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Corticotrophin-releasing factor mediates vasoactive intestinal peptide-induced hypophagia and changes in plasma parameters

Marcela Cristina Garnica-Siqueira[a], Andressa Busetti Martins[a], Dimas Augusto Morozin Zaia[b], Cristiane Mota Leite[c], Ernane Torres Uchôa[a,*], Cássia Thaïs Bussamra Vieira Zaia[a,*]

[a] Laboratory of Neuroendocrine Physiology and Metabolism, Department of Physiological Sciences, State University of Londrina, Londrina, PR, Brazil
[b] Laboratory of Prebiotic Chemistry, Department of Chemistry, State University of Londrina, Londrina, PR, Brazil
[c] Universidade Norte do Paraná, Londrina, PR, Brazil

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ABSTRACT

Vasoactive intestinal peptide (VIP) and corticotrophin-releasing factor (CRF) are anorexigenic neuropeptides that act in the hypothalamus to regulate food intake. Intracerebroventricular (ICV) microinjection of VIP promotes increased plasma adrenocorticotrophic hormone (ACTH) and corticosterone, indicating that VIP activates hypothalamic-pituitary-adrenal axis. The aim of this study was to evaluate the interaction between VIP and CRF, by verifying the effects of ICV administration of VIP on the activity of neurons and CRF mRNA expression in paraventricular nucleus of hypothalamus (PVN). In addition, it was evaluated the effects of pretreatment with CRF type 1 receptor (CRFR1) antagonist (Antalarmin, ANT) or CRF type 2 receptor (CRFR2) antagonist (Antisauvagine-30, AS30) on VIP-induced changes on food intake and plasma parameters of male rats. Compared to Saline group, VIP increased not only the number of *Fos-related antigens* (FRA)-immunoreactive neurons in the PVN but also CRF mRNA levels in this nucleus. Both ANT and AS30 treatment attenuated the inhibition of food intake promoted by VIP, ANT showing a more pronounced effect. Both antagonists also attenuated VIP-induced reduction and enhancement of free fatty acids and corticosterone plasma levels, respectively, and only AS30 was able to attenuate the hyperglycemia. These results suggest that CRF is an important mediador of VIP effects on energy balance, and CRFR1 and CRFR2 are involved in these responses.

1. Introduction

Vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide widely distributed in the central nervous system (CNS) and it acts as neuromodulator (Schutzberg et al., 1980). Neurons expressing VIP are distributed in the CNS, such as the cortex, hippocampus, amygdaloid nucleus, thalamus and hypothalamus. VIP binds with high affinity to its receptors VPAC1, primarily expressed in the hippocampus and cortex, and in a lesser extent in the hypothalamus (Joo et al., 2004), and VPAC2, expressed in the thalamus, hypothalamus, brain steam and amygdala (Sheward et al., 1995). In the hypothalamus, VPAC2 is found in supraoptic nucleus (SON), suprachiasmatic nucleus, arcuate nucleus, parvocelular region of the paraventricular nucleus (PVN) and anterior pituitary (Gerhold et al., 2001), areas associated with neuroendocrine functions in the CNS (Usdin et al., 1994).

It is known that VIP is released in the gastrointestinal tract in response to feeding (Miskowiak et al., 1985), suggesting that peripheral VIP might influence feeding behavior, since VIP can cross brain-blood-barrier (Dogrukol-Ak et al., 2003; Dogrukol-Ak et al., 2004). According to this, peripheral injection of VIP was shown to reduce food intake in different species (Matsuda et al., 2005; Matsuda et al., 2006; Yu et al., 2011), reinforcing this concept. Additionally, intracerebroventricular microinjection of VIP in rats promotes increase of glucose plasma concentrations and decreased food intake (Ghourab et al., 2011; Martins et al., 2018), as well as increased plasma adrenocorticotrophic hormone (ACTH) and corticosterone in a dose-dependent manner, indicating tha VIP regulates the hypothalamic-pituitary-adrenal axis (HPA), probably by activating corticotrophin-releasing factor (CRF) neurons (Alexander and Sander, 1994; Wang et al., 1998). In addition, although the hypothalamic release of VIP in response to feeding is not evidenced in the literature, it can be suggested that VIP is released in the hypothalamus, or at least in part involved in feeding-induced changes in the PVN, since Alexander et al. (1995) demonstrated that VIP release in the PVN mediates food-induced ACTH and CORT

