reduced body weight gain by 39.4% following once-daily administration for 7 days (figure 3). It was also intriguing that the endogenously secreted PYY, PYY1-36, enabled equipotent and possibly sustained food intake suppression following pulmonary delivery (figure 2). The mechanisms for these effects involved the reduced orexigenic NPY in the hypothalamus ARC, accompanied with the neuronal c-Fos activation, as a result of the increased systemic levels through lung absorption with 12–14% of bioavailability (figures 4–6). Although it is still premature to predict similar success in humans, this study serves as proof-of-concept for needle-free systemic delivery of PYY through the lung for the effective appetite and body weight control, potentially for the future development of new treatment options for the management of obesity.

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Conflict of Interest

P. P. N., R. M. C. and M. S. designed the study. P. P. N. and M. S. conducted, collected, analysed the data, and wrote the manuscript. R. M. C. also wrote the manuscript. The authors do not declare any conflict of interest relevant to this manuscript.

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