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ORIGINAL ARTICLE

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Adrenalectomy impairs vasoactive intestinal peptide-induced changes in food intake and plasma parameters

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Abstract

Purpose The aim of this study is to evaluate the effects of adrenalectomy (ADX) and glucocorticoid in the changes induced by intracerebroventricular (ICV) administration of vasoactive intestinal peptide (VIP) on food intake and plasma parameters, as well as VIP receptor subtype 2 (VPAC2) mRNA expression in different hypothalamic nuclei of male rats.

Methods Male Wistar rats (260–280 g) were subjected to ADX or sham surgery, 7 days before the experiments. Half of ADX animals received corticosterone (ADX + CORT) in the drinking water. Animals with 16 h of fasting received ICV microinjection of VIP or saline (0.9% NaCl). After 15 min: (1) animals were fed, and the amount of food ingested was quantified for 120 min; or (2) animals were euthanized and blood was collected for biochemical measurements. Determination of VPAC2 mRNA levels in LHA, ARC, and PVN was performed from animals with microinjection of saline.

Results VIP treatment promoted the anorexigenic effect, which was not observed in ADX animals. Microinjection of VIP also induced an increase in blood plasma glucose and corticosterone levels, and a reduction in free fatty acid plasma levels, but adrenalectomy abolished these effects. In addition, adrenalectomy reduced mRNA expression of VPAC2 in the lateral hypothalamic area and arcuate nucleus, but not in the paraventricular nucleus.

Conclusions These results suggest that adrenal glands are required for VIP-induced changes in food intake and plasma parameters, and these responses are associated with reduction in the expression of VPAC2 in the hypothalamus after adrenalectomy.

Keywords VIP · Glycemia · Corticosterone · Free fatty acids · Arcuate nucleus of the hypothalamus · VPAC2 receptor expression

Introduction

The vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide widely expressed in the nervous system and belongs to the family of regulatory peptides, glucagon–secretin. Neurons expressing VIP are distributed in the central nervous system (CNS), such as the cortex, hippocampus, amygdaloid nucleus, thalamus, and hypothalamus [1]. VIP actions are mediated by specific receptors that are coupled to adenyl cyclase to increase intracellular cAMP [1, 2]. There are two VIP receptor subtypes, VPAC1, widely distributed in the CNS, most abundantly expressed in the hippocampus and cortex, and to a lesser extent in the hypothalamus [3], and VPAC2, expressed in the thalamus, hypothalamus, brainstem, and amygdala [4]. In the hypothalamus, VPAC2 is found in the supraoptic nucleus, suprachiasmatic nucleus, arcuate nucleus (ARC), parvocellular region of the

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paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and anterior pituitary [3–6], areas associated with neuroendocrine functions in the CNS [7].

Intracerebroventricular (ICV) microinjection of VIP in rats promotes an increase in glucose plasma concentrations and a decrease in food intake [8, 9], as well as increased in plasma adrenocorticotrophic hormone (ACTH) and corticosterone in a dose-dependent manner, indicating that VIP regulates the hypothalamic–pituitary–adrenal axis (HPA) [10, 11].

Bilateral adrenalectomy (ADX) decreases food intake and body weight, whereas CORT replacement was demonstrated to stimulate food intake [12–14] and to attenuate increases in ACTH secretion and corticotrophin-releasing factor (CRF) expression in the PVN [15, 16], where glucocorticoid receptors are highly expressed [17]. Ceccatelli et al. [18] recognized co-localization of VIP and CRF in PVN parvocellular neurons of rats, and an increase in VIP expression was observed in the pituitary gland after ADX, while treatment with dexamethasone abolished this effect [19, 20].

As both VIP and glucocorticoids are involved in the control of food intake, VIP activates the HPA axis and glucocorticoids are known to induce hyperglycemia; the present study was designed to evaluate the effects of the presence or absence of glucocorticoids on ICV administration of VIP-induced changes on food intake, glucose, free fatty acids (FFA), and corticosterone plasma levels, as well as the VPAC2 mRNA expression in different hypothalamic nuclei after ADX.

