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Effects of albumin-conjugated PYY on food intake: the respective roles of the circumventricular organs and vagus nerve

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Abstract

The mechanism and routes through which peptide tyrosine-tyrosine (PYY) exerts its anorectic effects are still largely unknown. In the present study, we investigated the roles of the area postrema (AP), subfornical organ (SFO) and vagus nerve in mediating the anorectic effect of PYY using PYY_{3-36} conjugated to human serum albumin (PYY_{3-36}-HSA) in rats. PYY_{3-36}-HSA is a large molecule that does not penetrate the blood–brain barrier, and thus provides a useful tool to discriminate between the central (brain) and peripheral actions of this peptide. PYY_{3-36}-HSA induced significant reductions in food and body weight gain up to 24 h after administration. The anorectic effect of PYY_{3-36}-HSA was delayed for 2 h in rats in which both AP and SFO were ablated, while lesion of either of these circumventricular organs in isolation did not influence the feeding responses to PYY_{3-36}-HSA. The PYY_{3-36}-HSA-induced anorectic effect was also reduced during the 3- to 6-h period following subdiaphragmatic vagotomy. Lesions of AP, SFO and AP/SFO as well as subdiaphragmatic vagotomy blunted PYY_{3-36}-HSA-induced expression of c-*fos* mRNA in specific brain structures including the bed nucleus of stria terminalis, central amygdala, lateral–external parabrachial nucleus and medial nucleus of the solitary tract. In addition, subdiaphragmatic vagotomy inhibited the neuronal activation induced by PYY_{3-36}-HSA in AP and SFO. These findings suggest that the anorectic effect and brain neuronal activation induced by PYY_{3-36}-HSA are dependent on integrity of AP, SFO and subdiaphragmatic vagus nerve.

Introduction

Peptide tyrosine-tyrosine (PYY) is a member of the neuropeptide Y (NPY) family, produced by the L endocrine cells of the distal gut (Bottcher & Alumets, 1986; Greeley *et al.*, 1989). PYY is released postprandially into the circulation in proportion to energy intake (Adrian *et al.*, 1985). Two forms of the peptide are found in blood, PYY_{1-36} and the amino-terminally truncated PYY_{3-36} (Eberlein *et al.*, 1989; Grandt *et al.*, 1994). Recently, peripheral administration of PYY_{3-36} was reported to reduce food intake in rodents, rabbits, monkeys and humans (Batterham *et al.*, 2002; Challis *et al.*, 2003; Koegler *et al.*, 2005; Moran *et al.*, 2005; Sileno *et al.*, 2006; Chelikani *et al.*, 2007).

The precise mechanism for the anorectic effect of PYY_{3-36} is still not completely understood. A direct action of circulating PYY_{3-36} through Y_2 receptors located in specific brain regions involved in the control of food intake, including the arcuate nucleus (Arc), has been proposed. Consistent with this hypothesis, intra-Arc injection of PYY_{3-36} was shown to reduce food intake in rats (Batterham *et al.*, 2002). Initially, the postulated mechanism underlying the Arc-related anorectic effect of PYY_{3-36} was the activation of the anorexigenic pro-opiomelanocortin (POMC) neurons in the Arc (Batterham *et al.*, 2002). Subsequently, data showed that PYY_{3-36} inhibited both POMC and NPY neurons, suggesting that the anorectic action of PYY_{3-36} was possibly mediated by blocking NPY neuron activity (Acuna-Goycolea & van den Pol, 2005) rather than being dependent on the melanocortin system (Challis *et al.*, 2004; Halatchev *et al.*, 2004). One has to bear in mind that the auto-inhibitory pre-synaptic Y_2 receptor is not only expressed in Arc neurons but also in numerous other brain sites including the amygdaloid nuclei, medial preoptic area, hypothalamus, anterodorsal thalamus, midbrain structures and brainstem (Gustafson *et al.*, 1997; Parker & Herzog, 1999; Stanic *et al.*, 2006). Intraperitoneal administration of PYY_{3-36} was shown to induce Fos-immunoreactivity in the Arc and also in two brainstem regions, the nucleus of the solitary tract (NTS) and area postrema (AP) and in the central nucleus of amygdala (CeA) (Bonaz *et al.*, 1993; Halatchev & Cone, 2005).

In addition to exerting a direct central action, PYY could also exert its anorectic effect on the peripheral side of the blood–brain barrier

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(BBB) by acting on either vagal afferents or brain structures devoid of the BBB, namely the circumventricular organs (CVOs). The presence of the mRNA encoding the Y_2 receptor has been detected in the nodose ganglion, implying that this receptor could be found at the level of the vagal afferents emerging from the gut (Zhang *et al.*, 1997). Consistently, the anorectic effect of PYY_{3-36} and activation of the Arc neurons induced by peripheral administration of PYY_{3-36} is impeded by truncal vagotomy or transection of the brainstem–hypothalamic relay (Abbott *et al.*, 2005; Koda *et al.*, 2005). Y_1 and Y_2 receptors are also found in the AP (Dumont *et al.*, 1996), while Y_1 receptor mRNA has recently been reported in the subfornical organ (SFO) (Hindmarch *et al.*, 2008). Both of the CVOs have thus been suggested to play potentially important roles in sensing circulating PYY (Fry *et al.*, 2007).

The goal of this study was to further investigate the effects of circulating PYY_{3-36} on energy metabolism and the activation of specific brain regions by studying the effects of an albumin-conjugated form of PYY_{3-36} (PYY_{3-36}-HSA). PYY_{3-36}-HSA consists of PYY_{3-36} covalently bound at its N-terminus to human serum albumin (HSA) via a maleimide residue (Supporting InformationFig. S1) that confers good plasma stability (Supporting Information Fig. S2). PYY_{3-36} and N-terminus HSA-conjugated PYY_{3-36} show similar Y_2 receptor specificity (Supporting Information Fig. S3A) with greater affinity for the Y_2 than for the Y_1 receptor (Supporting Information Fig. S3B). Large molecules such as albumin and albumin-conjugated peptides cannot readily cross intact BBB (Chuang *et al.*, 2002).

In this study we examined the effect of acute peripheral administration of PYY_{3-36}-HSA on food and water intake as well as body weight gain and subsequently analysed the expression of c-*fos* mRNA, a marker of neuronal activation, in specific brain regions. We also investigated the influence of the AP, SFO and vagus nerve on the anorectic effect and neuronal activation induced by PYY_{3-36}-HSA in CVO-lesioned and vagotomized rats.

Materials and methods

Animals and diet

Male Wistar rats weighing 225–250 g were purchased from Charles River Canada (St-Constant, Canada). All rats were cared for and handled according to the Canadian Guide for the Care and Use of Laboratory Animals and our institutional animal care committee approved the present protocol. The animals were housed individually in wire-bottom cages suspended above absorbent paper and, unless otherwise specified, fed *ad libitum* with a stock diet (Rodent Laboratory CHOW Purina # 5075; Strathroy, ON, Canada). They were subjected to a 12-h dark–light cycle and kept at an ambient temperature of 23 ± 1°C. Rats were acclimatized to these conditions for a period of 7 days prior to experimental procedures.

In a first series of experiments we assessed the effects of an acute peripheral administration of PYY_{3-36}-HSA on food intake, body weight gain and brain expression of c-*fos* mRNA. In a second series of experiments, we investigated the influence of the AP, SFO and vagus nerve lesions on the anorectic effect and c-*fos* expression induced by PYY_{3-36}-HSA.

Electrolytic CVO lesions

Rats (n = 91) were subjected to electrolytic lesions, which were carried out under ketamine (60 mg/kg)/xylazine (7.5 mg/kg) anesthesia. In sham-lesioned rats, an electrode was introduced in either the SFO or AP, or SFO and AP but no current was passed.

AP lesion

Each rat was placed in a stereotaxic apparatus. Its head was flexed and the cisterna magna was opened to permit access to the fourth ventricle. Rats were then subjected to the electrolytic (Keithley Instruments 225 Current Source, 0.5 mA for 20 s) lesion of the AP using a monopolar, parylene-C-insulated tungsten electrode (MicroProbes, Gaitersburg, MD, USA) with a tip exposure of 100 μm.

SFO lesion

Each rat was placed in a stereotaxic apparatus. Its head was horizontally fixed and a midline incision of the soft tissues above the skull was made. A small hole was drilled in the cranium so that the electrode could be advanced into the region of SFO using the following stereotaxic coordinates: midline, 0.8 mm caudal to bregma (β), and 4.5 mm below dura. Electrolytic lesions were then achieved as described above for the AP.

Double AP/SFO lesion

Lesions of these two CVOs were carried out as described above and, in all cases, the SFO lesion was carried out first and the AP lesion second.

