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EFFECTS OF VISFATIN/PBEF/NAMPT ON FEEDING BEHAVIOR AND HYPOTHALAMIC NEUROMODULATORS IN THE RAT

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Visfatin, also known as pre-B cell colony enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT), is a cytokine that is produced by adipose tissue, skeletal muscle, liver and immune cells. We studied the effects of visfatin/PBEF/NAMPT on feeding behavior, hypothalamic steady state concentrations of aminergic neurotransmitters and hypothalamic mRNA levels of anorexigenic peptides, such as cocaine- and amphetamine-regulated transcript (CART) peptide, corticotropin-releasing hormone (CRH), proopiomelanocortin (POMC), and orexigenic peptides, such as agouti-related peptide (AgRP) and neuropeptide Y (NPY). Forty-eight rats were injected in the arcuate nucleus (ARC) of the hypothalamus with either saline or visfatin/PBEF/NAMPT (3 µg). Food intake was recorded 1, 2 and 24 h following injection, and either dopamine (DA), norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT) or peptide gene expression were evaluated 2 and 24 h after visfatin/PBEF/NAMPT administration. Compared to vehicle, visfatin/PBEF/NAMPT significantly increased food intake, as evaluated 1, 2 and 24 h post-injection. Visfatin/PBEF/NAMPT treatment led to a significant decrease of DA steady state concentration, CART and CRH mRNA levels. Consequently, visfatin/PBEF/NAMPT could play an orexigenic role in the ARC, and the effect could be mediated by modulation of DA, CART and CRH activity in the hypothalamus.

Feeding behavior is finely modulated by a complex interplay of neurotransmitters, neuropeptides, cytokines and hormones in the central nervous system, where the arcuate nucleus (ARC) of the hypothalamus is known to play a pivotal role in this cross-talk signalling (1). The ARC includes two populations of first-order neurons expressing either neuropeptide Y (NPY)/ agouti-related peptide (AgRP), which enhance food intake, or proopiomelanocortin (POMC)/ cocaine- and amphetamine-regulated transcript (CART) peptide, which have an inhibitory effect on food intake. Both NPY/AgRP and POMC/CART peptide neurons project to second-order neurons, located partially in the paraventricular nucleus (PVN), producing anorexigenic peptides such as corticotropin-releasing hormone (CRH) (2). The ARC neurons, lying above the median eminence, where the blood-brain barrier is not complete, can be modulated by hormones involved in both short- and long-term regulation of energy homeostasis (3). Moreover, the hypothalamic control of feeding by gastrointestinal or adipose tissue-derived hormones (adipokines) has also been shown to be modulated by aminergic neurotransmitters, such as dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT), which play a pivotal role in the transduction of peripheral afferents into

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satiety and feeding signals (4). Adipose tissue, besides its energy-storage role, is now regarded as a dynamic endocrine organ secreting a variety of adipokines, which are able to affect energy homeostasis, both peripherally and at hypothalamic level (5). In particular, adipokines might provide a plausible link between obesity, insulin resistance, endothelial dysfunction, and atherosclerosis (6).

Visfatin has been described as a 52 kDa peptide hormone secreted by adipose tissue, in particular visceral adipose tissue (7), even if other studies found no differences between visceral and subcutaneous fat depots (8, 9). The same peptide was previously identified as pre-B cell colony enhancing factor (PBEF), a cytokine stimulating the maturation of B cell precursors, that is produced in several tissues, including liver, bone marrow and skeletal muscle (10). It was also identified as nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis from nicotinamide in mammals (11). Visfatin/PBEF/NAMPT was initially shown to exert insulin-mimetic effects by binding to and activating insulin receptors (7), although its physiological relevance remains controversial. Revollo et al. (12) failed to confirm the insulin-mimetic effects of visfatin/PBEF/NAMPT, but a significant physiological role in the regulation of pancreatic islet B-cell function through the NAD biosynthetic activity was reported, suggesting a connection between this peptide and glucose metabolism. Although elevated circulating visfatin/PBEF/NAMPT concentrations were reported to be associated with type 2 diabetes (13), other clinical studies did not find the same association (14-15). Moreover, even more conflicting results with regard to correlation of visfatin/PBEF/NAMPT with obesity have been reported. Higher plasma visfatin/PBEF/NAMPT levels were found in obese subjects (8). Conversely, other studies indicated opposite results (16) or no association (14).