Materials and methods

Animals

Male Wistar rats weighing 260–280 g (total number of animals = 130), from the Central Animal Care Facility of the State University of Londrina (UEL), were housed in cages in a temperature-controlled room at 22 ± 2 °C, and kept on a 12:12-h light–dark cycle with lights on at 6:00 a.m. Animals had ad libitum access to pelleted rat chow and water, unless otherwise specified. To improve adaptation to the laboratory environment, rats were handled daily before experiments. All experimental procedures were conducted between 7:00 a.m. and 12:00 p.m. and were approved by local Ethics Commission on the Use of Animals of UEL (protocol number 14371201744).

Bilateral ADX and sham surgeries were performed with an association of ketamine (K, 100 mg/kg, Agener União, 10%) and xylazine hydrochloride (X, 20 mg/kg, Anasedan®, Vetbrands, Jacareí, Brazil, 2%) intraperitoneally, via single dorsal midline incision on the skin and a bilateral small cut through the muscle layer. After the surgery and during all the experimental periods, ADX animals were given 0.9% saline with 0.5% ethanol, without glucocorticoid (ADX) or with glucocorticoid (corticosterone, Sigma Co., CA) at the concentration of 25 mg/L (ADX + CORT). Sham-operated animals underwent similar surgical procedures without removal of adrenal glands and were given tap water with 0.5% ethanol to drink. To ensure completeness of ADX surgery and adequacy of glucocorticoid replacement, corticosterone plasma level was determined by a fluorometric method of Guillemin et al. [21].

Intracerebroventricular (ICV) surgery

Animals were anesthetized with an intraperitoneal K + X injection, placed in a stereotaxic instrument (David Kopf Instruments, model 900) with bregma and lambda in the same 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 [22] atlas coordinates: 0.8 mm caudal to bregma, 3.6 mm below the skullcap, and 1.5 mm lateral to the sagittal suture. A 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 injections. After surgery, rats received a 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 in all groups by sectioning the brain in a cryostat at the end of the experiment.

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

Microdissections of LHA, ARC, and PVN were obtained using a stainless punch needle 1.5 mm in diameter from coronal sections (1200 µm), in a cryostat according to coordinates from −0.92 to −2.12 for PVN and −2.12 to −3.32 for LHA and ARC, from bregma. Total RNA was isolated from each micropunched hypothalamic tissue sample using Trizol reagent (Invitrogen®, New Zealand) according to the manufacturer's protocol. RNA concentration in each sample was determined using Multi-detection micro plate 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 Applied Biosystems 7500 real-time PCR system. The TaqMan® Gene Expression Assay (Applied Biosystems) used in this study was Rn 00568267_m1 (VPAC2). 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. 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 Ct of the housekeeping gene ($\Delta Ct = Ct_{Unknown} - Ct_{Housekeepinggene}$). Fold-change of mRNA expression in unknown sample relative to control group was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{Unknown} - \Delta Ct_{Control}$ [23]. Data are shown as mRNA expression relative to control group (Sham).

Experimental protocols

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

The drug ICV microinjected for experiments was VIP (Sigma Co., CA; dose of 40 ng/g body weight: 2.7–3.4 nmol of VIP for 220–280 g body weight) in 6 µL microinjected in 1 min, and sterile saline (0.9% NaCl, 6 µL in 1 min) was microinjected as a vehicle. The dose of VIP was chosen based on different works from literature [8–10, 24].

Experiment 1: effects of microinjection with VIP on food intake in Sham, ADX, and ADX + CORT animals

Animals received ICV microinjection of VIP or Saline. After 15 min, all animals had access to food (50 g) and food intake was quantified (g/100 g body weight) after 120 min.