Histological analysis of AP and SFO lesions

Validation of lesion sites was carried out by histological examination of the coronal brain sections. Only animals having complete lesions (Supporting Information Fig. S4) were included in the statistical analyses. AP-lesioned groups excluded rats with any AP remaining or rats with damage to the adjacent NTS. In AP sham-lesioned animals, no damage was present in the AP tissue or the surrounding NTS. The SFO lesion group included only animals in which the structure was totally destroyed or rats with the rostral SFO and rostroventral stalk sufficiently damaged to disconnect the SFO from its targets in the ventral forebrain, as SFO efferent projections to the preoptic region and hypothalamus leave the structure via its rostroventral stalk (Miselis, 1981). Data from rats with partial damage to SFO without destruction of the rostroventral pole were not included in the analyses. SFO sham-lesioned animals had evidence of electrode tracks through the hippocampal commissure, but no evidence of damage to the SFO. After histological evaluation of AP and SFO, rats were distributed in experimental groups as follows: AP lesion (n = 18), SFO lesion (n = 11), AP/SFO lesion (n = 9), AP sham-lesion (n = 19), SFO sham-lesion (n = 20) and AP/SFO sham-lesion (n = 14).

Postoperative care

After electrolytic or sham lesions, rats were returned to their cages with water and food given *ad libitum*. Each animal's health status was carefully monitored. During the first two postoperative days, all rats were carefully followed and fed *ad libitum* with a 'recovery' diet (liquid chocolate flavoured *Ensure* and *Pablum* baby cereals, containing 10.45 kJ/g with 14.7% of energy as fat, 70.6% as carbohydrate and 14.4% as protein) and given antibiotic/anti-inflammatory treatment (Baytril 5 mg/kg/Ketoprofen 5 mg/kg subcutaneously injected, daily). The 'recovery' diet was a palatable, well-balanced diet which was provided to promote resumption of normal eating and drinking after the lesions. On the third postoperative day, animals were returned to a normal laboratory chow diet (containing 12.9 kJ/g with 4.5% of energy as fat, 77.5% as

carbohydrate and 18.0% as protein). Rats were allowed 2 weeks to recover prior to any experimental procedures.

Subdiaphragmatic vagotomy

Subdiaphragmatic vagotomy and pyloroplasty were carried out in 23 rats, under isoflurane anesthesia. Rats were fasted overnight before surgery. The stomach and lower esophagus were exposed after an upper midline laparotomy. The stomach was gently retracted down beneath the diaphragm and, under microscopic control, the anterior and posterior vagal trunks were visualized and isolated from the esophagus about 2 cm above the stomach. Two sutures were tied around each vagal trunk with sterile 4.0 silk (Ethicon Inc., Markham, ON, Canada) immediately above the hepatic branch and beneath the diaphragm, and at least 1 cm of the nerves was dissected between the sutures. In addition, all the neural and connective tissue surrounding the esophagus was removed. A pyloroplasty was performed in order to prevent gastric stasis. The pyloric sphincter was severed by an incision parallel to its axis and reconstructed with coated vicryl 8.0 taper-point sutures (Ethicon Inc.) perpendicular to the pylorus axis. The stomach was returned to its normal position and the muscular abdominal wall and skin were successively closed with a sterile 4.0 silk suture. In sham rats ($n = 15$), a similar abdominal incision was made, the stomach was retracted, the vagal trunks were identified and manipulated, and pyloroplasty was performed. Six of 23 vagotomized rats died shortly after the surgery due to post-surgical complications. The remaining 17 vagotomized rats survived and maintained good health. All operated rats were allowed 1 week to recover prior to any experimental procedure. During that period all animals were handled each day to habituate them to injection procedures.

Assessment of vagotomy

Immediately the animals were killed, we performed visual inspection of vagal nerve integrity to verify each vagotomy. The ends of the vagal trunks were identified, and the integrity of the peri-esophageal nervous tissue, the appearance of the esophagus and stomach, the presence of the food in the stomach and the esophageal reflux were recorded. The stomachs were weighed and size measured. The criteria for complete vagal transection were the inability to find continuity of the nervous tissue and the presence of gastric distension (Supporting Information Table S1) and esophageal reflux. All vagotomized rats ($n = 15$) complied with all criteria. All sham-operated rats ($n = 17$) had intact vagal innervation and normal stomach sizes. The ratio of stomach weight to body weight was used to confirm the effectiveness of the vagotomy (Supporting Information Table S1). It has been previously shown that a stomach to body weight ratio greater that 0.02 in fasted rats is a good indicator of a sustained extensive vagal destruction (Martin *et al.*, 1977). The stomach to body weight ratio in our vagotomized rats was 0.07 ± 0.008 vs. 0.01 ± 0.001 in shams ($t_{30} = 6.81$, $P < 0.0001$, non-directional Student's *t*-test). Also, coronal brainstem sections were stained with cresyl violet (for cell body) and Luxol fast blue (for myelin) in order to evaluate cell loss in dorsal motor nucleus of the vagus (DMX) after vagotomy (Cox & Powley, 1981). Reduction in neuronal soma was observed in DMX of vagotomized rats compared with shams (Supporting Information Fig. S5). DMX neurons were counted in brainstem sections from 13.76 to 14.16 mm caudal to bregma and an average of four measurements was taken for each animal. Neurons were easily distinguished from glia by their size. A significant reduction of DMX cells was observed in vagotomized rats compared with shams (25.10 ± 1.051 vs. 34.30 ± 1.035, respectively, $t_{30} = 6.23$, $P = 0.0002$, non-directional Student's *t*-test).

Postoperative care

Following surgery rats were carefully monitored. For the first three post-operative days, an anti-inflammatory treatment (Ketoprofen 5 mg/kg) was subcutaneously administered daily to rats. Liquid diet (Clinical Resource Standard, Novartis Nutrition, Whitby, ON, Canada) was allowed *ad libitum* to rats for 24 h following surgery. On the second post-operative day, the animals were returned to the rat laboratory chow. Rats were allowed 1 week to recover before any investigation.

PYY administration

Rats were subjected to subcutaneous injections using a 1-mL syringe connected to a 30-gauge needle. The injected volume of solution was adjusted according to the weight of each animal (2.86 mL/kg). Albumin-conjugated $PYY_{3\text{-}36}$ (CJC-1682) (Conjuchem Biotechnologies, Inc., Montreal, QC, Canada) was dissolved in a solution of isotonic pyrogen-free saline. The dose of 350 nmol/kg (24.7 mg/kg) was used for food intake measurements and for brain activation. Control animals were injected with isotonic pyrogen-free saline.

Food intake measurements

The effect of $PYY_{3\text{-}36}$-HSA on food intake was assessed between the third and fifth postoperative weeks (in CVO-lesioned rats and their shams) and during the second postoperative week (in vagotomized rats and their shams). Following a period of fast for 20–22 h, rats were subcutaneously injected with $PYY_{3\text{-}36}$-HSA or saline and then given free access to food. Injections were performed between 08:00 and 09:00 h. Food intake was measured at 1, 2, 3, 6 and 24 h after injection, while water intake was measured at 6 and 24 h after injection.

Brain in situ *hybridization histochemistry*

At the end of the seventh postoperative week (in CVO-lesioned rats and their shams) and the third postoperative week (in vagotomized rats and their shams), after a 20-h fast, rats were subcutaneously injected with $PYY_{3\text{-}36}$-HSA or saline and 60 min later they were killed. Injections were performed between 07:30 and 10:30 h.

All rats were killed in the morning, between 08:30 and 11:30 h. Rats were anesthetized with ketamine (60 mg/kg)/xylazine (7.5 mg/kg), given intraperitoneally, and, without delay, were intracardially perfused with 200 mL of ice-cold isotonic saline followed by 500 mL of paraformaldehyde (4%) solution. The brains were then removed and kept in paraformaldehyde (4%) for 7 days. They were then transferred to a solution containing paraformaldehyde (4%) and sucrose (10%) before being cut 12 h later using a sliding microtome (Histoslide 2000; Reichert-Jung, Heidelberger, Germany). Brain sections (30 μm) were taken from the olfactory bulb to the brainstem and stored at −30°C in a cold cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%) and glycerol (20%).

In situ hybridization histochemistry was used to localize c-*fos* mRNA in brain sections. The expression of the c-*fos* gene (mRNA or protein) has been extensively and reliably used to estimate brain activation (Sagar *et al.*, 1988; Hoffman *et al.*, 1993). The protocol for

in situ hybridization was adapted from the technique described by Simmons *et al.* (1989). Briefly, brain sections (one out of every six brain sections) were mounted onto poly-L-lysine-coated slides and allowed to dessicate overnight under vacuum. The sections were then successively fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37°C with proteinase K (10 μg/mL in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M trietholamine, pH 8.0) and dehydrated through graded concentrations (50, 70, 95 and 100%) of alcohol. After vacuum drying for at least 2 h, 90 μL of the hybridization mixture, containing an antisense ^{35}S-labeled cRNA probe (10^7 cpm/mL), was spotted on each slide. The slides were coverslipped and incubated overnight at 60°C in a slide warmer. The next day, coverslips were removed and the slides were rinsed four times with 4 × saline-sodium citrate (0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7.0) containing 1 mM 1,4-dithiothreitol, digested for 30 min at 37°C with RNAse-A (10 mg/mL, Roche Diagnostics, IN, USA), washed in descending concentrations of saline-sodium citrate (2 ×, 10 min; 1 ×, 5 min; 0.5 ×, 5 min; 0.1 ×, 30 min at 60°C) containing 1 mM 1,4-dithiothreitol and dehydrated through graded concentrations of alcohol. After a 2-h period of vacuum drying, the slides were exposed on an X-ray film (Eastman Kodak, Rochester, NY, USA) for 24 h. Once removed from the autoradiography cassettes, the slides were defatted in toluene and dipped in NTB2 nuclear emulsion (Kodak). The slides were exposed for 7 days before being developed in D19 developer (Kodak) for 3.5 min at 14–15°C and fixed in rapid fixer (Kodak) for 5 min. Finally, tissues were rinsed in running distilled water for 1–2 h, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in toluene and coverslipped with dibutylphtalate-xylol mounting medium.

