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LETTER TO THE EDITOR

CENTRAL APELIN-13 ADMINISTRATION MODULATES HYPOTHALAMIC CONTROL OF FEEDING

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The 77 amino prepropeptide apelin has been isolated from bovine stomach tissue and several smaller fragments, including apelin-13, showed high affinity for the orphan APJ receptor. The distribution of apelinergic fibers and receptors in the hypothalamus may suggest a role of apelin-13 on energy balance regulation, albeit the studies reporting the acute effects of apelin on feeding control are inconsistent. Considering the possible involvement of apelinergic system on hypothalamic appetite controlling network, in the present study we evaluated in the rat the effects of intrahypothalamic apelin-13 injection on food intake and the involvement of orexigenic and anorexigenic hypothalamic peptides and neurotransmitters. Eighteen rats (6 for each group of treatment) were injected into the ARC with either vehicle or apelin-13 (1-2 μg/rat). Food intake and hypothalamic peptide and neurotransmitter levels were evaluated 2 and 24 h after injection. Compared to vehicle, apelin-13 administration increased food intake both 2 and 24 h following treatment. This effect could be related to inhibited cocaine- and amphetamine-regulated transcript (CART) gene expression and serotonin (5-hydroxytryptamine, 5-HT) synthesis and release, and increased orexin A gene expression in the hypothalamus.

To the Editor,

Hypothalamic feeding control is the result of an articulated interplay of neurotransmitters, neuropeptides, and peripheral signals, which could cross the blood-brain barrier, either by saturable transport systems or by lipophilic diffusion (1). The 77 aminoacid prepropeptide apelin has been isolated from bovine stomach tissue as the endogenous ligand for the orphan APJ receptor, and several smaller peptide fragments, including apelin-13, showed high affinity for this receptor (2). Multiple studies on fish also suggested a stimulatory effect of apelin-13 on food intake, with the possible involvement of hypothalamic neuropeptides (3, 4). This is consistent with the expression of apelin-13 and APJ receptor in the hypothalamus (3). In addition, apelin-13 could also modulate feeding through inhibition of serum leptin levels (5). As regards to food intake control, the hypothalamic arcuate nucleus (ARC) is a key site of interactions between peripheral hormones and neuropeptides. In the ARC, short- and long-term satiety signals arising from gastrointestinal tract and adipose tissue modulate the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) co-expressing neurons, as well as the anorexigenic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) peptide co-expressing neurons. These ARC neurons project to paraventricular nucleus (PVN) and

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lateral hypothalamic area (LHA), where corticotropin releasing hormone (CRH) and orexin-A producing neurons further transduce central feeding signaling (1).

Hypothalamic aminergic neurotransmitters have also been involved in feeding control (1). In this context, we evaluated the effect of apelin-13 on food intake, and the role of NPY, AgRP, CART, POMC, CRH, orexin-A, and dopamine (DA), norepinephrine (NE), and serotonin (5-hydroxytryptamine, 5-HT) in the hypothalamus.

MATERIALS AND METHODS

Animals and drugs

Thirty-six male adult chow-fed Sprague-Dawley rats (290-340 g) were randomized for the experimental paradigms and housed in plexiglas cages (40 cm × 25 cm × 15 cm), one rat per cage, in accordance with the European Community ethical regulations (EU Directive 2010/63/EU) on the care of animals for scientific research. Rat apelin-13 (5 mg/vial, purchased from Vinci-Biochem, Italy) was diluted in saline and dosages selected on the basis of previous experiments (6).