Experiment 2: effects of microinjection with VIP on plasma parameters in Sham, ADX, and ADX + CORT animals

Animals received ICV microinjection of VIP or Saline. After 15 min, all animals were decapitated, and trunk blood was collected in heparinized tubes and centrifuged at 14,000 × *g* for 20 min. Blood plasma was stored at –20 °C and used for biochemical dosages of glucose by spectrophotometric determination based on peroxidase reaction [25] using the BioLiquid Glucose Commercial Kit (Laborclin, PR, Brazil), corticosterone (modified fluorometric method of Guillemin et al. [21]), and FFA (modified spectrophotometric method of Falholt et al. [26]).

Experiment 3: effects of ADX and glucocorticoid replacement on mRNA expression of VPAC2 in the LHA, PVN, and ARC

Fasted Sham, ADX, and ADX + CORT were decapitated 7 days after surgery, and brains were collected under RNAse-free conditions, immediately frozen on dry ice, and stored at –80 °C for determination of VPAC2 mRNA levels in LHA, ARC, and PVN.

Statistical analysis

Data are expressed as means ± SEM. Normal distribution and homogeneity of data were tested. One-way ANOVA was performed to evaluate the differences among Sham, ADX, and ADX + CORT groups on VPAC2 mRNA expression in LHA, PVN, and ARC (experiment 3). Two-way ANOVA, followed by Student Newman–Keuls post hoc test, was performed to evaluate the interaction between the variable group (according to the presence or absence of glucocorticoids—Sham, ADX, or ADX + CORT) and the variable treatment (Saline or VIP) on food intake and plasma parameters (experiments 1 and 2). Differences were considered significant at $P < 0.05$.

Results

Experiment 1: effects of microinjection with VIP on food intake in Sham, ADX, and ADX + CORT animals

In Sham groups, VIP treatment showed reduction ($P < 0.001$) of about 60% in food intake compared with Saline treatment, but food intake of VIP did not differ from Saline in ADX and ADX + CORT groups. In saline-treated animals, the ADX group showed reduced ($P = 0.04$) food intake compared with the Sham group, while food intake of the ADX + CORT group did not differ from Sham and ADX (Fig. 1). In VIP-treated animals, there was no difference among the three experimental groups. There was an

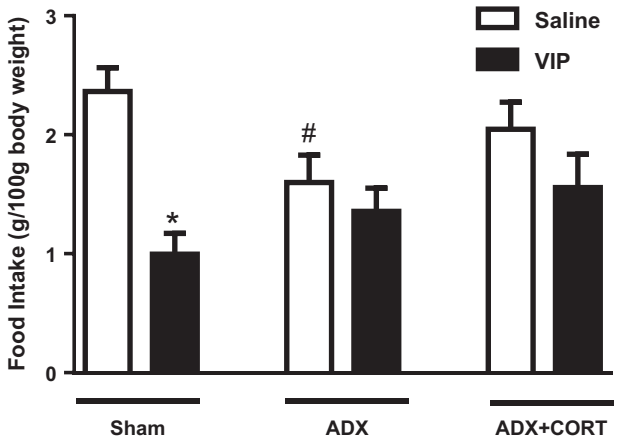

Fig. 1 Food intake (g/100 g of body weight) for 120 min of Sham, ADX, and ADX + CORT groups after ICV microinjection of saline (0.9% NaCl, 6 µL in 1 min) or vasoactive intestinal peptide (VIP; 40 ng/g body weight, 6 µL in 1 min). Data are shown as means ± SEM ($n = 9$–13 rats/group). *$P < 0.001$ vs. the respective Saline group. #$P = 0.04$ vs. Sham/Saline group