Antisense ^{35}S-labeled riboprobes

The c-*fos* cRNA probe was generated from the *Eco*RI fragment of rat c-*fos* cDNA (Dr. I. Verma, The Salk Institute, La Jolla, CA, USA) subcloned into a pBluescript SK-1 plasmid (Stratagene, La Jolla, CA, USA), and linearized with *Sma*I and *Xho*I (Pharmacia Biotech Inc., Oakville, ON, Canada) for antisense and sense probes, respectively. Radioactive riboprobe was synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, 10 mM dithiothreitol, 6 mM MgCl, 40 mM Tris (pH 7.9), 0.2 mM ATP/GTP/CTP, 100 μCi α-^{35}S-UTP (Perkin Elmer, Montreal, QC, Canada), 40 U RNase inhibitor (Roche Diagnostics) and 20 U of RNA polymerase (T7 or T3 for antisense and sense probes, respectively) for 60 min at 37°C. The DNA templates were treated with 100 μL of DNAse solution containing 0.1 U/mL DNAse (Roche Diagnostics), 0.25 mg/mL tRNA and 50 mM Tris/10 mM $MgCl_2$. The riboprobe was purified using an RNeasy Mini Kit (Qiagen Inc., Missisauga, ON, Canada), eluted in 150 μL of 10 mM Tris/1 mM EDTA buffer and incorporated in a hybridization solution containing (per mL) 10^7 cpm of ^{35}S probe, 52% formamide, 330 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8, 1 × Denhart's solution, 10% dextran sulfate, 0.5 mg/mL tRNA, 10 mM 1,4-dithiothreitol and diethyl pyrocarbonate-treated water. This solution was mixed and heated at 65°C before being spotted on slides. The specificity of the probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

Quantitative analysis of the hybridization signals

The hybridization signals revealed on NTB2 dipped nuclear emulsion slides were examined under dark-field microscopy using an Olympus BX51 microscope (Olympus America, Melville, NY, USA). Images were acquired with an Evolution QEi camera and analysed with ImagePro plus version 5.0.1.11 (MediaCybernetics, Silver Spring, MD, USA). Saturation of the hybridization signal was avoided by adjusting the exposure time for the image with the strongest hybridization signal sampled for each region in every series. The luminosity of the system was set to the maximum and the saturation warning option was used to visualize saturated regions in the image preview. Thereafter, according to the pixel distribution histogram, the exposure time was adjusted in order to reduce to zero the number of saturated (pure white) pixels. The same exposure time was used throughout the analysis of the entire series.

The oval part of the bed nucleus of stria terminalis (BSTov, 0.10–0.26 mm caudal to *β*), SFO (0.83–1.08 mm caudal to *β*), suprachiasmatic nucleus (SCN, 1.30–1.40 mm caudal to *β*), paraventricular nucleus of thalamus (PVT, 1.40–1.80 mm caudal to *β*), parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVH, 1.80–2.00 mm caudal to *β*), CeA (2.00–2.45 mm caudal to *β*), lateral hypothalamus (LH, 2.30–2.56 mm caudal to *β*), Arc (2.54–3.25 mm caudal to *β*), lateral–external part of the parabrachial nucleus (PBle, 9.16–9.25 mm caudal to *β*), medial part of NTS (NTSm – at the level at which the NTS contacts the fourth ventricle, 13.28–13.60 mm caudal to *β*) and caudal part of NTS (NTSc – caudal to the obex, 13.68–14.36 mm caudal to *β*), AP and DMX (13.76–14.16 mm caudal to *β*) were outlined and the measurements of the pixel density of the hybridization signal were performed on both hemispheres of 2–4 brain sections for each animal assigned to a given treatment. When no hybridization signal was visible under dark-field illumination, the brain structures of interest were outlined under bright-field illumination and then subjected to densitometric analysis under dark-field illumination. The pixel density for each specific region was corrected by subtraction of background readings taken from areas immediately surrounding the region analysed. The brain sections from the different groups of rats were matched for rostrocaudal levels as closely as possible.

Plasma determinations

At the time the animals were killed, blood was collected by cardiac puncture, centrifuged (1500***g***, 15 min at 4°C), and the separated plasma was stored at −20°C until later biochemical measurements. Plasma glucose concentrations were determined using an automated glucose analyser (YSI 2300 Stat Plus; YSI Inc., Yellow Springs, OH, USA). A commercially available radioimmunoassay kit was used for the measurement of plasma insulin (Linco Research, St. Charles, MO, USA) levels.

Statistical analysis

Statistical analyses were done with StatView v5.0 software (SAS Institute Inc., Cary, NC, USA). Results are presented as mean values ± SEM. Statistical differences in body weight gain and food intake between sham and lesioned rats were determined using repeated-measures ANOVA in a mixed-model analysis with a heterogeneous autoregressive covariance structure. Multivariate normality was verified with Mardia's test. For all other variables, statistical differences between groups were determined by non-directional Student's *t*-test or two-way ANOVA for variables measured in this study. When necessary, data were log-transformed in order to satisfy the variance normality criterion. Fisher's protected least significance difference test or Bonferonni–Dunn *post hoc* tests were used to

identify the intergroup differences in cases of significant interactions. Results were considered significant at $P < 0.05$.

Results

Postoperative body weight gain and food intake

In CVO-lesioned and sham-operated rats, body weight and food and water intake were monitored daily during the first 8 postoperative days and every second day during the subsequent 44 days. During the first 2 post-surgical days, AP- and AP/SFO-lesioned rats showed a significant reduction in food intake compared with their shams. Concurrently, AP- and AP/SFO-lesioned rats drank more water than their shams, but afterwards no difference in water intake was observed between lesioned and sham-operated animals. After the first 2 post-surgical days, food intake in AP-lesioned rats was restored to the sham level, while AP/SFO-lesioned rats continued to eat less than shams. This led to a slower body weight regain in AP/SFO-lesioned rats compared with their respective shams during the first 30 days of the experimental period. They then slowly regained weight and, at the end of the experimental period, the difference in body weight between AP/SFO-lesioned and sham rats was no longer statistically significant. Rats having single lesions of AP or SFO did not show any significant difference in body weight gain compared with their respective shams (Supporting Information Fig. S6).

In vagotomized and sham-operated animals, body weight and food intake were measured daily during all experimental period. During the first 3 post-operative days, food intake and body weight gain in the vagotomized rats were less than those of shams. Food intake in the vagotomized rats then began to increase and, in the second part of the experimental period, their daily body weight gain became similar to shams (Supporting Information Fig. S7).

Effects of acutely injected PYY_{3-36}-HSA on body weight, food and water intake

As shown in Fig. 1, subcutaneous injection of PYY_{3-36}-HSA in rats deprived of food for 20–22 h induced a significant decrease in food intake that persisted for 24 h (food intake, percentage of saline; 0–1 h, 94 ± 2%, $t_{53} = 1.83$, $P = 0.06$; 1–2 h, 55 ± 5%, $t_{54} = 5.69$, $P < 0.0001$; 2–3 h, 35 ± 5%, $t_{54} = 6.07$, $P < 0.0001$; 3–6 h, 51 ± 5%, $t_{53} = 4.39$, $P < 0.0001$; 6–24 h, 71 ± 2%, $t_{53} = 9.93$, $P < 0.0001$) (Fig. 1A). Water intake was also significantly reduced by PYY_{3-36}-HSA (water intake, percentage of saline; at 6 h, 82 ± 2%, $t_{54} = 2.61$, $P = 0.01$; at 24 h, 86 ± 2%, $t_{54} = 4.06$, $P < 0.0001$, non-directional Student's *t*-test) (Fig. 1C). In addition, body weight gain during the day after injection was smaller in PYY_{3-36}-HSA-treated rats (body weight at 24 h, 71 ± 1% of saline, $t_{54} = 10.55$, $P < 0.0001$, non-directional Student's *t*-test) than in saline-injected rats (Fig. 1B).