* Corresponding authors at: Laboratório de Fisiologia Neuroendócrina e Metabolismo, Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, UEL, Rodovia Celso Garcia Cid, PR 445, km 380, Campus Universitário, 860570-970 Londrina, PR, Brazil.
E-mail addresses: euchoa@uel.br (E.T. Uchôa), ctbvzaia@uel.br (C.T.B.V. Zaia).

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secretion.

CRF reduces food intake (Arase et al., 1988) and it is classically known as the main secretagogue of the HPA axis, which can also be regulated by vasopressin, a neuropeptide co-localized with CRF neurons especially in the medial parvocellular PVN (Piekut and Joseph, 1986) that stimulates ACTH secretion in the presence of CRF (Rivier and Vale, 1983). CRF acts through specific receptors, type 1 (CRFR1) and type 2 (CRFR2). CRFR1 has a large CNS distribution, with significant expression in the cerebellum, limbic structures, dorsal and medial raphe nuclei, PVN and anterior and intermediate pituitary (Chalmers et al., 1995; Van Pett et al., 2000), being related to the activation of the HPA axis by its presence in the corticotrophs (Gutman et al., 2003). CRFR2 is expressed in the olfactory bulb, amygdala, nucleus of the solitary tract, hippocampus, raphe nuclei, and hypothalamus, in ventromedial nucleus, SON, and PVN (Lovenberg et al., 1995; Van Pett et al., 2000), hypothalamic areas related to food intake control and the anorexic effects of CRF (Vaughan et al., 1995).

Thus, as VIP activates HPA axis and both VIP and CRF are anorexigenic neuropeptides, it was hypothesized that CRF acts as mediator of VIP-induced changes on food intake and plasma metabolic parameters. For this purpose, the present study aimed to evaluate the effects of VIP on the activity of PVN neurons and CRF mRNA expression in the PVN, as well as the involvement of CRFR1 and CRFR2 on VIP-induced changes on food intake and corticosterone, glucose and free fatty acids plasma parameters.

2. Material and methods

2.1. Animals

Male Wistar rats (260–280 g, $n = 224$), from the Central Animal Care Facility of the State University of Londrina (UEL), were housed in cages at controlled temperature (22 ± 2 °C) with a fixed light-dark cycle (light from 6:00 AM to 6:00 PM). Animals had ad libitum access to pelleted rat chow and water, unless otherwise specified. To improve adaptation to the laboratory environment, rats were daily handled before the experiments. All experimental procedures were conducted between 7:00 AM and 12:00 PM and were approved by local Ethics Commission on the Use of Animals (protocol number 14371.2017.44).

2.2. Intracerebroventricular (ICV) surgery

Animals were anesthetized with an association of ketamine (K, 100 mg/kg, Agener União, 10%)/xylazine hydrochloride (X, 20 mg/kg, Anasedan®, Vetbrands, Jacareí, Brazil, 2%) intraperitoneally, placed in a stereotaxic instrument (David Kopf Instruments, model 900) with bregma and lambda in a horizontal plane. A stainless-steel guide cannula (0.7 mm external diameter, 0.4 mm internal diameter and 10 mm length) was implanted in the right lateral ventricle according to Paxinos and Watson's (1997) atlas coordinates: 0.8 mm caudal to bregma, 3.6 mm below the skullcap and 1.5 mm lateral to the sagittal suture. Cannula was fixed to the cranium using dental acrylic resin and two jeweller's screws. Within the implanted cannula, a 30-gauge metal obturator filled the cannula except during the microinjections. After surgery, rats received prophylactic dose of antibiotic (50,000 units of penicillin G: 0.1 mL per 100 g of body weight, intramuscularly) and paracetamol (200 mg/kg, orally). Animals were kept in collective cages containing a maximum of three animals, for better surgery recovery. Three days before the experiment, they were accommodated in individual cages for adaptation. Cannula placement was verified by sectioning the brain in a cryostat at the end of the experiment, in all groups.