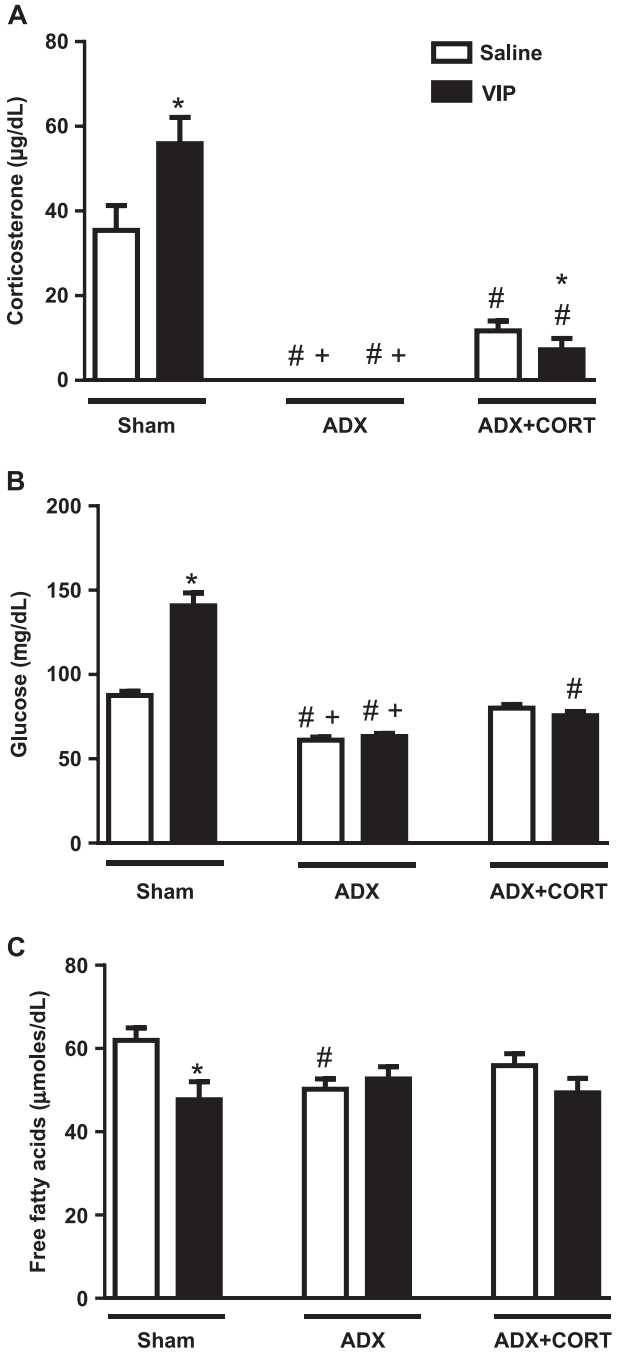

Fig. 2 Blood plasma concentration of corticosterone (µg/dL) (**a**), glucose (md/dL) (**b**), and free fatty acids (µmole/dL) (**c**) of Sham, ADX, and ADX + CORT groups after ICV microinjection of saline (0.9% NaCl, 6 µL in 1 min) or vasoactive intestinal peptide (VIP; 40 ng/g body weight, 6 µL in 1 min). Data are shown as means ± SEM ($n = 8$–13 rats/group). *$P < 0.001$ vs. the respective Saline group. #$P < 0.05$ vs. the respective Sham. +$P < 0.001$ vs. the respective ADX + CORT

interaction between group (Sham, ADX, or ADX + CORT) and treatment (Saline or VIP) on food intake [$F(5,63) = 3.6$, $P < 0.05$]. We observed no effect of group [$F(5,63) = 1.13$, $P = 0.33$] on food intake, with an effect of treatment [$F(5,63) = 15.08$, $P < 0.001$].

Experiment 2: effects of microinjection with VIP on plasma parameters in Sham, ADX, and ADX + CORT animals

In Sham group, VIP treatment induced an increase ($P < 0.001$) in corticosterone plasma levels compared to Saline, while in ADX + CORT group VIP reduced ($P < 0.05$) corticosterone plasma levels. In animals treated with both Saline and VIP, corticosterone plasma concentrations were higher in the Sham group than in ADX + CORT. The ADX group did not show detectable concentrations of plasma corticosterone (Fig. 2a), confirming the effectiveness of the surgery. There was an interaction between group (Sham, ADX, or ADX + CORT) and treatment (Saline or VIP) on corticosterone plasma levels [$F(5,63) = 6.5$, $P = 0.003$]. We observed an effect of group [$F(5,63) = 230.4$, $P < 0.001$] on corticosterone plasma levels, with no effect of treatment [$F(5,63) = 0.107$, $P = 0.75$].