Effects of PYY_{3-36}-HSA on body weight and food and water intake in CVO-lesioned (AP and/or SFO) and vagotomized rats

Subcutaneous injection of PYY_{3-36}-HSA induced similar reductions in food intake up to 24 h after injection in SFO-lesioned and sham rats (cumulative food intake, percentage of saline; at 1 h, 106 ± 4 vs. 99 ± 3%; at 2 h, 83 ± 2 vs. 88 ± 3%; at 3 h, 73 ± 2 vs. 80 ± 2%; at 6 h, 64 ± 2 vs. 73 ± 2%; at 24 h, 62 ± 2 vs. 71 ± 2%, in SFO-lesioned vs. sham rats) (Fig. 2A). Significant main effects of drug were detected on cumulative food intake of SFO-lesioned and respective sham rats at 2 h ($F_{1,54} = 13.27$, $P = 0.0006$), 3 h ($F_{1,54} = 46.87$, $P < 0.0001$), 6 h ($F_{1,54} = 110.85$, $P < 0.0001$) and 24 h ($F_{1,54} = 199.26$, $P < 0.0001$) after injection (Fig. 2A). PYY_{3-36}-HSA injection also caused similar decreases in food intake in AP-lesioned and in sham-operated rats (cumulative food intake, percentage of saline; at 1 h, 88 ± 4 vs. 93 ± 3%; at 2 h, 82 ± 4 vs. 85 ± 2%; at 3 h, 82 ± 3 vs. 77 ± 2%; at 6 h, 72 ± 3 vs. 74 ± 3%; at 24 h, 72 ± 2 vs. 71 ± 2%, in AP-lesioned vs. sham rats) (Fig. 2C). Significant main effects of drug were detected on cumulative food intake of AP-lesioned and respective sham rats at 2 h ($F_{1,64} = 20.66$, $P < 0.0001$), 3 h ($F_{1,64} = 31.02$, $P < 0.0001$), 6 h ($F_{1,64} = 49.07$, $P < 0.0001$) and 24 h ($F_{1,64} = 134.14$, $P < 0.0001$) after injection (Fig. 2C). Interestingly, the anorectic effect of PYY_{3-36}-HSA was delayed in AP/SFO-lesioned rats for the first 2 h after injection. Cumulative food intake (percentage of saline) in lesioned vs. sham-operated rats at 1 h was 105 ± 5 vs. 88 ± 3% (interaction between effects of lesion and drug: $F_{1,41} = 3.99$, $P = 0.05$) and at 2 h was 96 ± 5 vs. 79 ± 2% (interaction between effects of lesion and drug: $F_{1,41} = 5.57$, $P = 0.02$) (Fig. 2E). Later, PYY_{3-36}-HSA-induced reduction in food intake became comparable again in AP/SFO-lesioned and sham-operated rats (cumulative food intake, percentage of saline; at 3 h, 86 ± 5 vs. 74 ± 2%; at 6 h, 80 ± 4 vs. 70 ± 2%; at 24 h, 75 ± 2 vs. 72 ± 2%, in lesioned vs. sham rats) (Fig. 2E). Significant main effects of drug were detected on cumulative food

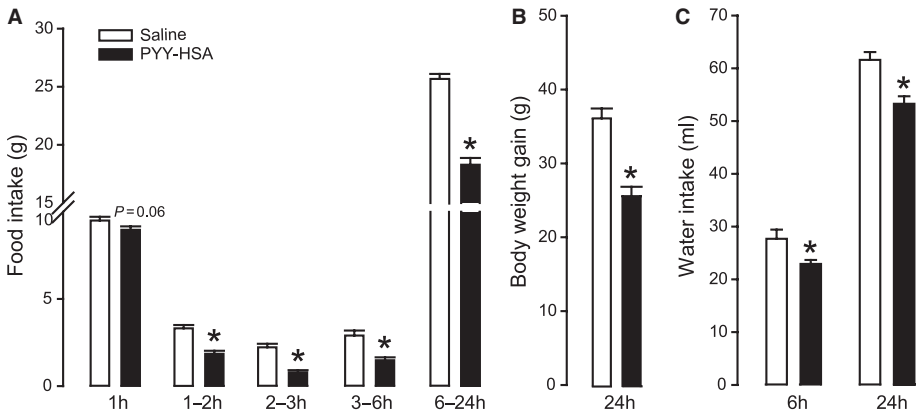

FIG. 1. Food intake (A), body weight gain (B) and cumulative water intake (C) during re-feeding in rats subcutaneously injected with albumin-conjugated PYY (PYY_{3-36}-HSA) (350 nmol/kg) or saline. Measurements were taken at 1, 2, 3, 6 and 24 h after injection for food intake, at 6 and 24 h for water intake, and at 24 h for body weight. Data represent means ± SEM. *Significant effect of drug assessed by non-directional Student's *t*-test within time interval, $P < 0.05$.

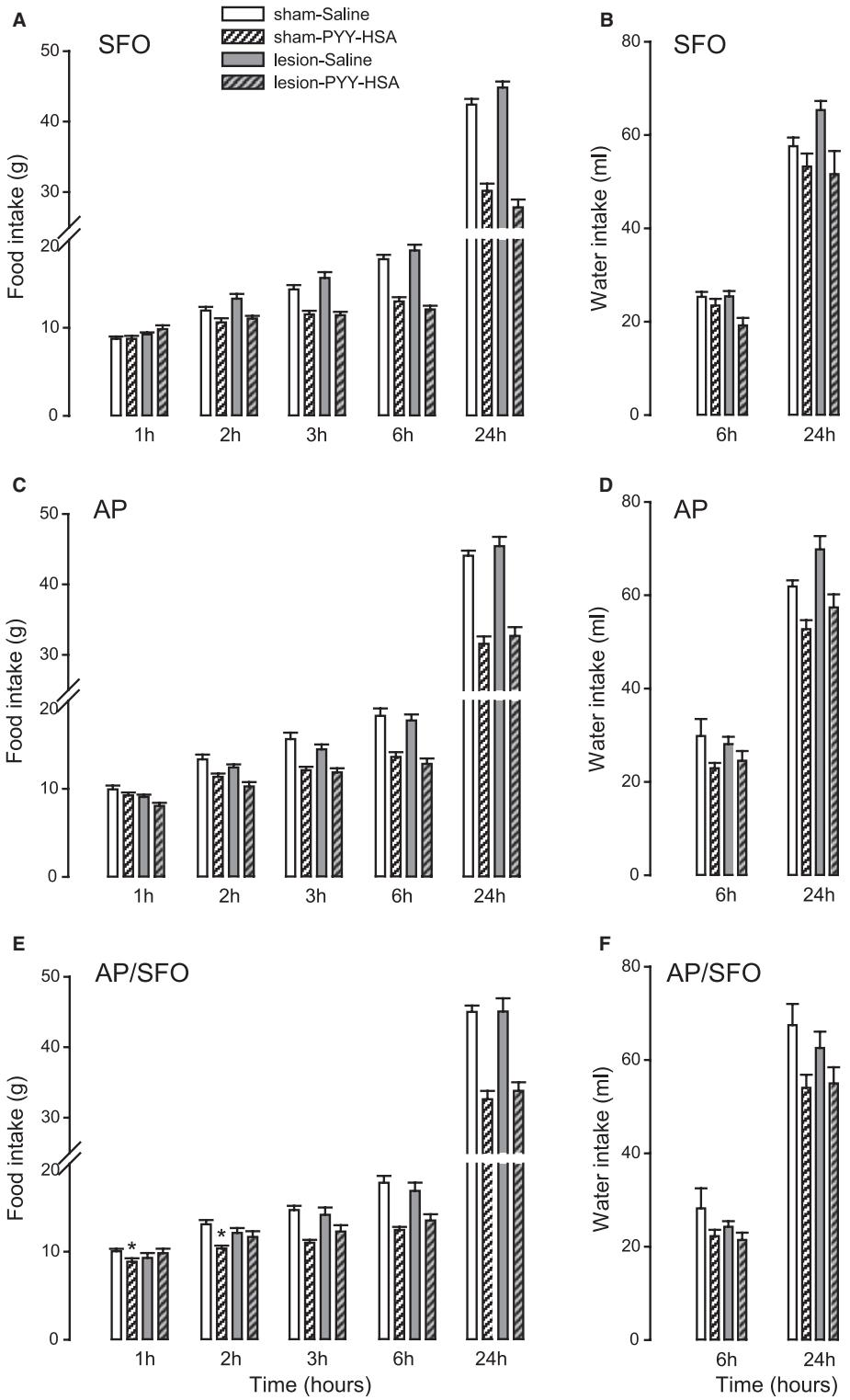

FIG. 2. Cumulative food (A, C, E) and water intake (B, D, F) during re-feeding in SFO-lesioned (A, B), AP-lesioned (C, D) and AP/SFO-lesioned rats (E, F) after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Measurements were taken at 1, 2, 3, 6 and 24 h after injection for food intake and at 6 and 24 h for water intake. Data represent means ± SEM. *Significant effect assessed by Bonferonni–Dunn *post-hoc* test following two-way ANOVA, $P < 0.05$.

intake of AP/SFO-lesioned and respective sham rats at 3 h ($F_{1,41} = 25.35$, $P < 0.0001$), 6 h ($F_{1,41} = 42.13$, $P < 0.0001$) and 24 h ($F_{1,41} = 80.66$, $P < 0.0001$) after injection (Fig. 2E).

