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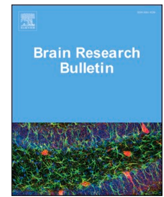

Drastic decline in vasoactive intestinal peptide expression in the suprachiasmatic nucleus in obese mice on a long-term high-fat diet

Domingo Afonso-Oramas [a,b,c,*,1], Laura Santana-Cordón [a,1], Alejandro Lemus-Mesa [a], Silvia Teixidó-Trujillo [d], Ana Elena Rodríguez-Rodríguez [e], Ignacio Cruz-Muros [a,b], Miriam González-Gómez [a,b,c], Pedro Barroso-Chinea [a,b,c,*]

[a] *Departamento de Ciencias Médicas Básicas, Facultad de Medicina, Universidad de La Laguna, Tenerife, Spain*
[b] *Instituto de Tecnologías Biomédicas de Canarias (ITB), Universidad de La Laguna, Tenerife, Spain*
[c] *Instituto Universitario de Neurociencias. Universidad de La Laguna, Tenerife, Spain*
[d] *Departamento de Medicina Interna, Dermatología y Psiquiatría. Facultad de Medicina, Universidad de La Laguna, Tenerife, Spain*
[e] *Unidad de Investigación, Hospital Universitario de Canarias (HUC), Tenerife, Spain*

A R T I C L E I N F O

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A B S T R A C T

The suprachiasmatic nucleus (SCN*)* is the main region for the regulation of circadian rhythms. Although the SCN contains a heterogeneous neurochemical phenotype with a wide variety of neuropeptides, a key role has been suggested for the vasoactive intestinal neuropeptide (VIP) as a modulator circadian, reproductive, and seasonal rhythms. VIP is a 28-amino acid polypeptide hormone that belongs to the secretin-glucagon peptide superfamily and shares 68 % homology with the pituitary adenylate cyclase-activating polypeptide (PACAP). VIP acts as an endogenous appetite inhibitor in the central nervous system, where it participates in the control of appetite and energy homeostasis. In recent years, significant efforts have been made to better understand the role of VIP in the regulation of appetite/satiety and energy balance. This study aimed to elucidate the long-term effect of an obesogenic diet on the distribution and expression pattern of VIP in the SCN and nucleus accumbens (NAc) of C57BL/6 mice. A total of 15 female C57BL/6J mice were used in this study. Female mice were fed ad libitum with water and, either a standard diet (SD) or a high-fat diet (HFD) to induce obesity. There were 7 female mice on the SD and 8 on the HFD. The duration of the experiment was 365 days. The morphological study was performed using immunohistochemistry and double immunofluorescence techniques to study the neurochemical profile of VIP neurons of the SCN of C57BL/6 mice. Our data show that HFD-fed mice gained weight and showed reduced VIP expression in neurons of the SCN and also in fibres located in the NAc. Moreover, we observed a loss of neuropeptide Y (NPY) expression in fibres surrounding the SCN. Our findings on VIP may contribute to the understanding of the pathophysiological mechanisms underlying obesity in regions associated with uncontrolled intake of high-fat foods and the reward system, thus facilitating the identification of novel therapeutic targets.

1. Introduction

The neuroendocrine system plays a crucial role in the control of food intake and body energy balance, closely interacting with certain hormones, neurotransmitters and neuropeptides implicated in body weight control. Currently, there is extensive literature on the physiological role of neuropeptide Y (NPY), somatostatin (SST), vasoactive intestinal peptide (VIP), leptin, orexin, ghrelin, agouti-related protein (AgRP), melanocyte-stimulating hormone (MSH), galanin, among others, in those processes (Casanueva and Dieguez, 1999; Clark et al., 1984; Kalra, 1997; Kalra et al., 1999; Heiman and Statnick, 2003; Yang and Harmon, 2003; Bjorbaek and Kahn, 2004; Costentin, 2004). VIP is a 28 amino acids polypeptide hormone that belongs to the secretin-glucagon superfamily of peptides and shares 68 % homology with PACAP (Vu et al., 2015a,b). VIP is widely distributed in the peripheral and central nervous systems, but it is particularly abundant in the gastrointestinal tract and

* Correspondence to: Departamento de Ciencias Médicas Básicas (Anatomía), Facultad de Ciencias de la Salud (Medicina), Universidad de La Laguna, 38207 La Laguna, Tenerife, Spain.
E-mail addresses: daforam@ull.es (D. Afonso-Oramas), pbarroso@ull.es (P. Barroso-Chinea).