VIP microinjection induced an increase ($P < 0.001$) in glucose plasma levels (Fig. 2b) in the Sham group when compared with Saline microinjection, while VIP treatment did not promote any change in ADX and ADX + CORT groups. In Saline-treated animals, ADX group had lower glucose plasma values ($P < 0.001$) than Sham and ADX + CORT groups, and in VIP-treated animals, ADX and ADX + CORT groups showed reduced glycemia ($P < 0.05$) compared with the Sham group. There was an interaction between group (Sham, ADX, or ADX + CORT) and treatment (Saline or VIP) on glucose plasma levels [$F(5,63) = 46.4$, $P < 0.001$]. We observed an effect of group [$F(5,63) = 132.2$, $P < 0.001$] and treatment [$F(3,63) = 41.6$, $P < 0.001$] on glucose plasma levels.

FFA plasma concentrations (Fig. 2c) were decreased ($P = 0.003$) by VIP microinjection in the Sham group. Nevertheless, in ADX and ADX + CORT groups, there was no difference between Saline and VIP treatments. In addition, ADX decreased ($P < 0.050$) FFA plasma concentrations in Saline-microinjected animals, and no difference was observed among Sham, ADX, and ADX + CORT groups in VIP-microinjected animals. There was an interaction between group (Sham, ADX, or ADX + CORT) and treatment (Saline or VIP) on FFA plasma levels [$F(5,63) = 6.5$, $P < 0.05$]. We observed no effect of group [$F(5,63) = 0.54$, $P = 0.588$] on FFA plasma levels, with an effect of treatment [$F(5,63) = 5.25$, $P = 0.03$].

Experiment 3: effects of ADX and glucocorticoid replacement on mRNA expression of VPAC2 in the LHA, ARC, and PVN

ADX and ADX + CORT groups showed a reduction in VPAC2 mRNA expression in the LHA [$F(2,31) = 7.8$, $P =$