The decrease in water intake caused by PYY_{3-36}-HSA during the 24-h period after injection was not significantly changed by the CVO lesions, AP and/or SFO (SFO-lesioned vs. sham, water intake, percentage of saline, at 6 h, 75 ± 6 vs. 93 ± 5% and at 24 h, 79 ± 7 vs. 92 ± 5%, Fig. 2B; AP-lesioned vs. sham, water intake, percentage of saline, at 6 h, 87 ± 7 vs. 77 ± 4% and at 24 h, 82 ± 4 vs. 85 ± 3%, Fig. 2D; AP/SFO-lesioned vs. sham, water intake;

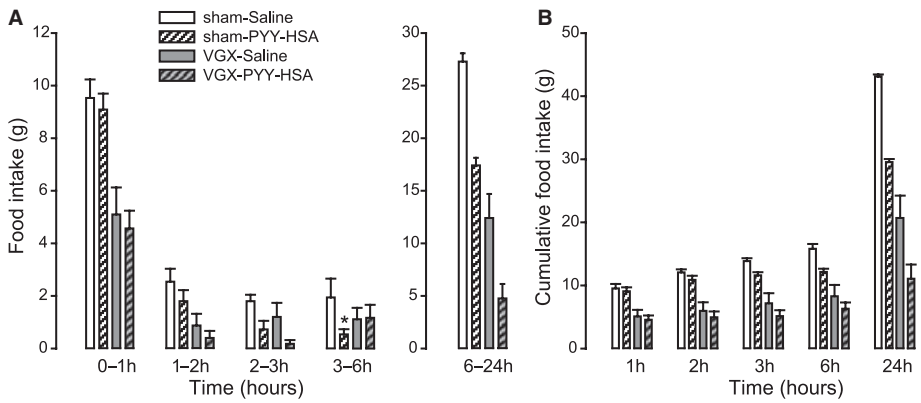

FIG. 3. Food intake during successive intervals of time (A) and cumulative food intake (B) during re-feeding in vagotomized (VGX) and sham-operated rats after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Measurements were taken at 1, 2, 3, 6 and 24 h after injection. Data represent means ± SEM. *Significant effect assessed by Bonferonni–Dunn *post-hoc* test following two-way ANOVA, $P < 0.05$.

percentage of saline, at 6 h, 88 ± 6 vs. 79 ± 5% and at 24 h, 88 ± 5 vs. 80 ± 4%, Fig. 2F). Significant main effects of drug were detected on water intake of SFO-lesioned and their shams at 6 h ($F_{1,54}$ = 8.12, $P = 0.006$) and 24 h ($F_{1,54} = 9.06$, $P = 0.004$) (Fig. 2B), in AP-lesioned and their shams at 6 h ($F_{1,64} = 4.79$, $P = 0.03$) and 24 h ($F_{1,64} = 23.17$, $P < 0.0001$) (Fig. 2D), as well as in AP/SFO-lesioned and their shams at 6 h ($F_{1,41} = 2.75$, $P = 0.05$) and 24 h ($F_{1,41} = 7.95$, $P = 0.003$) after injection (Fig. 2F).

The reduced body weight gain observed in PYY_{3-36}-HSA-treated rats during the 24 h after injection was not affected by the ablation of AP or SFO (SFO-lesioned vs. sham: 62 ± 5 vs. 74 ± 3% of saline; AP-lesioned vs. sham, 75 ± 3 vs. 68 ± 3% of saline) or by AP/SFO lesions (AP/SFO-lesioned vs. sham, 75 ± 3 vs. 71 ± 3% of saline) (data not shown).

Subdiaphragmatic vagotomy blunted the reduction in food intake induced by PYY_{3-36}-HSA between 3 and 6 h after injection. Food intake (percentage of saline) in vagotomized vs. sham PYY_{3-36}-HSA-treated rats at 3–6 h was 106 ± 44 vs. 27 ± 10% (interaction between effects of surgery and drug: $F_{1,28} = 5.07$, $P = 0.03$) (Fig. 3A). However, vagotomy did not block the hypophagia during the first 3 h and after 6 h following the PYY_{3-36}-HSA injection. Main effects of PYY_{3-36}-HSA were present at 2–3 h ($F_{1,28} = 9.35$, $P = 0.004$) and 6–24 h ($F_{1,28} = 35.49$, $P < 0.0001$) after injection (Fig. 3A). However, the diminution induced by PYY_{3-36}-HSA in cumulative

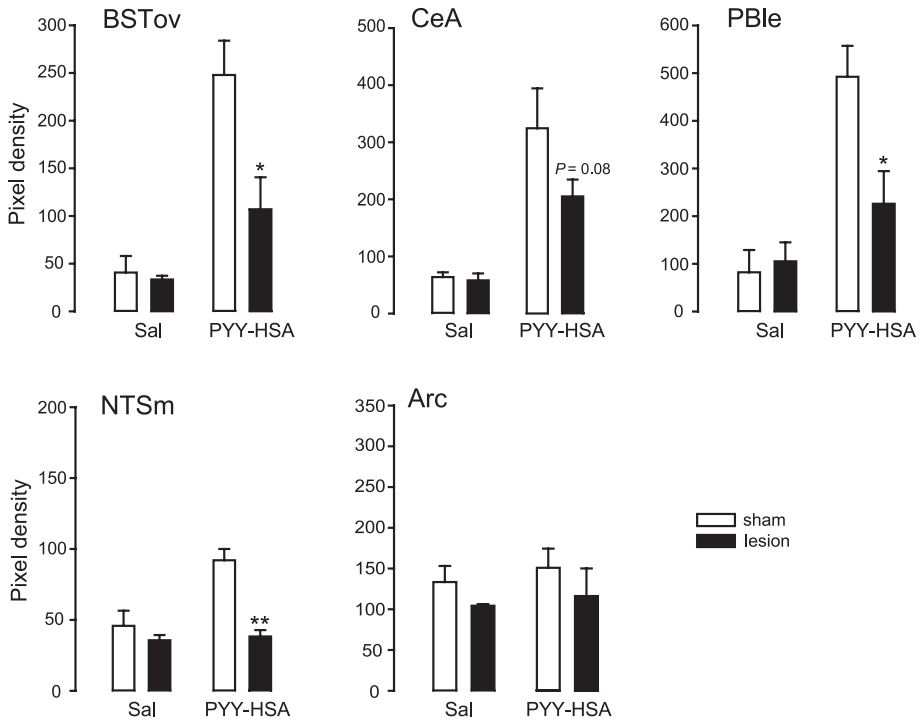

FIG. 4. Mean pixel density of the hybridization signal for c-*fos* mRNA expression in brain of AP/SFO-lesioned and sham-operated rats, 60 min after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Bed nucleus of the stria terminalis, oval part (BSTov), central nucleus of amygdala (CeA), parabrachial nucleus, lateral–external part (PBle), nucleus of the solitary tract, medial part (NTSm) and arcuate nucleus (Arc). Data represent means ± SEM. Significant effect of surgery *$P < 0.05$, **$P < 0.001$, assessed by Ficher's PLSD test following two-way ANOVA.

food intake [main effect of drug at 3 h ($F_{1,28}$ = 4.49, P = 0.04), 6 h ($F_{1,28}$ = 6.07, P = 0.02) and 24 h ($F_{1,28}$ = 27.71, P < 0.0001); Fig. 3B] and 24-h body weight gain (data not shown) was not altered by the subdiaphragmatic vagotomy.

Neuronal activation following acute administration of PYY_{3-36}-HSA in the presence or absence of the CVOs (AP and/or SFO) and the vagus nerve

The hybridization signal for c-*fos* mRNA at 60 min after the subcutaneous injection of PYY_{3-36}-HSA was quantified in brain nuclei involved in various behaviors including food intake control: BSTov, SFO, SCN, PVT, parvocellular and magnocellular PVH, CeA, LH, Arc, PBle, NTSm, NTSc, DMX and AP. In the SCN, PVT, PVH, LH, NTSc and DMX the neuronal activation induced by PYY_{3-36}-HSA was similar to that observed in saline-injected rats (data not shown). In sham-operated rats, PYY_{3-36}-HSA induced a significant increase in c-*fos* mRNA expression in the CVOs, SFO and AP, in the limbic structures, BSTov and CeA, and in the hindbrain nuclei including the PBle and NTSm (see Figs 4 and 6 for pixel density; Figs 5B, E, H and K, and 7C, B, E, H, K, N and R for darkfield photomicrographs). Significant main effects of drug were observed in AP/SFO-sham operated rats (BSTov: $F_{1,12}$ = 23.43, P = 0.0004; CeA: $F_{1,12}$ = 15.84, P = 0.001; PBle: $F_{1,12}$ = 15.71, P = 0.001; NTSm: $F_{1,12}$ = 6.54, P = 0.02; Fig. 4) as well as in sham-vagotomized rats (BSTov: $F_{1,25}$ = 77.89, P < 0.0001; CeA: $F_{1,25}$ = 68.58, P < 0.0001; PBle: $F_{1,25}$ = 91.82, P < 0.0001; NTSm: $F_{1,25}$ = 11.10, P = 0.002; SFO: $F_{1,25}$ = 4.38, P = 0.04; AP: $F_{1,25}$ = 13.66, P = 0.001; Fig. 6).