In vivo intrahypothalamic treatment

Eighteen rats were anesthetized by intraperitoneal injection of ketamine-xylazine (50 and 5 mg/kg, respectively), treated with carprofen (4 mg/kg) and subjected to stereotaxic procedure, as previously reported (7). After a midline incision, the skull was scraped clean, washed with sterile saline solution, and disinfected with iodopovidone 10% solution. A drop of lidocaine 2% solution was applied into both the surgical incision and intraperitoneal injection sites. Coordinates for placement of a cannula into the ARC were as follows: anteroposterior (AP), −3 mm (or +3 mm posterior to the bregma); mediolateral (ML), 0.22 mm; dorsoventral (DV), −9 mm (7). 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 then intraperitoneally treated with piperacillin (20 mg/kg/day) (Teva SRL, Italy). During 72 h after surgery and before irisin administration, rats were carefully controlled: they made a rapid recovery and went back to sniff, eat and drink, groom themselves, explore the cage and the sawdust bedding and stand on hind legs as before surgery. There were no signs of hemorrhage. After the recovery period, we did not observe any significant alteration in basal food intake (Table I) in respect to six sham-operated animals, not included in the present experimental paradigm [Food intake (g): 10.66±0.41]. Furthermore, preliminary measurements on spontaneous physical activity, recorded by a video camera (SSC-DC378P, Biosite, Stockholm, Sweden) positioned on the top-center of the cage and connected to a computer, indicated negligible alterations in respect to sham animals. Beginning 72 h after surgery, at 9:00 a.m., rats (6 animals for each group) were injected into the ARC with either vehicle (saline) or apelin-13 (1-2 μg). The cannula was connected with a 10 μl syringe (Hamilton, Switzerland), 1 μl of drug solution or vehicle were injected into the arcuate nucleus. Food was recorded 24 h after apelin-13 administration, as previously reported (7), and finally animals were sacrificed by CO_2 inhalation. In preliminary experiments performed in our laboratories on rats of the same age and weight, we confirmed the location of the cannula tip by injecting dye (Evans blue 0.5% and Zelatin 5%) and histological examinations of frozen hypothalamic sections.

RNA extraction

Immediately after sacrifice, brains (N = 9) were rapidly removed, individual hypothalami were immediately dissected and stored in RNAlater solution (Ambion, Austin, TX) at −20°C until further processing. Total RNA was extracted from the hypothalamus using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. 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, 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, USA) 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, Rn00565995_m1 for orexin-A gene). β-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 $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and then determine the relative changes in individual gene expression (relative quantification) (7). 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.

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

Immediately after sacrifice, brains (N = 9) were rapidly removed and individual hypothalami dissected and subjected to biogenic amine extractive procedures. Briefly, hypothalamic tissues were homogenized in an 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 microdialysis cell (ESA-5014b) porous graphite working electrode and solid state palladium reference electrode. The analytical conditions for biogenic amine identification and quantification were selected as previously reported (7).

In vitro hypothalamic perfusion

Hypothalamic synaptosomes were obtained from 18 male adult Sprague-Dawley rats (290-340 g), as previously described (12). Then, the synaptosome suspension was incubated at 37°C, under O_2/CO_2 95%/5%, pH 7.35–7.45, in Krebs-Ringer buffer (mM: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, Tris–HCl 10, glucose 10, ascorbic acid 1). After the incubation period, identical aliquots of synaptosome suspension (1.24 mg protein determined by bicinchoninic acid protein assay) were layered onto 0.8 µm Millipore filters, placed into 37°C water-jacketed superfusion chambers [18 different chambers for each experiment and perfused with Krebs-Ringer buffer (0.6 ml/min)], and perfusate was collected (2 min fractions) to detect released neurotransmitters by HPLC coupled to electrochemical detection, following two experimental protocols. The analytical conditions for amine identification and quantification in the perfusate fractions were selected on the basis of previous experiments performed on hypothalamus homogenate (7). In a first set of experiments, apelin-13 (1–10 nM) was added to the perfusion buffer for 10 min (stimulus period), followed by 8 min with Krebs-Ringer buffer alone (return to basal period). A second set of experiments was run to evaluate the effects of apelin-13 (1–10 nM) on neurotransmitter release induced by a mild depolarizing