Regarding the feeding behavior, intracerebroventricular (ICV) injection of visfatin/PBEF/NAMPT has been shown to increase food intake in chicks, where it seems to be more potent than other central orexigenic factors, such as NPY (17). On the other hand, ICV administration of visfatin/PBEF/NAMPT has been found to exert anorexigenic effects in rats (18).

In the present study, we aimed to investigate the feeding modulatory effect of visfatin/PBEF/NAMPT acutely injected into the ARC of the rat, and the hypothalamic DA, NE and 5-HT activity, by measuring their steady state concentrations. In addition, we evaluated the effects of visfatin/PBEF/NAMPT on gene expression of hypothalamic neuropeptides which play key roles in feeding regulation, such as CART peptide, CRH, POMC, AgRP, and NPY.

MATERIALS AND METHODS

Animals and drugs

Male adult Wistar rats (200-250 g) were housed in plexiglas cages (40 cm × 25 cm × 15 cm), one rat per cage, in acclimatized colony rooms (22±1°C; 60% humidity), on a 12/12 h light/dark cycle (light phase: 07:00-19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Community ethical regulations on the care of animals for scientific research.

Rat recombinant visfatin/PBEF/NAMPT was purchased from Enzo Life Sciences AG, Lausen, Switzerland.

In vivo intrahypothalamic treatment

Forty-eight rats were anesthetized by intraperitoneal injection of chloral hydrate (400mg/kg) (Farmalabor, Milano, Italy), treated with ketoprofen (1.4mg/kg) (Dompé farmaceutici, Milano, Italy) to sustain analgesia and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). After a midline incision, the skull was scraped clean, washed with a sterile saline solution, and disinfected with iodopovidone 10% solution. A drop of lidocaine 2% solution (Farmalabor, Milano, Italy) was applied into the incision. Coordinates for placement of a cannula into the ARC of the hypothalamus were as follows: anteroposterior (AP), -3 mm, (or +3 mm posterior to the bregma); mediolateral (ML), 0 mm; dorsoventral (DV), -7 mm (19). The site for the cannula was marked. Small holes were drilled into the skull and stainless-steel screws were placed. A hole was drilled through the skull, in the marked site, for the placement of the 21-gauge stainless-steel cannula (1.7 cm long), which was stereotaxically implanted into the ARC. The

lateral coordinate was 0 mm, but the hole drilled through the skull for the placement of the cannula was larger than a point due to drill-bit. Hence, we carefully and slowly implanted the cannula into the hole, sideways rather than in the midpoint. We paid attention to avoid the sagittal sinus: not a drop of blood was spilled during cannula placement in the rats enrolled in our study.

Once in place, the cannula was attached to the skull using dental cement (Formatray, Salerno, Italy). Sterile obturators were inserted into the cannulas to prevent them from clogging and to reduce the potential for brain infection.

Immediately after surgery, rats were injected subcutaneously with 1 ml of sterile saline solution and 1 ml of 5% glucose solution (Galenica Senese, Siena, Italy) and intraperitoneally with amoxicillin (20mg/kg) (Farmalabor, Milano, Italy).

Seventy-two hours after surgery, rats (24 animals for each group) were injected, in the ARC, at 09:00 h, with 10 µl of either vehicle (saline) or visfatin/PBEF/NAMPT (3 µg). The rats were gently hand-held while the obturators were removed.