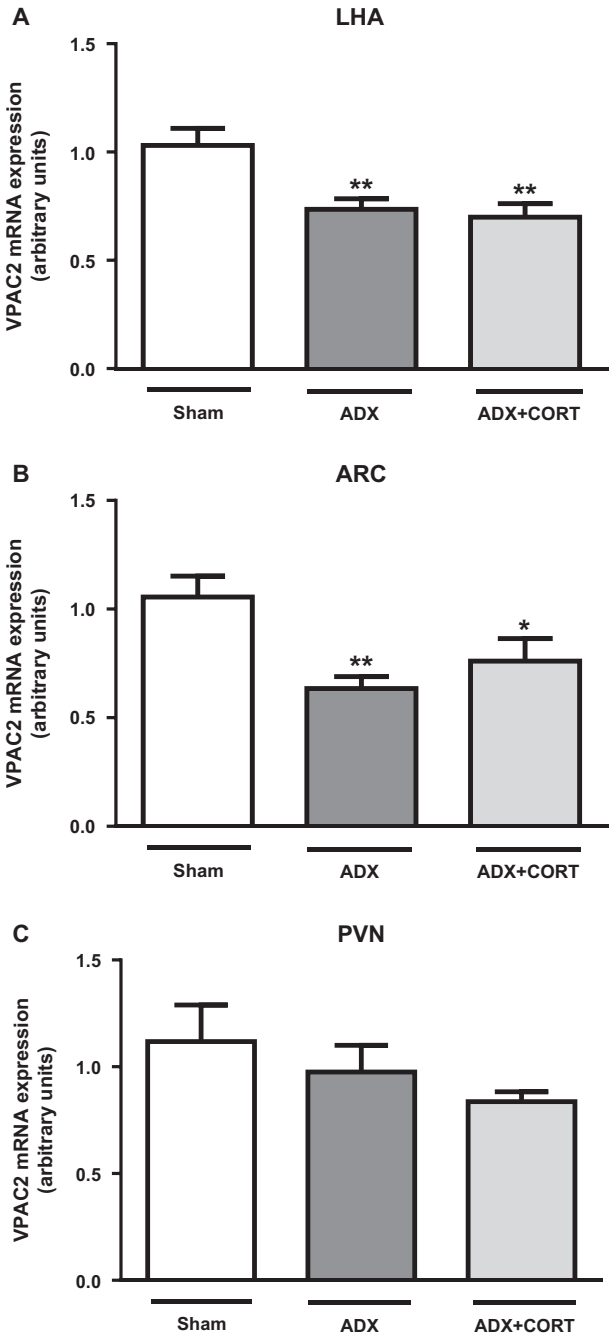

Fig. 3 Relative VIP receptor type 2 (VPAC2) mRNA expression in the lateral hypothalamic area (**LHA; a**), arcuate nucleus (**ARC; b**), and paraventricular nucleus (**PVN; c**) of Sham, ADX, and ADX + CORT groups treated with ICV microinjection of saline (0.9% NaCl, 6 µL in 1 min). Data are shown as mean ± SEM ($n = 9$–13 rats/group). *$P <$ 0.05 vs. Sham group. **$P = 0.008$ vs. Sham group

0.002] and ARC [$F(2,31) = 5.6$, $P = 0.008$] compared with the Sham group (Fig. 3a, b). In PVN, there was no difference in VPAC2 mRNA expression among Sham, ADX, and ADX + CORT groups (Fig. 3c).

Discussion

The present study investigated the effects of ADX and glucocorticoid replacement on VIP-induced changes on food intake and plasma parameters of rats. Further, VPAC2 mRNA expression in LHA, ARC, and PVN was also investigated in Sham, ADX, and ADX + CORT animals. VIP treatment reduced food intake and promoted changes in plasma parameters, but adrenalectomy was shown to abolish these effects, since such responses were not observed in adrenalectomized animals. In addition, adrenalectomy reduced mRNA expression of VPAC2 in LHA and ARC but not in PVN.

To evaluate the effect of glucocorticoid removal on VIP-induced changes, ADX rats with and without corticosterone replacement in drinking fluid were used. Corticosterone plasma levels have a circadian variation, and replacement in the drinking water results in increase of plasma corticosterone after lights off, corresponding to the feeding period, which could benefit the control of HPA axis activity [14, 27]. As expected, both groups showed higher values of plasma corticosterone than the ADX group, but a lower plasma concentration of corticosterone in the ADX + CORT group than the Sham group is probably because animals were fasted prior to the experiment and consequently ingested less fluid during this period [14]. In addition, increased corticosterone plasma levels after ICV microinjection of VIP in Sham animals corroborate the previous data in the literature [9, 28, 29], as well as Alexander and Sander [10], who observed an increase in plasma corticosterone after microinjection of VIP in PVN of rats.

Hypophagia promoted by microinjection of VIP in the lateral ventricule of Sham group corroborates the established anorexigenic effect of VIP, as observed by reduction of food intake after VIP microinjection in different species [8, 9, 24, 30, 31]. The reduction of food intake after ADX in Saline-treated animals is in accordance with the well-known effect of ADX to decrease food intake and body weight [14, 27], which is mediated by an increase of CRF mRNA expression in the PVN, due to the absence of negative feedback of glucocorticoids in this hypothalamic nucleus, as well as by enhancement of oxytocin mRNA expression in the PVN [27, 32, 33]. In addition, ADX is also associated with reduced expression of neuropeptide Y (NPY) and agouti-related protein in the ARC [34]. Besides that, Alexander and Sander [35] observed that microinjection of VIP in the PVN stimulates the secretion of ACTH and corticosterone, and pretreatment with CRF antagonist inhibits these responses. Previous work from our group showed that ICV microinjection of VIP promotes increase on CRF mRNA expression in PVN and that activation of both CRF receptors contribute to VIP-induced hypophagia [29],