2.3. Perfusion, tissue preparation, and Immunohistochemistry

Animals were anesthetized with an intraperitoneal K + X injection and were transcardially perfused with 200 mL of cold isotonic saline (0.15 M) containing heparin (1:80), followed by 400 mL of cold 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB, pH 7.4), using a peristaltic bomb with a speed of 10 mL per minute. The brain was removed, fixed in 4% paraformaldehyde for 1 h and stored at 4 °C in PB containing 30% sucrose. Coronal sections of 30 μm were obtained in a cryostat (Leica), collected in PB and stored in cryoprotectant solution at −20 °C. Sections were processed for FRA (*Fos-related antigens*) immunoreactivity by incubating for 48 h at 4 °C with an anti-FRA antibody (K25, Santa Cruz) diluted 1:2.000 in 0.1 PB containing 1.5% normal goat serum and 0.3% Triton. Free-floating sections were then washed with 0.01 PB and incubated with biotin-labelled anti-rabbit immunoglobulin (Vector Inc., Ca, USA, 1:200 dilution in 1.5% normal goat serum-PB) and after with the avidin-biotin-peroxidase complex (Vectastain, 1:200 in PB), for 1 h each at room temperature. For detection FRA labelling, diaminobenzidine hydrochloride (DAB, Sigma CO., Ca, USA, 0.01%) was used and intensified with 1% nickel ammonium sulfate. Thereafter, sections were mounted on gelatinized slides, air-dried overnight, dehydrated in xylene and placed under a cover slip with Ethelan (New Jersey, USA).

The PVN was identified according to Paxinos and Watson (1997) brain rat atlas. Medial (PaMP) and ventral (PaV) subdivisions of the PVN were considered at −1.80 mm, and posterior parvocellular (PaPo) subdivision of the PVN was considered at −2.12 mm from bregma. FRA immunoreactive neurons were quantified with the aid of an image system attached to a microscope (Motic). FRA-immunoreactivity neurons, indicated in black staining, were identified when the nuclear structure demonstrated a clear immunoreactivity compared with the background level. Sections were counted for PaMP, PaV and PaPo, and the visual counting of neurons expressing FRA was performed in one or two sections from ten to twelve animals of each experimental group by participants blind to the experimental protocols.

2.4. Microdissection, total RNA isolation and quantitative real-time PCR

Microdissections of the PVN were obtained using a stainless punch needle 1.5 mm in diameter, in a cryostat according to coordinates from −0.92 to −2.12 for PVN (1200 μm) from bregma. Total RNA was isolated from each micropunched hypothalamic tissue sample using Trizol reagent (Invitrogen®, New Zealand) according to the manufacturer's protocol. The RNA concentration in each sample was determined using a Multi-detection microplate reader (Synergy HT, BioTek), and 500 ng of RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. The TaqMan® Gene Expression Assay (Applied Biosystems) used in this study was Rn 01462137_m1 (CRF). Each PCR reaction was performed in duplicate. Water (instead of cDNA) was used as negative control. Housekeeping gene, beta actin, was run for each cDNA sample. The determination of gene transcript levels in each sample was obtained by the ΔΔCT method. For each sample, the threshold cycle (Ct) was determined and normalized to the average of the housekeeping gene ($\Delta Ct = Ct_{Unknown} - Ct_{Housekeepinggenes}$). The fold-change of mRNA expression in the unknown sample relative to the control group was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{Unknown} - \Delta Ct_{Control}$ (Livak and Schmittgen, 2001). Data are shown as mRNA expression relative to the control group (Saline).

2.5. Experimental protocols

All animals were subjected to ICV surgery. After 6 days of recovery, they were fasted for 16 h, and on the seventh day after surgery, the experimental tests were performed.

The drugs ICV microinjected for the experiments were: VIP (Sigma Co., CA), 40 ng/g body weight (BW) in 6 μL microinjected in 1 min, and sterile saline (0.9% NaCl, 6 μL in 1 min) was microinjected as vehicle.