[1] These authors contributed equally to this work.

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pancreas, where it exerts various physiological functions. Indeed, its primary functions include stimulating the intestinal secretion of ions and water, inhibiting gastric acid secretion, as well as inducing systemic vasodilatation, increasing cardiac output, bronchodilatation and hyperglycaemia, among others effects (Bloom et al., 1973; Dickson and Finlayson, 2009; Harmar, 2012; Vu et al., 2014). At the central nervous system, VIP is mainly expressed in the cerebral cortex, forebrain limbic structures (septum, amygdala, hippocampus), thalamus and hypothalamic areas such as the SCN and the arcuate nucleus (ARC) (Lam, 1991; Klein et al., 1991; Benagiano et al., 2009; Mazuski et al., 2020; Todd et al., 2020). In particular, VIP is expressed at high levels in the neurons of the SCN (Kudo et al., 2013; Vosko et al., 2015). In the central nervous system, VIP plays an important role as a neurotransmitter and neuroendocrine factor. The synergistic coactivation of VIP and GABAergic pathways in the SCN has been identified as a key step in priming the molecular oscillation responsible for the circadian rhythm (Fan et al., 2015). Thus, suprachiasmatic VIP-neurons are required for normal circadian rhythmicity (Aton et al., 2005; Vosko et al., 2007; Kudo et al., 2013; Kingsbury and Wilson, 2016; Jones et al., 2018; Todd et al., 2020). VIP is known to play a significant role in the input and output pathways from the SCN. It regulates the long-term firing rate of SCN neurons by increasing specific membrane receptors in the cAMP pathway (Kudo et al., 2013). In addition to adenylate cyclase action, these actions of VIP in the SCN have been shown to depend on the activities of phospholipase C (PLC) or protein kinase A (PKA) (An et al., 2011). The factors that regulate both VIP synthesis and release are currently unknown. However, there is evidence that VIP release by VIPergic neurons is specifically regulated by acetylcholine, dopamine, GABA, serotonin and SST, highlighting the existence of interactions between different neurotransmitters and neuropeptides in the central nervous system (Newmyer et al., 2019).

VIP receptors are widely distributed throughout the body, being particularly abundant in endocrine organs, immune tissues and blood vessels (Laburthe et al., 2007).

VIP exerts its wide range of biological functions through specific membrane receptors, VPAC1, VPAC2 and PAC1, which belong to the class II G-protein-coupled receptor family (Said and Mutt, 1970; Harmar et al., 2012; Couvineau and Laburthe, 2011; Sanford et al., 2022).

The binding of VIP to its receptors mainly triggers the cAMP/protein kinase A pathway, which is considered an immunosuppressive signalling pathway (Dickson and Finlayson, 2009).

While VPAC1 receptor is primarily found in the cerebral cortex and hippocampus (Joo et al., 2004; Delgado and Ganea, 2013; Cunha-Reis et al., 2017), VPAC2 is mainly localised in the SCN, and to a lesser extent in the thalamus, hippocampus, cerebral cortex, periventricular nucleus, hypothalamus, spinal cord and dorsal root ganglia (An et al., 2012; Delgado and Ganea, 2013). Likewise, PAC1 is found in the olfactory bulb, thalamus, hypothalamus, dentate gyrus of the hippocampus and granule cells of the cerebellum (Delgado and Ganea, 2013). The neurotransmitter and neuromodulatory functions of VIP include rhythm generation in the SCN, regulation of neuroendocrine secretion in the hypothalamus and energy metabolism in glial cells (Mazuski et al., 2020; Todd et al., 2020). The interaction between VIP and its receptors plays a crucial role in regulating these functions. Thus, evidence suggests that VIP/VPAC1 binding induces neuroprotection and regulates energy and glucose homeostasis (Sanford et al., 2022), while VIP/VPAC2 interaction normalizes circadian rhythm, contributes to insulin sensitivity and regulates proinflammatory cytokine production. Additionally, VIP/PAC1 interaction mediates light-induced behaviour and gene expression, facilitates glucose homeostasis, as well as regulates inflammatory mediators (Laburthe et al., 2007; Couvineau and Laburthe, 2011).