Food intake was recorded 1, 2 and 24 h after treatment. Twenty-four animals (12 for each group) were sacrificed by decapitation, 2 and 24 h after administration, for neurotransmitter activity assessment and gene expression analysis, respectively. Immediately after sacrifice, we carefully examined the skull, brain and blood vessels of each rat, and found no damage; we observed only the small hole produced by the cannula on the upper surface of the ARC. The lower surface was intact.

Hypothalamic neurotransmitter extraction and high performance liquid chromatography (HPLC) determination

After sacrifice, brains (n=24) were rapidly removed and individual hypothalami dissected. Hypothalamic tissues were homogenized in ice bath for 2 min with Potter-Elvehjem homogenizer in 1 ml of 0.05 N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite. The homogenate was 5-fold diluted in chromatographic mobile phase and centrifuged at 4,500 x *g* for 10 min. The supernatant was filtered on 0.45 µm PTFE sterile filters (Whatman) and directly injected for HPLC. Neurotransmitter recovery was satisfactory (≥90%) and reproducible, with percentage relative standard deviation ≤10%.

The HPLC apparatus consisting of a Jasco (Tokyo, Japan) PU-2080 chromatographic pump and an ESA (Chelmsford, MA, USA) Coulochem III coulometric detector, equipped with micro dialysis cell (ESA-5014b) porous graphite working electrode and solid state palladium reference electrode. The analytical cell was set at −0.150 V, for detector 1 and at +0.300 V, for detector 2, with a range of 100 nA. The chromatograms were monitored at the analytical detector 2. Integration was performed by Jasco Borwin Chromatography software, version 1.5. The chromatographic separation was performed by isocratic elution on Phenomenex Kinetex reverse phase column (C18, 150 mm×4.6 mm i.d., 2.6 µm). The mobile phase was (10:90, v/v) acetonitrile and 75 mM pH 3.00 phosphate buffer containing octanesulfonic acid 1.8 mM, EDTA 30 µM and triethylamine 0.015% v/v. Flow rate was 0.6 ml/min and the samples were manually injected through a 20 µl loop. Neurotransmitter peaks were identified by comparison with pure standard retention time. Neurotransmitter concentrations in the samples were calculated by linear regression curve (y = bx + m) obtained with standard. Neither internal nor external standard were necessary for neurotransmitter quantification, in the hypothalamus homogenate, and all tests performed for method validation yielded results in accordance to limits indicated in official guidelines for applicability in laboratory trials. The standard stock solutions of DA, NE and 5-HT at 2 mg/ml were prepared in bidistilled water containing 0.004% EDTA and 0.010% sodium bisulfite. The stock solutions were stored at 4°C. Work solutions (1.25-20.00 ng/ml) were daily obtained progressively diluting stock solutions in mobile phase.

RNA extraction

After sacrifice, brains (n=24) were rapidly removed and individual hypothalami immediately dissected and stored in RNA*later* solution (Ambion, Austin, TX, USA), for gene expression analysis, at -20°C until further processed.

Total hypothalamic RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. In brief, each rat hypothalamus was homogenized in 1 ml of TRI Reagent. The homogenized samples were centrifuged at 12,000 x *g* for 10 min at 4°C to remove the insoluble material. The supernatant was added to 0.2 ml of chloroform and the samples were centrifuged at 12,000 x *g* for 15 min at 4°C. The aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at 12,000 x *g* for 10 min at 4°C, the gel-like RNA pellet was washed with 1 ml of 75% ethanol and dissolved in RNase-free water. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX), according to the manufacturer's instructions. The RNA solution was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels

and staining with ethidium bromide, under UV light.

Reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)

One μg of total RNA extracted from each sample in a 20 μl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) initially at 25°C for 10 min, then at 37°C for 120 min and finally at 85°C for 5 s.

Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA, USA). Reactions were performed in MicroAmp Fast Optic 96-well Reaction Plates (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR primers and TaqMan probes were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products, Rn00567382_m1 for CART gene, Rn01462137_m1 for CRH gene, Rn00595020_m1 for POMC gene, Rn01431703_g1 for AgRP gene, Rn00561681_m1 for NPY gene). B-actin (Applied Biosystems, Foster City, CA, USA, Part No. 4352340E) was used as the housekeeping gene. In accordance with the manufacturer's instructions, each amplification reaction was performed with 10 μl of TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG (Applied Biosystems, Foster City, CA, USA), 1 μl of primer probe mixture, 1 μl of cDNA, 8 μl of nuclease-free water. The thermal cycling conditions (fast operational mode) were: 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 1 s and 60°C for 20 s. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. In addition to samples, individual runs included no-template controls (one for each Assay-on-Demand Gene Expression Product) and a reverse transcriptase minus control (for AgRP gene Assay-on-Demand Gene Expression Product). Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Applied Biosystems, Foster City, CA, USA). The comparative $2^{\Delta\Delta}$ method was used to quantify the relative abundance of mRNA and then determine the relative changes in individual gene expression (relative quantification) (20). This method uses a calibrator sample to enable a comparison of gene expression levels in different samples. The values obtained indicate the changes in gene expression in the sample of interest by comparison with the calibrator sample, after normalization to the housekeeping gene.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Food intake, gene expression data, deriving from relative quantification, and hypothalamic steady state concentrations of DA, NE, 5-HT were collected from each of the 48 animals used in the experimental procedure and means ± S.E.M. were determined for each experimental group and analyzed by unpaired *t* test. As for gene expression analysis, 1.00 (calibrator sample) was considered the theoretical mean for the comparison. Differences were considered to be significant when *P* was less than 0.05.

RESULTS

Compared to vehicle, intrahypothalamic injection of visfatin/PBEF/NAMPT (3 μg) induced a significant increase in food intake, as evaluated 1, 2, and 24 h after treatment (Fig. 1).

When we evaluated hypothalamic aminergic neurotransmitter levels, we found a significant reduction of hypothalamic DA steady state concentration after visfatin/PBEF/NAMPT treatment, without any effect on NE and 5-HT (Table I).

Furthermore, we found a significant reduction of CART and CRH gene expression in visfatin/PBEF/NAMPT treated rats, while POMC, AgRP and NPY gene expression was not modified in respect to vehicle (Fig. 2).

DISCUSSION

The regulation of food intake and energy expenditures is integrated in the hypothalamus, where peripheral hormone and neurotransmitter signallings convey timely updated information about energy needs and metabolic substrate availability (1). To date, clinical studies have provided conflicting results concerning the possible associations between circulating visfatin/PBEF/NAMPT levels and anthropometric and biochemical parameters in obesity and type 2 diabetes (8, 13-16).

Recently, visfatin/PBEF/NAMPT was shown to be present in human cerebrospinal fluid at 10% plasma concentrations and it has been suggested that this peptide could get through the blood-brain barrier, possibly via an active transport mechanism (21). However, visfatin/PBEF/NAMPT also seems to be expressed in the brain (22).