indicating that the anorexigenic effect of VIP is mediated by its action through CRF neurons in the PVN. Overall, these data suggest that lack of VIP-induced reduction of food intake in ADX rats is likely because VIP microinjection may not be able to further increment CRF expression in the PVN induced by ADX and consequently potentiate this anorexigenic response. In addition to this, VIP-induced hypophagia seems to be mediated by stimulation of hypothalamic melanocortin, while anorexigenic effect of ADX is not associated with increases in proopiomelanocortin (POMC) in the ARC, where, in fact, ADX reduces POMC mRNA expression [34, 36]. Accordingly, it is possible that the absence of anorexigenic response of VIP after ADX may also be ascribed to these opposite effects of VIP and ADX on POMC neurons in ARC, since VIP would have to recruit melanocortin system downregulated by ADX. Though glucocorticoid replacement was effective in restoring food intake of ADX to similar values of Sham animals, the absence of VIP-induced reduction in food intake of ADX animals with glucocorticoid replacement suggests that VIP actions on food intake depends on the intact adrenal glands, not only the presence of glucocorticoids. Regarding plasma parameters, increased glucose plasma concentrations in the Sham group after microinjection of VIP is a well-known effect observed in previous studies [9, 29, 37]. In addition, lower values of glucose plasma levels after ADX demonstrate the importance of adrenal glands to control glucose levels [38, 39]. Accordingly, impairment of VIP to increase glycemia after ADX reinforces that glucocorticoids and sympathetic activation are required for the regulation of this parameter [40, 41]. Furthermore, reduced FFA plasma concentrations induced by VIP microinjection have also been observed in previous works [9, 29], and decreased values of circulating FFA after ADX are in accordance with previous works in the literature, which showed that glucocorticoids are important for mobilization of this energy substrate [42, 43]. As observed for food intake, the lack of effects of VIP on FFA may be due to the fact that VIP can not potentiate ADX-induced reduction on FFA plasma levels.

It is known that bilateral adrenalectomy in rodents is a well-established experimental model to investigate the mechanisms underlying classical symptoms observed in primary adrenal insufficiency in humans, such as hypophagia and loss of body weight [14, 33, 44, 45]. Concerning VIP, due to methodological limitations, human studies focus on the evaluation of VIP concentrations in the periphery than in the CNS after any challenge [46–51]. It should be noted that, as far as we know, there is no study in the literature that evaluated the interaction between adrenal glands and VIP effects, both in rodents and humans. Thus, the present study was the first to demonstrate that adrenalectomy promoted a decrease in mRNA expression of VPAC2 in ARC and LHA. VPAC2, a receptor that has a high affinity for VIP and pituitary adenylate ciclase-activating polypeptide, is extensively distributed in the hypothalamus and expressed in different nuclei [52–54]. It was demonstrated that VPAC2 mRNA is expressed in proopiomelacortin (POMC) [55] and NPY [56] neurons of the ARC. In accordance with this, the modulation of glucocorticoids on VPAC2 expression obtained in the current study may be supported by the work of Wiik [57], which demonstrated an increase in the number of binding sites for VIP after in vitro exposure of leukocytes to glucocorticoids. Thus, impairment of VIP-induced hypophagia and changes in glucose and FFA plasma levels in ADX animals is associated with lower expression of VPAC2 mRNA in the hypothalamus after adrenalectomy.

In summary, it is noteworthy in the present study that adrenalectomy impairs VIP-induced hypophagia, hyperglycemia, and reduction of FFA plasma levels, and these effects are associated with the reduction of VPAC2 mRNA expression in ARC and LHA induced by adrenalectomy. These data suggest that adrenal glands are required for VIP-induced changes on food intake and plasma parameters, and this response seems to be, at least in part, due to the modulation of glucocorticoids in the expression of VPAC2 in the hypothalamalus.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the local Ethics Commission on the Use of Animals of UEL (protocol number 14371201744).

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