The literature concerning the association between VIP and obesity is very scarce. However, some evidence shows that VIP centrally regulates feeding behaviour in different vertebrate species (Jung et al., 2008; Vu et al., 2015a,b). Interestingly, mice genetically lacking VIP showed a reduction in body weight and an increase in lean mass. In addition, these mice had an altered circadian feeding behaviour. However, other studies have shown that direct administration of VIP to the ARC nucleus in mice is able to reduce hyperphagia induced by fasting and food intake, reducing total lipids and increasing plasma concentrations of free fatty acids (Martins et al., 2022). In addition, these mice presented hyperglycemia associated with a reduction in total cholesterol. On the other hand, other human studies have indicated a significant correlation between triglycerides and VIP, with high levels of both factors observed in patients suffering from obesity (Tomkin et al., 1983). All these data suggest that endogenous VIP is involved in appetite/satiety control and even VIP may play an important role in the development of obesity (Vu et al., 2015b).

In this study, we aimed to investigate the basal expression and neurochemical profile of VIP in the SCN of the hypothalamus. Subsequently, we examined the neurochemical changes that occur under an obesogenic or high-fat diet, using a specialised feed containing an approximate lipid content of 60 %, in mice with a C57BL/6 J genetic background.

Our analysis indicates that mice treated with a high-fat diet showed weight gain and a decrease in VIP expression in the SCN of the hypothalamus, as well as a reduction in VIP fibres around the NAc. We also observed a loss of neuropeptide Y (NPY) expression in fibres surrounding the SCN. After studying their neurochemical profile, we observed that VIP-expressing neurons are predominantly GABAergic.

2. Materials and methods

2.1. Animal Model

A total of 15 female C57BL/6J mice were used in this study. Mice were approximately 6 weeks old at the start of the study and were housed in cages at 22ºC under a 12 h light-dark cycle and relative humidity of 50% in the animal house of the University of La Laguna (ULL). Animal care was performed in accordance with institutional guidelines in compliance with Spanish (Real Decreto 53/2013, February 1. BOE, February 8, 2013, n: 34, p. 11370–11421) and international laws and policies (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes) and were approved by the Institutional Animal Care and Use Committee (Comité de Ética de la Investigación y de Bienestar Animal (CEIBA) of University of La Laguna, Spain).

2.2. Experimental design

Female mice were fed ad libitum with water and, either a standard diet (SD) or a high-fat diet (HFD) to induce obesity, following previous studies (Rodriguez-Rodriguez et al., 2021). Briefly, in the HFD, calories are derived from protein (20%), carbohydrate (20%), and fat (60%), including 232 mg cholesterol (lard and blue dye); for a total of 5.24 kcal per gram (Research Diets, D12492). In SD, calories come from protein (20%), carbohydrate (67%), and fat (13%) (Teklad Global diet, Envigo).

Female mice on SD were 7 and those on HFD were 8. The duration of the experiments was 365 days. Animals were weighted twice per week and before sacrifice. Female mice weighing in the range of 16.2 ± 0.9 g were selected for this study. At the end of the experiment, animals in HFD incremented their weight reaching values of 54.9 ± 8 g and animals in SD weighing in the range of 23 ± 4 g, values considered normal for their age (control mice group).

All animals were sacrificed and processed equally on the same day. Mice were deeply anaesthetised with an overdose of pentobarbital sodium and transcardially perfused with heparinised ice-cold 0.9% saline (20 ml) followed by 4% paraformaldehyde in phosphate-buffered saline pH 7.4 (PBS, 50 ml).