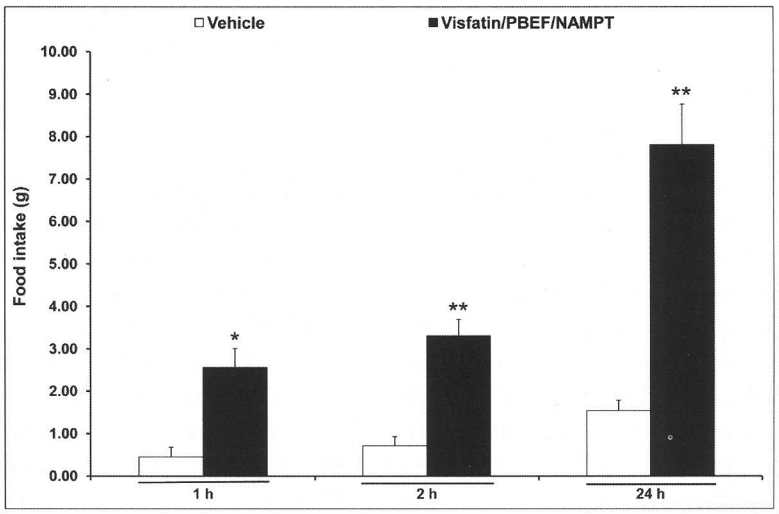

Fig. 1. *Food intake (in grams) in rats fed a standard laboratory diet and treated with either vehicle or visfatin/PBEF/NAMPT (3 μg). Vehicle or visfatin/PBEF/NAMPT was administered by intrahypothalamic injections, during the light phase, at 09:00 h. Food intake was recorded 1, 2 and 24 h after treatment in each group of rats. Values represent the means ± S.E.M. Compared to vehicle, visfatin/PBEF/NAMPT significantly increased food intake, as evaluated 1, 2, and 24 h post-injection. *P<0.001 vs vehicle; **P<0.0001 vs vehicle.*

Table I. *Hypothalamic amine steady state concentrations (ng/mg wet tissue).*

	Vehicle	Visfatin/PBEF/NAMPT
Dopamine	0.39 ± 0.05	0.12 ± 0.04*
Norepinephrine	1.59 ± 0.09	1.51 ± 0.15
Serotonin	0.80 ± 0.08	0.79 ± 0.07

*Hypothalamic dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine) steady state concentrations (ng/mg wet tissue) 2 h after treatment with visfatin/PBEF/NAMPT (3 μg), as determined by HPLC. Values represent the means ± S.E.M. Compared to vehicle, visfatin/PBEF/NAMPT significantly decreased DA steady state concentration. *P<0.05 vs vehicle.*

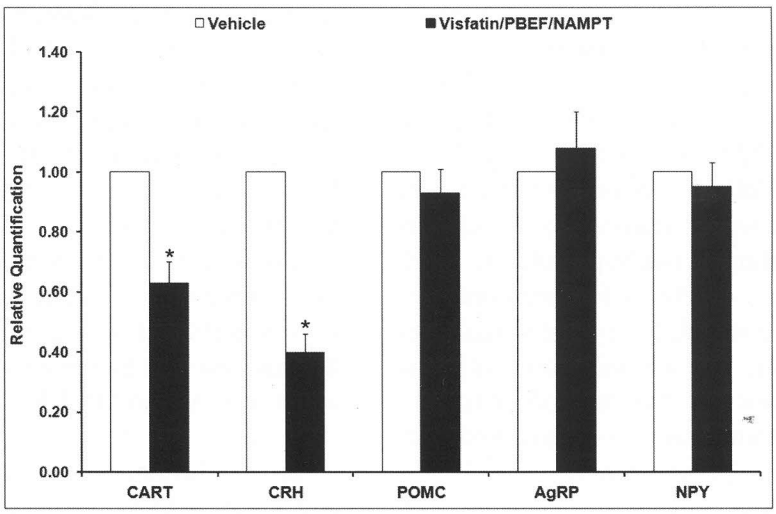

Fig. 2. *Relative gene expression of hypothalamic anorexigenic and orexigenic neuropeptides 24 h after treatment with visfatin/PBEF/NAMPT (3 μg), as determined by real-time RT PCR. Data were calculated using the $2^{\Delta\Delta}$ method; they were normalized to B-actin mRNA levels and then expressed as relative to vehicle (calibrator sample, defined as 1.00). Values represent the means ± S.E.M. Compared to vehicle, visfatin/PBEF/NAMPT significantly decreased cocaine- and amphetamine-regulated transcript (CART) and corticotropin-releasing hormone (CRH) gene expression. *P<0.0001 vs vehicle.*

Previous data on the role of visfatin/PBEF/NAMPT in feeding modulation have been inconsistent. In chicks, ICV injection of visfatin/PBEF/NAMPT stimulated feeding (17), while in rats ICV administration of visfatin/PBEF/NAMPT decreased food intake and body weight (18).