stimulus (K^+ 15 mM). After a 15 min equilibration perfusion with buffer alone, a 23 min perfusion with apelin-13 (1–10 nM) was started, where in the final 3 min (depolarization period), K^+ concentration in the perfusion buffer was elevated to 15 mM (after removal of equimolar concentrations of Na^+), followed by 8 min with Krebs-Ringer buffer alone (return to basal period). Perfusate aliquots were stored at −80°C, then lyophilized and finally suspended in 1 ml HPLC grade water for neurotransmitter quantification. Neurotransmitter release was expressed as ng/ml recovered in the perfusate samples.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA), through analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. Gene expression measurements were performed according to the relative comparative quantification method ($2^{-\Delta\Delta Ct}$), as previously described. Gaussian distribution of data was assessed by D'Agostino and Pearson omnibus normality test. Statistical significance was set at $P < 0.05$. With regard to the animals randomized for each experimental group, the number was calculated on the basis of the "Resource Equation" $N=(E+T)/T$ ($10 \leq E \leq 20$) elaborated by the "National Centre for the Replacement, Refinement and Reduction of Animals in Research" (NC3RS) and reported on the following web site: https://www.nc3rs.org.uk/experimental-designstatistics.

RESULTS

Intrahypothalamic administration of apelin-13 stimulated food intake 2 h and 24 h after peptide administration, in a dose-independent manner [Table I: (2 h post-injection data: ANOVA, $F=5.68$, $P< 0.05$; post-hoc, $*P<0.05$ *vs* vehicle) (24 h post-injection data: ANOVA, $F=28.72$, $P< 0.0001$; post-hoc, $***P<0.001$ *vs* vehicle)], without any significant effect on body weight (data not shown).

This is consistent with previous observations of increased food intake following peripheral and central apelin-13 administration, at different times points (3, 4).

In addition, intrahypothalamic apelin-13 administration significantly increased orexin A gene expression (ANOVA, $F=11.64$, $P< 0.01$; post-hoc, $*P<0.05$, $**P<0.01$ *vs* vehicle) and inhibited CART gene expression (ANOVA, $F=10.42$, $P< 0.01$; post-hoc, $*P<0.05$; $**P<0.01$ *vs* vehicle), while POMC, CRH, NPY and AgRP mRNA levels were not affected.

Apelin-13 treatment also decreased 5-HT levels (ng/mg wet tissue) in the hypothalamus (Vehicle: 1.32±0.04; Apelin-13 1 µg: 0.96±0.02**; Apelin-13 2 µg: 1.11±0.07**; ANOVA, $F=9.58$, $P< 0.01$; post-hoc, $**P<0.01$ *vs* vehicle), while NE and DA levels (ng/mg wet tissue) were not modified [NE: (Vehicle: 1.61±0.04; Apelin-13 1 µg: 1.70±0.07; Apelin-13 2 µg: 1.68±0.04); DA:(Vehicle: 0.58±0.04; Apelin-13 1 µg: 0.57±0.03**; Apelin-13 2 µg: 0.52±0.03)].

Finally, we tested apelin-13 activity on 5-HT release (ng/ml) from hypothalamic synaptosomes *in vitro*. We found an inhibitory effect on both basal (Vehicle: 1.01±0.06; Apelin-13 1 nM:0.62±0.04***;

Table I. *Food intake (in grams) in rats fed a standard diet and treated with either vehicle or apelin-13 (1-2 µg/rat).*

	Vehicle	Apelin-13 1 µg	Apelin-13 2 µg
2 h post-injection	0.74±0.02	0.86±0.04*	0.86±0.02*
24 h post-injection	10.44±0.41***	16.66±0.40***	16.13±0.19***

*Vehicle or apelin-13 were administered by intrahypothalamic injection, during the light phase, at 9:00 a.m. Food intake was evaluated 2 and 24 h after treatment in each group of rats. Values represent the means ± SEM. Compared to vehicle, apelin-13 significantly stimulated food intake [(2 h post-injection data: ANOVA, F=5.68, P< 0.05; post-hoc, *P<0.05 vs vehicle) (24 h post-injection data: ANOVA, F=28.72, P< 0.0001; post-hoc, ***P<0.001 vs vehicle)].*