2.3. Brain section preparation

Brains were removed and stored overnight at 4ºC in the same fixative. They were then cryoprotected in a graded series of sucrose-PBS solutions and stored at −80ºC until processing. Considering that mouse SCN is a bilateral structure located in the ventral portion of the anterior hypothalamus, ventrolateral to the optic recess of the third ventricle and dorsal to the optic chiasm, the brains were dissected into several blocks. Only those sections containing the forebrain, including the SCN and the NAc, were used. The Paxino and Franklin mouse brain stereotaxic coordinate atlas was used as a reference to guide the dissection of the blocks (between +1.18 mm and −1.70 mm to Bregma) (Paxinos and Franklin, 1997). Forebrains were immersed in a cryoprotective solution containing 30% sucrose in the same buffer and kept overnight at 4 °C. Coronal sections of 20 μm thickness were cut using a freezing microtome. These sections were collected in parallel series of 8–10 and processed for VIP immunohistochemistry or single immunofluorescence staining. In addition, VIP was combined with GAD67 (the rate-limiting enzyme in GABA synthesis), somatostatin (SST) and neuropeptide Y (NPY) for double immunofluorescence analysis.

2.4. Immunostaining

2.4.1. Immunohistochemical staining for anti-VIP

The floating sections were treated with 3% H_2O_2 for 30 min to inactivate endogenous peroxidase activity. Subsequently, they were incubated in a solution of 4% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA) in PBS containing 0.05% Triton X-100 (TX-100, Sigma) for 60 min at RT. Afterwards, the sections were incubated overnight in PBS with 2% NGS and a rabbit anti-VIP polyclonal antibody (1:500; PA5–78224-Invitrogen), whose antibody has been previously tested by several authors (Hwang and Hashimoto-Torii, 2022; Ang et al., 2021; Semaniakou et al., 2021). Following several rinses, the sections were incubated for 2 h in biotinylated goat anti-rabbit antiserum (1:1000; Jackson ImmunoResearch) and 1:200 NGS in PBS.

Immunoreactions were visualised by incubating for 1 h at RT with ExtrAvidin-peroxidase (1:5000, Sigma) in PBS, and then for 10 min in a solution of 0.005% 3′−3′-diamiobenzidine tetrahydrochloride (DAB, Sigma), 0.05 % ammonium nickel (II) sulfate and 0.001% H_2O_2 in cacodylate buffer 0.05 N pH 7.6. After several rinses in PBS, the sections were dehydrated, cleared in xylene, and coverslipped with DPX (BDH Chemicals, Poole, England). In parallel, mouse brain tissue was incubated with the antibody diluent alone, without the addition of the primary antibody. Subsequently, the tissue was incubated with secondary antibodies and detection reagents to assess the specificity of the antibody. Following the immunohistochemistry development, no labelling was observed in the mouse SCN, NAc, or other adjacent regions.

VIP-immunostained neurons and dense fibers were observed in the mouse SCN, as well as fibres within the NAc. Following the immunohistochemical procedure, the number of positive VIP neurons in each section was counted under 40X magnification using an optical photomicroscope (Olympus BX61; Olympus, San Diego, CA, USA). In our evaluation, the quantitative analysis of the total number of VIP-neurons in each forebrain section was conducted by calculating from seven randomly chosen optical fields. Five thick sections per condition (HDF vs SD), spaced 50 μm apart from each other along the rostrocaudal axis, were randomly selected in each mouse. The number of labelled neurons was expressed as a percentage of VIP-positive cells in the mouse SCN in each section. The mean number of VIP-neurons in the mouse SCN at basal level was 69 ± 7 in each hemisphere and section (control group of mice), with no significant differences between them. In addition, densitometric analysis was performed to assess the staining intensity of VIP neurons in the SCN and positive fibres in the NAc. The analysis included 5 fields per section, 5 sections per mouse (50 μm between sections) and 5 mice per group. An average of 340 neurons and 235 fibres were analysed for each mouse. The acquired RGB image was converted to a 32-bit greyscale image and inverted.