Visfatin/PBEF/NAMPT levels seem to follow the pattern of feeding stimulatory hormone. In the liver, both the mRNA and peptide levels increase upon fasting (23), which is typical of feeding stimulating hormones such as ghrelin, which displays increased mRNA in gastric cells and plasma peptide levels during fasting (24). Conversely, the concentrations of fasting serum visfatin/PBEF/NAMPT decrease after 7 days of overfeeding (25), mimicking the decrease of plasma ghrelin levels observed following 2 weeks of overfeeding in healthy men (26).

Our current findings showing that the acute administration of visfatin/PBEF/NAMPT into the ARC significantly increases food intake in rats (Fig. 1) support a feeding stimulatory role of this peptide in the rat, in agreement with previous data on chicks (17). The conflicting results of ours in respect to Park et al. (18), which showed an anorectic effect of visfatin/PBEF/NAMPT in the rat, could be partially explained by the different routes of administration (we injected rats directly in the ARC, while Park et al. injected intracerebroventricularly) and by the different time of administration (2 h into the light phase in our model *vs* 2 h before the dark phase in Park et al. experiments). Regarding the site of injection, the administration of visfatin/PBEF/NAMPT directly into the ARC, as effected in our experiments, could better target the hypothalamic nuclei controlling feeding behavior. Catecholamines and serotonin can be regarded as central transducers of peripheral signalling at the hypothalamic level. In particular, the role of DA in feeding control is still unsettled, with both inhibitory and stimulatory effects. On one side, DA administration into the perifornical hypothalamus inhibits food intake (4), and increased DA transmission is associated with the anorectic effects of amphetamines (27). On the other hand, DA is able to stimulate feeding, after its injection into the lateral hypothalamus and higher DA levels are found in the brain of obese rats (4). In our experiment we have found a significant reduction in hypothalamic DA steady state concentrations after visfatin/PBEF/NAMPT treatment (Table I), which could be related to the feeding stimulatory role of visfatin/PBEF/NAMPT observed *in vivo*. Actually, the reduction of DA levels induced by visfatin/PBEF/NAMPT could be related to NAMPT activity to induce NAD biosynthesis (11), and NAD has been found to increase dopamine release in rat striatal slices, with consequent decrease in DA steady state levels (28). POMC/CART peptide producing neurons are mainly located in the ARC, and they project to the PVN where they stimulate CRH producing neurons. The roles of CART peptide and CRH as central mediators of anorectic pathways is well established. Central administration of CART peptide decreases both normal and starvation-induced feeding in rats (29). In addition, chronic administration of CART peptide reduces food intake and causes weight loss (30), and neutralizing antibodies to the endogenous peptide cause an increase in feeding (31). Possible mediators of the anorectic effects of CART peptide in the hypothalamus include the stimulated secretion of CRH and thyrotropin-releasing hormone by second order neurons in the PVN (32). Leptin activates POMC/CART peptide expressing neurons (33). Interestingly, both fasted rats and rodent models of obesity, such as leptin receptor-deficient Zucker *fa/fa* rats and leptin-deficient *ob/ob* mice, have reduced CART mRNA levels in the ARC. Administration of leptin to *ob/ob* mice normalized CART mRNA content in the ARC, inhibiting food intake (29). Moreover, CRH has been reported to play a key role in modulating the anorexigenic effect of leptin (34) and central leptin injections increased CRH gene expression in the PVN of fasted rats (35).

Consequently, the significant reduction of the gene expression of the anorectic mediators CART peptide and CRH shown in our experiments (Fig. 2), is consistent with the observed increase in food intake following visfatin/PBEF/NAMPT administration (Fig. 1).

In conclusion, the modulation of DA, CART and CRH activity induced by intrahypothalamic injection of visfatin/PBEF/NAMPT could account for the feeding stimulatory effect of this peptide. On the other hand, NE, 5-HT, POMC, AgRP and NPY seem not to be involved in the modulation of feeding

induced by visfatin/PBEF/NAMPT.

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