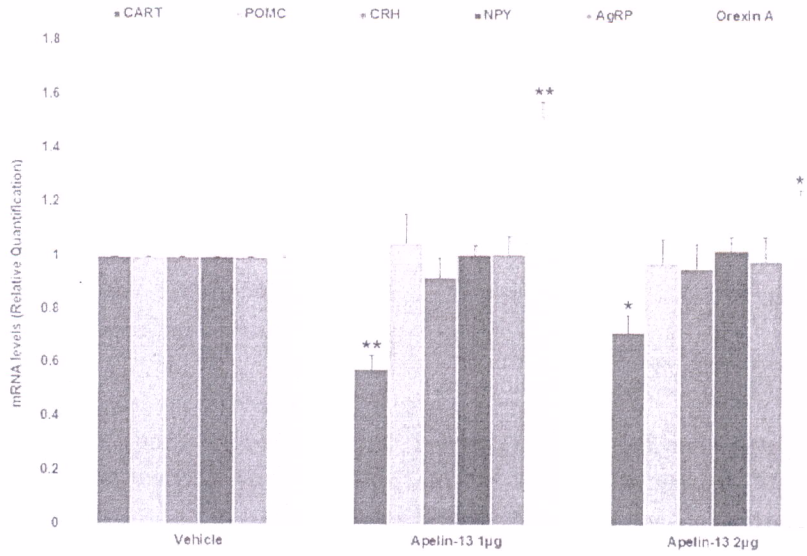

Fig. 1. *Relative gene expression of hypothalamic neuropeptides 24 h after vehicle or apelin-13 (1-2 µg/rat) administration, as determined by real-time RT-PCR. Data were calculated using the $2^{-\Delta\Delta Ct}$ method; they were normalized to β-actin mRNA levels and then expressed as relative to vehicle (calibrator sample, defined as 1.00±0.00). Compared to vehicle, apelin-13 significantly decreased CART gene expression (ANOVA, F=10.42, P< 0.01; post-hoc, *P<0.05, **P<0.01 vs vehicle) and increased orexin A gene expression (ANOVA, F=11.64, P< 0.01; post-hoc, *P<0.05, **P<0.01 vs vehicle).*

Apelin-13 10 nM: 0.63±0.03***; ANOVA, F=24.33, P< 0.0001; post-hoc, ***P<0.001 *vs* vehicle) and depolarization (K^+ 15 mM)-induced 5-HT release (Vehicle: 1.41±0.08; Apelin-13 1 nM:0.68±0.05***; Apelin-13 10 nM: 0.71±0.06***; ANOVA, F=33.08, P< 0.0001; post-hoc, ***P<0.001 *vs* vehicle). These inhibitory effects on both basal and depolarization-induced 5-HT release could be related to APJ receptor agonists binding to 5-HT1A autoreceptors (8).

DISCUSSION

Our findings showing stimulated feeding behavior 2 and 24 h after apelin-13 administration (Table I) adds to inconsistent results concerning the role of this peptide on feeding regulation. Previous works found either stimulatory or inhibitory effects, probably arising from differences in experimental conditions, including diet, dosages, time, and route of administration (9). With regard to this latter aspect, we may suggest that the direct administration of apelin-13 into the ARC, as performed in our experiments, provides a better targeting of the hypothalamic nuclei controlling feeding behavior. The observed inhibited gene expression of hypothalamic CART (which mediates anorectic effects) and stimulation of orexin-A (which plays an orexigenic role), as shown in Fig. 1, support a central effect of apelin-13 in the hypothalamic pathways that increases food intake. The present results are also consistent with the stimulation of orexin A and the inhibition of CART gene expression following apelin-13 treatment *in vitro* (10). We also found inhibition of hypothalamic 5-HT synthesis and release induced by apelin-13, which is also consistent with the orexigenic effect shown by apelin-13 *in vivo*. 5-HT release plays an anorectic role in the hypothalamus (1), and 5-HT involvement in mediating central apelin-13 behavioral effects has also been reported in mice (6). A recent study revealed anatomical and functional connections between CART and 5-HT neurons, possibly involved

in energy balance regulation (11). In addition, orexin A was found to inhibit 5-HT release, *in vitro* (12). In this context, we can hypothesize the serotoninergic system as being a key mediator of apelin-13-induced orexigenic effects.

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