AP/SFO lesion impeded the elevation in c-*fos* mRNA expression induced by PYY_{3-36}-HSA in the limbic structures (BSTov and CeA) and brainstem nuclei (PBle and NTSm) (see Fig. 4 for pixel density; Fig. C, F and I for darkfield photomicrographs). Significant interactions were detected between effects of lesion and drug (BSTov: $F_{1,12}$ = 5.20, P = 0.04; CeA: $F_{1,12}$ = 4.95, P = 0.04; PBle: $F_{1,12}$ = 4.47, P = 0.05; NTSm: $F_{1,12}$ = 5.19, P = 0.04). AP/SFO ablation did not affect c-*fos* mRNA expression levels in the Arc in saline- or PYY_{3-36}-HSA-injected rats (Figs 4 and 5L). Single lesions of AP or SFO did not affect the c-*fos* mRNA expression in any examined nuclei (data not shown).

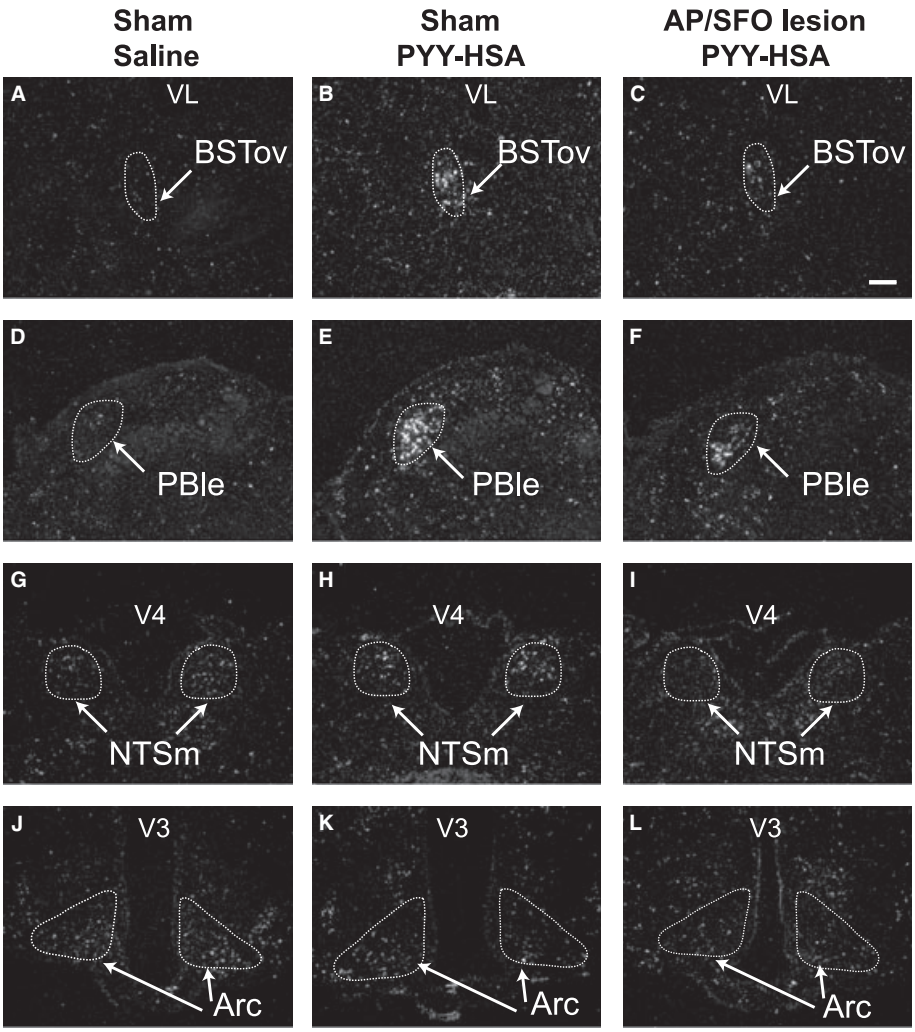

FIG. 5. Darkfield photomicrographs depicting c-*fos* mRNA expression in brain of AP/SFO-lesioned and sham-operated rats, 60 min after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Bed nucleus of the stria terminalis (BSTov; A–C), parabrachial nucleus, lateral–external part (PBle; D–F), nucleus of the solitary tract, medial part (NTSm; G–I), and arcuate nucleus (Arc; J–L) in sham rats injected with saline (A, D, G and J) or PYY_{3-36}-HSA (B, E, H and K) and in AP/SFO-lesioned rats injected with PYY_{3-36}-HSA (C, F, I and L). VL, lateral ventricle; V3, third ventricle; V4, fourth ventricle. Scale bar = 200 μm.

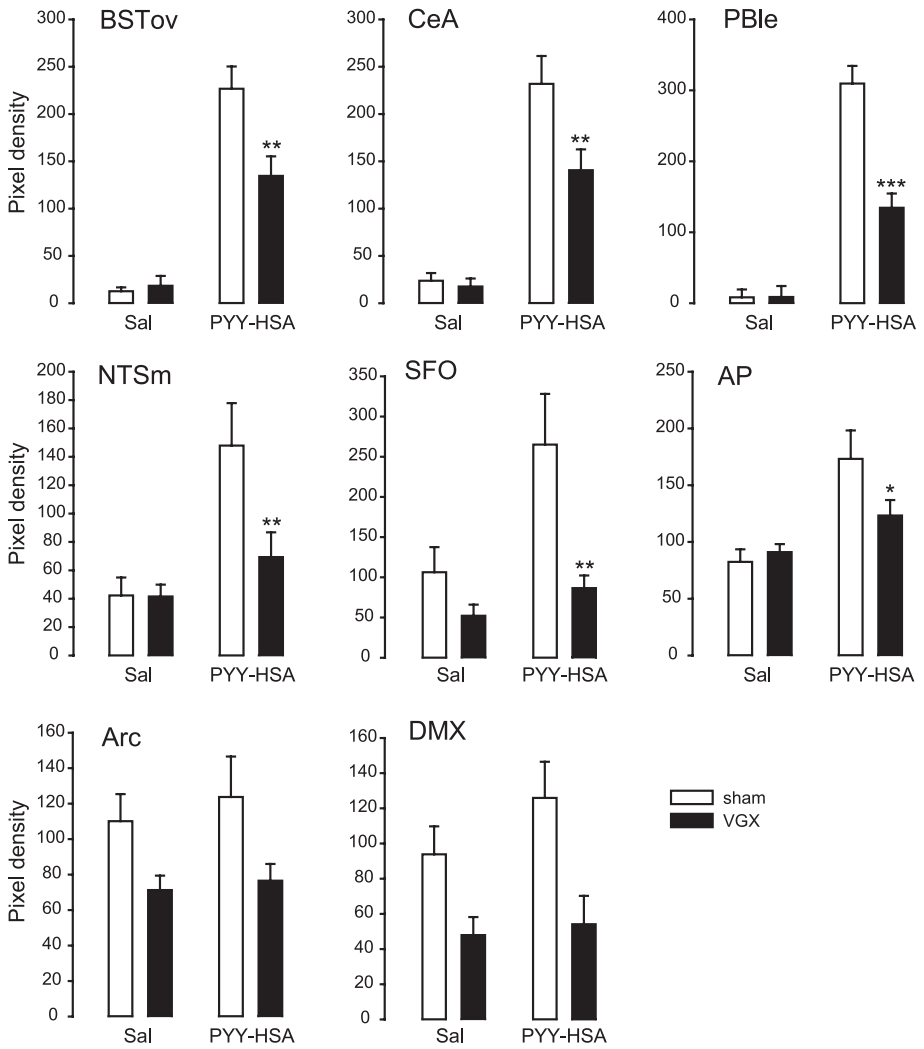

FIG. 6. Mean pixel density of the hybridization signal for c-*fos* mRNA expression in brain of vagotomized (VGX) and sham-operated rats, 60 min after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Bed nucleus of the stria terminalis, oval part (BSTov), central nucleus of amygdala (CeA), parabrachial nucleus, lateral–external part (PBle), nucleus of the solitary tract, medial part (NTSm), area postrema (AP) and arcuate nucleus (Arc). Data represent means ± SEM. Significant effect of surgery $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$, assessed by Ficher's PLSD test following two-way ANOVA.

Subdiaphragmatic vagotomy also reduced neuronal activation induced by PYY_{3-36}-HSA in the BSTov, CeA, PBle, NTSm, SFO and AP (see Fig. 6 for pixel density; Fig. 7C, F, I, L and O for darkfield photomicrographs). Significant interactions were detected between effects of surgery and drug (BSTov: $F_{1,25} = 6.19$, $P = 0.01$; CeA: $F_{1,25} = 4.22$, $P = 0.05$; PBle: $F_{1,25} = 13.70$, $P = 0.001$; NTSm: $F_{1,25} = 4.78$, $P = 0.04$; SFO: $F_{1,25} = 4.78$, $P = 0.04$; AP: $F_{1,25} = 4.98$, $P = 0.05$). Vagotomy similarly reduced c-*fos* mRNA expression levels in the Arc (Figs 6 and 7S) and DMX (Fig. 6) of either saline- or PYY_{3-36}-HSA-injected rats (main effect of vagotomy in Arc: $F_{1,25} = 7.98$, $P = 0.008$; and in DMX: $F_{1,25} = 12.89$, $P = 0.001$).