The representative histogram shows cell somas and fibres staining intensity scale, where 0 indicates no positivity and 200 indicates maximum positivity. Staining intensity for VIP was quantified using ImageJ version 1.53c software and expressed as the optical intensity normalised to the cross-sectional area close to the nucleus studied (SCN or NAc, respectively).

2.4.2. Double immunofluorescence

Given the pivotal role of GABAergic transmission in the SCN in restoring and modulating circadian synchrony (Fan et al., 2015), we aimed to investigate whether VIP-neurons co-expressed GABA. On the other hand, VIP and SST are neuropeptides involved in the control of hormone secretion as well as in appetite control (Baranowska et al., 2000). Thus, we analysed whether there is a co-expression of VIP with SST in the mouse SCN. Lastly, it is known that NPY levels undergo circadian variation in certain brain regions of rodents, such as the SCN (Calzá et al., 1990; Shinohara et al., 1993). Additionally, NPY plays an important role in energy balance and therefore in feeding regulation. Therefore, our objective was to investigate the presence of NPY-positive fibres towards the SCN. For this analysis, we employed specific commercial antibodies for the morphological study.

For single and double immunofluorescence labelling, forebrain sections were permeabilised following the same procedure as for immunohistochemistry. For single immunofluorescence targeting VIP, sections were incubated with a rabbit anti-VIP polyclonal antibody (PA5–78224-Invitrogen; 1: 1000) in the same buffer solution for 24 h at RT. After several rinses in PBS, the sections were then incubated for 2 h at RT in a solution of Alexa 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes-Invitrogen). Following several rinses in PBS, forebrain sections were mounted with Vectashield Mounting Medium with DAPI (Southernbiotech, Birmingham, AL). Immunofluorescence was examined using a confocal laser scanning microscope with appropriate filters (RRID: SC R_0168840; Olympus FV1000, Hamburg, Germany). Only cell profiles containing the whole nucleus were included in the analysis. The immunofluorescent labelling intensity from VIP neurons in the SCN and fibres in the NAc was quantified using the ImageJ version 1.53c software. Seven 220 μm x 220 μm fields were randomly selected from a minimum of five sections from each experimental condition and three different experiments. Images were acquired at 60X (1024 ×1024 pixels) and 3 μm x 3 μm square areas, including cell soma of at least 10 randomly selected cells or fibres positive to VIP per field were analysed.

The mean fluorescence labelling intensity is expressed as density/ μm^2 in cell bodies and per μm in fibers for each condition. Control experiments were performed by removing the primary antibodies, resulting in negative staining (*data not shown*).

To perform double immunofluorescence labelling, sections were incubated with the corresponding primary specific antibodies: a mouse anti-VIP monoclonal antibody (1: 500; [02] ab30680-ABCAM) was combined with a rabbit polyclonal anti-GAD67 antibody (1: 500; AB106-Chemicon), a rabbit polyclonal anti-somatostatin antibody (1: 500; AB5494 Millipore) and a rabbit monoclonal anti-NPY antibody [EPR21877] (1: 1000; AB221145-ABCAM) for 24 h at RT. These antibodies have been previously tested by several authors (Ma et al., 2020; Huang et al., 2021; Santana-Cordón et al., 2023). After several rinses in PBS, the sections were then incubated for 2 h at RT in a cocktail solution of fluorochrome-conjugated antibodies containing Alexa 546-conjugated goat anti-mouse IgG (1:200; Molecular Probes-Invitrogen) and Alexa 488-conjugated goat anti-rabbit IgG (1:200; Molecular Probes-Invitrogen). After several rinses in PBS, forebrain sections were mounted with Vectashield Mounting Medium with DAPI (Southernbiotech, Birmingham, AL) and then the immunofluorescence was examined using a confocal laser scanning microscopy system with appropriate filters (RRID: SC R_0168840; Olympus FV1000, Hamburg, Germany). Immunofluorescent intensity labelling of NPY fibres localised

in the SCN was performed in a similar manner to VIP signal analysis.