Plasma insulin and glucose following acute administration of PYY_{3-36}-HSA after CVO lesions (AP and/or SFO) and vagotomy

Plasma insulin and glucose did not show significant variations at 60 min after acute subcutaneous administration of PYY_{3-36}-HSA in either the presence or the absence of AP and SFO, or the subdiaphragmatic vagus nerve (Table 1).

Discussion

This study confirms the catabolic effect of the peripheral injection of PYY_{3-36}-HSA (Leger *et al.*, 2005). The latter caused a significant decrease in food intake and body weight gain that lasts for 24 h after its injection. Also, administration of PYY_{3-36}-HSA stimulated specific brain regions involved in energy homeostasis and ingestive behavior. The regions included the limbic structures (CeA and BSTov), hindbrain nuclei (PBle and NTSm) and the CVOs (SFO and AP). Importantly, the present results provide evidence that combined ablation of both AP and SFO as well as subdiaphragmatic vagotomy can modulate PYY_{3-36}-HSA-induced hypophagia and brain activation.

Previous experiments in a number of species have emphasized the role played by PYY in reducing food intake (Batterham *et al.*, 2003; Pittner *et al.*, 2004; Vrang *et al.*, 2006; Chelikani *et al.*, 2007; Parkinson *et al.*, 2008). Here, we demonstrate the anorectic effect of an acute peripheral administration of PYY_{3-36}-HSA. This conjugate does not cross the BBB (Banks, 2008) and has a prolonged half-life compared with PYY_{3-36} (Jette *et al.*, 2005). Additionally, the present data show that the effect of PYY_{3-36}-HSA on food intake was persistent for up to 24 h after its injection. As expected, body weight

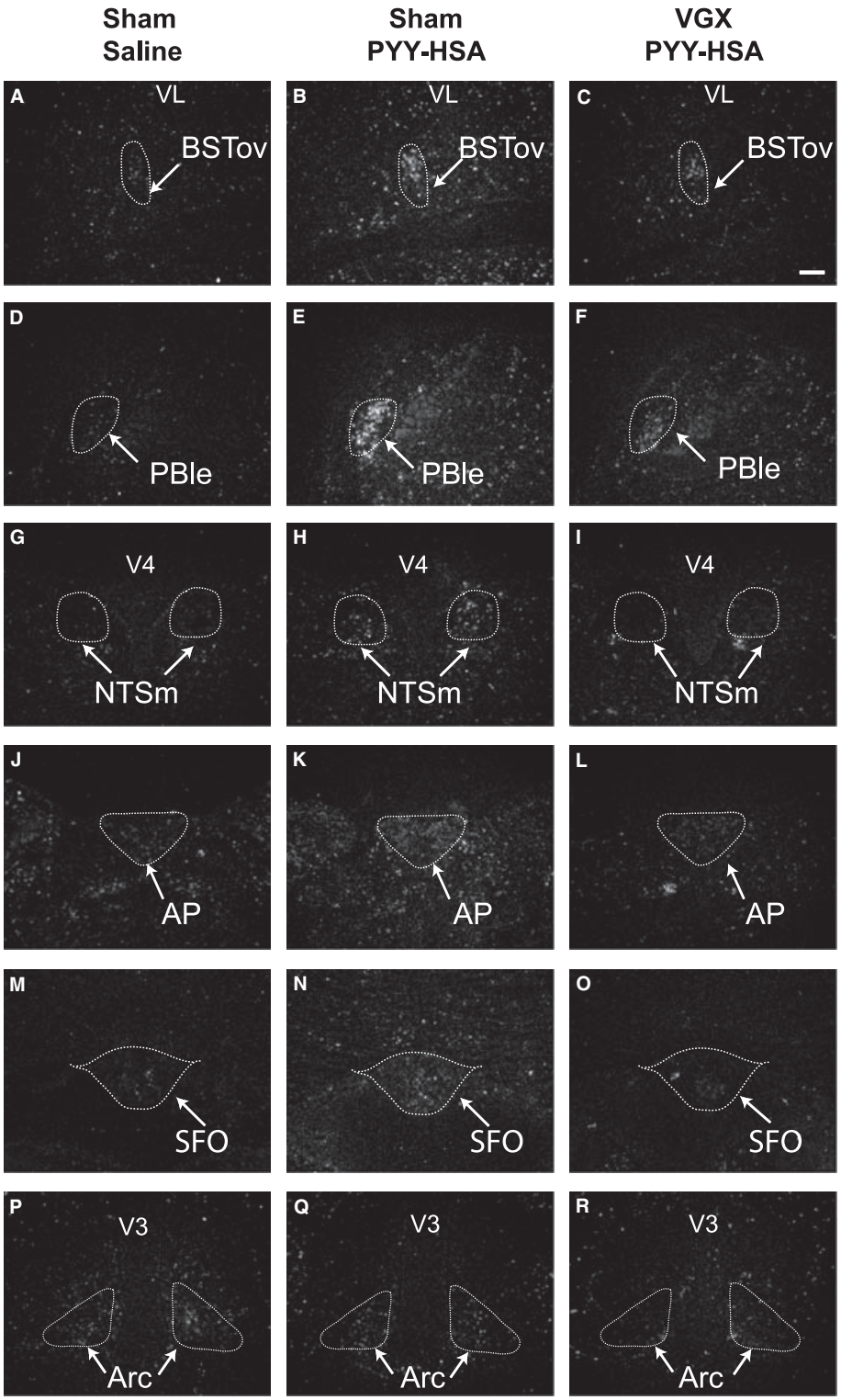

FIG. 7. Darkfield photomicrographs depicting c-*fos* mRNA expression in brain of vagotomized (VGX) and sham-operated rats, 60 min after subcutaneous injection of PYY_{3-36}-HSA (350 nmol/kg) or saline. Bed nucleus of the stria terminalis (BSTov; A–C), parabrachial nucleus, lateral-external part (PBle; D–F), nucleus of the solitary tract, medial part (NTSm; G–I), area postrema (AP; J–L), subfornical organ (SFO; M–O), and arcuate nucleus (Arc; panels P, Q and R) in sham rats injected with saline (A, D, G, J, M and P) or PYY_{3-36}-HSA (B, E, H, K, N and Q) and in VGX rats injected with PYY_{3-36}-HSA (C, F, I, L, O and R). VL, lateral ventricle; V3, third ventricle; V4, fourth ventricle. Scale bar = 200 μm.

gain dropped during that period, an effect which has also been observed following chronic administration of PYY_{3-36} in studies conducted in rodents (Pittner *et al.*, 2004; Vrang *et al.*, 2006; Chelikani *et al.*, 2007; Reidelberger *et al.*, 2008) and primates (Koegler *et al.*, 2005). We also observed that acute administration of PYY_{3-36}-HSA led to a reduction in water intake. This finding is in

TABLE 1. Plasma insulin and glucose in CVO-ablated rats and their controls, sham-operated rats, vagotomized (VGX) rats and their controls, following subcutaneous injection of albumin-conjugated PYY_{3-36} (PYY_{3-36}-HSA) (350 nmol/kg)

	Sham			Lesion				
	AP	SFO	AP+SFO	AP	SFO	AP+SFO	Sham	VGX
Insulin (nmol/l)								
Saline	0.066 ± 0.016	0.076 ± 0.012	0.113 ± 0.023	0.120 ± 0.014	0.103 ± 0.002	0.036 ± 0.022	0.035 ± 0.007	0.022 ± 0.005
PYY_{3-36}-HSA	0.081 ± 0.007	0.088 ± 0.028	0.070 ± 0.012	0.093 ± 0.012	0.107 ± 0.019	0.087 ± 0.016	0.027 ± 0.004	0.040 ± 0.008
Glucose (mmol/l)								
Saline	8.133 ± 0.385	8.319 ± 0.534	7.982 ± 0.321	7.686 ± 0.292	8.375 ± 0.775	7.385 ± 0.535	7.681 ± 0.555	7.040 ± 0.336
PYY_{3-36}-HSA	8.135 ± 0.187	7.348 ± 0.373	8.242 ± 0.157	8.013 ± 0.344	8.082 ± 0.390	7.285 ± 0.526	7.488 ± 0.613	7.757 ± 0.517

Data are means ± SEM. AP, area postrema; SFO, subfornical organ.

accordance with the previous demonstration of increases in water intake in Y_2 and Y_5 knockout mice (Thiele *et al.*, 2004; Wultsch *et al.*, 2006; Higuchi *et al.*, 2008) and, collectively, these data suggest a role for Y receptors in the regulation of water balance.

The anorectic effect of PYY_{3-36}-HSA was delayed for 2 h in AP/SFO-lesioned rats. Meanwhile, the single ablation of AP or SFO did not alter the effect of PYY_{3-36}-HSA on food intake, which is somewhat in line with previous results that showed that the AP lesion failed to lessen the anorectic effects of PYY_{3-36} (Cox & Randich, 2004). AP lesion had nonetheless been reported to attenuate the effects of PYY_{1-36} on basal (Deng *et al.*, 2001b) and CCK-8- or secretin-induced pancreatic secretion (Deng *et al.*, 2001a). Reasons as to why the hypophagic effect of PYY_{3-36}-HSA was only transiently inhibited by the combined AP/SFO lesion are not currently known, although this finding suggests that the two CVOs are involved in the early satiating effect of the peptide.

Our results also show that the anorectic effect of PYY_{3-36}-HSA is also blunted in vagotomized rats 3 and 6 h following injection which is, to some extent, consistent with previous investigations that have shown that subdiaphragmatic vagotomy attenuates the effects of PYY_{3-36} on food intake in rats (Abbott *et al.*, 2005; Koda *et al.*, 2005). Vagotomy has, however, been reported to be without effect in PYY_{3-36}-treated mice (Halatchev & Cone, 2005). It is noteworthy that the effect of the vagotomy is only seen at specific post-ingestive times, which suggest that the role of the vagus in modulating PYY action may be dependent on postprandial events.

The anorectic effects of PYY_{3-36} are believed to be mediated through distinct regions of the brain. Recently, it has been demonstrated that PYY_{3-36} administration to humans modulates neural activity within both corticolimbic and higher cortical areas as well as homeostatic brain regions including the hypothalamus (Batterham *et al.*, 2007). In rodents, the presence of Fos immunoreactivity has been observed following PYY_{3-36} administration in the Arc, AP, NTS, CeA and thalamus (periventricular and medial subnuclei) (Bonaz *et al.*, 1993; Batterham *et al.*, 2002; Halatchev *et al.*, 2004; Abbott *et al.*, 2005; Koda *et al.*, 2005; Blevins *et al.*, 2008). Immediate-early gene c-*fos* expression has been reliably used to assess neuronal activity in rats (Sagar *et al.*, 1988; Hoffman *et al.*, 1993). Our data show that PYY_{3-36}-HSA induced significant expression of c-*fos* mRNA in limbic regions (CeA and BSTov), brainstem nuclei (PBle and NTSm) and CVOs (AP and SFO). It is well known that these nuclei are brain regions that could be involved in energy balance regulation as most of them exhibit neuronal activation following food ingestion (Timofeeva *et al.*, 2002, 2005). Additionally, limbic structures (CeA and BST), and the PB nucleus are known to be involved in taste aversion learning (Reilly, 1999; St Andre *et al.*, 2007). In fact, there are studies that shed light on a dose-dependent aversive component of anorexia induced by PYY_{3-36} in mice (Halatchev & Cone, 2005), rats (Chelikani *et al.*, 2006) and humans (Degen *et al.*, 2005). In contrast, other studies do not support signs of taste aversion in rodents (Talsania *et al.*, 2005; Vrang *et al.*, 2006) or nausea in humans (Batterham *et al.*, 2002, 2003; le Roux *et al.*, 2006) and nonhuman primates (Koegler *et al.*, 2005; Moran *et al.*, 2005). We did not perform a conditioned taste aversion test with the dose of PYY_{3-36}-HSA used in our study, but the strong activation of the limbic structures caused by PYY_{3-36}-HSA may imply a potential aversive effect of PYY.

In other nuclei such as the SCN, PVT, PVH, Arc, LH, NTSc and DMX no specific activation was observed following PYY_{3-36}-HSA injection compared with saline. Peripheral administration of PYY_{3-36} has been shown to induce neuronal activation in the Arc (Batterham *et al.*, 2002; Halatchev *et al.*, 2004; Blevins *et al.*, 2008). PYY_{3-36}-HSA is a large molecule that does not penetrate the BBB of the Arc to reach Y receptors. The lack of neuronal activation in the Arc following PYY_{3-36}-HSA peripheral administration despite the fact that the Arc receives neuronal afferents from the limbic system, AP and lateral PB (Chronwall, 1985; Berthoud, 2002), which are activated by PYY_{3-36}-HSA, suggests that the activation of the Arc neurons may require a direct action of PYY on Arc-located Y receptor, and perhaps this is not essential for the inhibitory effects of this peptide on feeding.

We report here that specific brain neuronal activation induced by PYY_{3-36}-HSA was significantly affected by the double AP/SFO lesion and by vagotomy. In contrast, individual ablation of AP or SFO did not apparently lead to any modification in c-*fos* mRNA expression pattern evoked by PYY_{3-36}-HSA. Neuronal activation induced by PYY_{3-36}-HSA in CeA, BSTov, PBle and NTSm was blunted by the combined lesions of AP and SFO, and also by subdiaphragmatic vagotomy. It is known that BST receives afferents from the forebrain SFO (Swanson & Lind, 1986; Sunn *et al.*, 2003) and also from the hindbrain NTS (Ricardo & Koh, 1978). Medial CeA send very dense projections to the anterolateral BST, including its oval zone (Dong *et al.*, 2001). In the CeA, dense projections arrive from AP via neurons situated in the PBle (Saper & Loewy, 1980; van der Kooy & Koda, 1983) and from the NTS (Ricardo & Koh, 1978). NTSm and AP have been shown to be the main central targets for vagal afferent fibers (Sawchenko, 1983; Norgren & Smith, 1988). Our study shows that ablation of the vagal pathway altered neuronal activation caused by PYY_{3-36}-HSA in the AP and in the SFO as well. This finding is not unexpected as direct neuronal projection from the NTS to the SFO in rats has also been demonstrated (Zardetto-Smith & Gray, 1987).

Finally, we did not observe any variations in insulin and glucose plasma levels at 60 min after acute administration of PYY_{3-36}-HSA either in the presence or in the absence of the AP, SFO and vagus

nerve. Although studies made *in vivo* (Szecowka *et al.*, 1983; Bottcher *et al.*, 1989; Boey *et al.*, 2006a,b) and *in vitro* (Nieuwenhuizen *et al.*, 1994) have demonstrated that PYY_{1-36} has a role in glucose-stimulated insulin secretion, it appears that PYY_{3-36} has a lesser role in glucose metabolism. Plasma glucose concentration is not affected by acute or chronic intraperitoneal administration of PYY_{3-36} in obese and diabetic rodents (Pittner *et al.*, 2004). However, albeit to a lesser extent than PYY_{1-36}, PYY_{3-36} has been shown to reduce D-glucose-stimulated insulin release (Yoshinaga *et al.*, 1992). Other studies have demonstrated that PYY_{3-36} improved insulin sensitivity in diet-induced insulin-resistant mice (van den Hoek *et al.*, 2004) and rats (Vrang *et al.*, 2006). Recently, it has been shown that chronic PYY_{3-36} treatment ameliorates insulin resistance in mice (van den Hoek *et al.*, 2007). However, fasting glucose and insulin levels seem not to be affected by PYY_{3-36} treatment.

In conclusion, the present results demonstrate that PYY_{3-36}-HSA has a long-lasting suppressive effect on food intake and body weight gain. Although transitory, the delay caused by the AP/SFO lesion and subdiaphragmatic vagotomy on the PYY_{3-36}-HSA-induced anorexia suggests that both CVOs and vagus are synergistically involved in mediation of this effect of PYY_{3-36}. Anatomically specific neuronal activation induced by PYY_{3-36}-HSA appears also to be, at least partially, dependent on the presence of both AP and SFO and of subdiaphragmatic vagus nerve.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Structure of PYY_{3-36}-HSA (CJC-1682).

Fig. S2. Pharmacokinetic profile of PYY_{3-36}-HSA (CJC-1682) following a single subcutaneous or intravenous injection in normal Sprague-Dawley rats.

Fig. S3. CJC-1682 (PYY conjugated at N-terminus with HSA) and PYY_{3-36} exhibit more potency to human Y2 receptor that Y1 receptor *in vitro*.

Fig. S4. Photomicrographs of coronal sections through subfornical organ and area postrema showing complete lesion vs. respective sham operation.

Fig. S5. Photomicrographs of coronal sections through dorsal motor nucleus of vagus of sham-operated vs. vagotomized rats showing cell loss.

Fig. S6. Daily body weight gain and daily food intake in sham-operated and lesioned rats.

Fig. S7. Daily body weight gain and daily food intake in sham-operated and vagotomized rats.

Table S1. Stomach measurements in vagotomized (VGX) and sham-operated rats at the end of the experimental period.

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Abbreviations

AP, area postrema; Arc, arcuate nucleus; BBB, blood–brain barrier; BST, bed nucleus of stria terminalis; CeA, central nucleus of amygdala; CVOs, circumventricular organs; DMX, dorsal motor nucleus of vagus; LH, lateral hypothalamus; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PB, parabrachial nucleus; POMC, pro-opiomelanocortin; PVH, paraventricular hypothalamic nucleus; PVT, paraventricular nucleus of thalamus; PYY, peptide tyrosine-tyrosine; PYY_{3-36}-HSA, PYY_{3-36} conjugated to human serum albumin; SCN, suprachiasmatic nucleus; SFO, subfornical organ.

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