2.5. Statistical analysis

The data were analysed by using Graph Pad Prism software version 5.0c (RRID: SCR_002798; San Diego, CA) and presented as mean ± SEM. Firstly, the Kolmogorv-Smirnov test was performed to assess whether changes in cell number and the signal from VIP, as well as from NPY fibres around the SCN followed a normal distribution in the control group of mice (n = 6 fed a SD) and obese mouse group (n = 8 fed a HFD). In cases of normal distribution, Student's T-test was used to compare means, while the Mann-Whitney U was employed for nonparametric data. A significance level of *p < 0.05 and * *p < 0.01 was considered statistically significant.

3. Results

3.1. Downregulation of expression and number of VIP neurons in the suprachiasmatic nucleus and fibres located in the nucleus accumbens in obese mice

Our morphological study revealed VIP immunoreactivity in the cell body and fibres of neurons of the SCN of the hypothalamus, mainly located in the ventrolateral and ventromedial part (Fig. 1 A), and also in fibres reaching the NAc (Fig. 1 B) in control mice fed a SD. This finding is consistent with previous studies performed on different species, including rodents, non-human primates and humans (Chang and Tian, 1991; Klein et al., 1991; Hirunagi et al., 1993; Zhou et al., 1995; Hofman et al., 1996; Tanaka et al., 1997; Romijn et al., 1999; Cayetanot et al., 2005; Kingsbury and Wilson, 2016; Todd et al., 2020). We found no differences in the number of VIP neurons per section in the SCN in each hemisphere, similar to previous studies in humans (Hofman et al., 1996). Densitometric analysis of VIP neurons and signal in the cell body of this hypothalamic nucleus and the fibres around the NAc in obese mice, revealed a decrease in the number of VIP-positive neurons in each hemisphere (86.9 ± 8%; p < 0.01; n = 47 (right side) and n = 39 (left side)) compared to control mice (n = 334 (right side) and n = 327 (left side)) and expression ((32.3 ± 2 (right side) and 36.1 ± 5 (left side) in obese mice vs. (147.3 ± 2 (right side) and 145.3 ± 6 (left side) in control mice; p < 0.01)) along the rostrocaudal axis of the hypothalamus was found in both hemispheres (Fig. 1 A, C and E). In addition, a significant reduction in the signal from the VIP fibres located in the NAc was observed in obese mice subjected to a HFD (37.5 ± 3 (right side) and 32.3 ± 2 (left side)) compared to control mice (110.5 ± 2 (right side) and 105.3 ± 6 (left side); p < 0.01; Fig. 1 B, D and E). It is important to note that the origin of VIP fibres in the NAc remains unknown. In an attempt to determine whether VIP fibres observed in the NAc belong to SCN projection neurons, we injected mice unilaterally within the NAc (n = 3) with Fluoro-Gold (FG), a retrograde tracer. One week after FG injection and after processing for the morphological study, we analysed double immunofluorescence for VIP and FG in SCN neurons. In this case, we did not observe VIP-FG double labelling in any SCN neuron, thereby excluding its direct involvement in the reward-motivation system (*data not included in this work*). These findings are consistent with the current literature, as there is no evidence to date for a direct pathway from the SCN to the NAc, although there is an indirect circuit to the Ventral Tegmental Area (VTA) through the medial preoptic nucleus (MPON) (Luo and Aston-Jones, 2009).

Considering that there are direct connections from the VTA and NAc to the SCN (Grippo et al., 2017), it would be interesting to explore the origin of VIP fibres located in the NAc in future works and determine its impact on the reward system and its role in obesity.

In general, these findings were confirmed by immunofluorescence for VIP in neurons located mainly in the ventrolateral and ventromedial part of the SCN in the same study animals (obese mice vs control mice). Mice fed a HFD showed a decrease in signal for VIP-neurons at the cytoplasmic level in both parts of the SCN (23.5 ± 2 (right side) and 20.4 ± 7 (left side)) compared to control mice (101.5 ± 3 (right side) and 96.8 ± 4 (left side); p < 0.01; Fig. 2A and C) and also for fibres around the NAc in mice fed a HFD (48.6 ± 5 (right side) and 43.2 ± 7

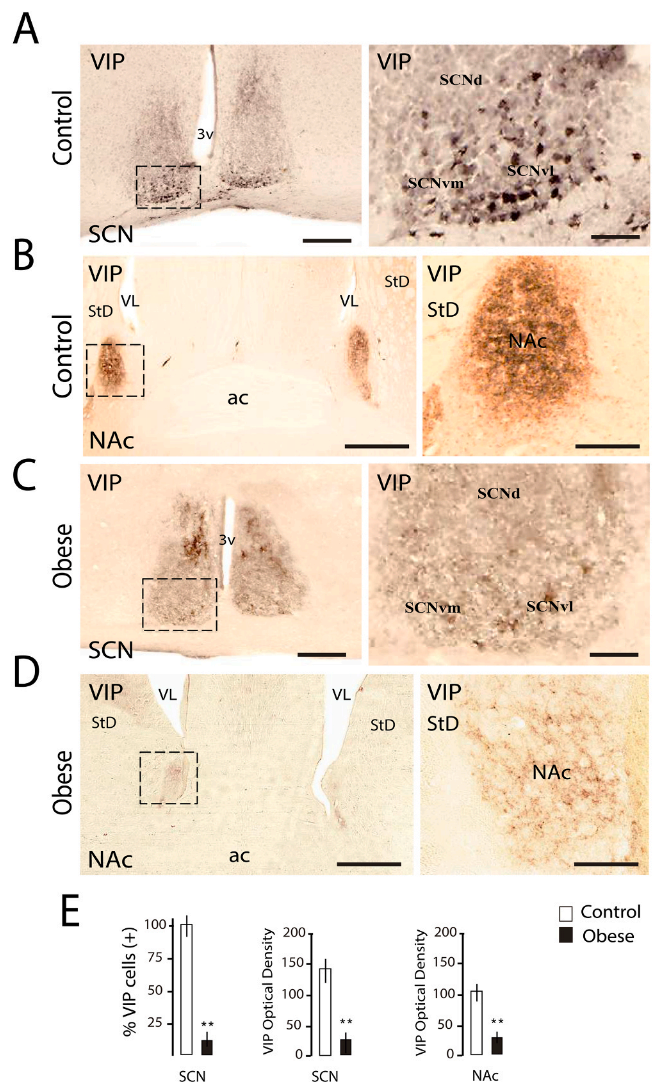

Fig. 1. A, VIP immunoreactivity in the SCN of healthy control mice, mainly in the ventromedial and ventrolateral parts, in both hemispheres. Note that these are oval neurons with staining mainly in the soma and dense staining in the fibres. The dashed box represents the magnified area (right) for morphological analysis of VIP-positive neurons in the SCN. B, VIP immunoreactivity in fibres located in the NAc of control mice. C, VIP immunoreactivity in the SCN of obese mice, a drastic decrease in VIP expression was observed in neurons distributed along the SCN, with a scarce number of neurons immunoreactive for VIP. Note the dark colour of the VIP signal within the cytoplasm in control mice compared to obese mice, whose labelling is weaker. D, VIP immunoreactivity in the NAc of the obese mice; a decrease in immunoreactivity for VIP in fibres located in the NAc was also observed in HFD-treated obese mice compared to SD-treated control mice. E, VIP-positive neuron count and densitometric analysis of the VIP expression in the soma of the SCN neurons and fibres located in the NAc in healthy and obese mice. This study shows a decrease in the number of VIP-positive neurons (86.9 ± 7%; p < 0.01) as well as their intracytoplasmic signal in the SCN (p < 0.01) and fibre signal in the NAc (p < 0.01) in long-term HFD-fed mice compared to SD-fed mice. SCN: suprachiasmatic nucleus; SCNd: Suprachiasmatic nucleus, dorsal part; SCNvm: suprachiasmatic nucleus, ventromedial part; SCNvl: suprachiasmatic nucleus, ventrolateral part; 3 v: third ventricle; VL: lateral ventricle; StD: dorsal striatum; NAc: nucleus accumbens; ac: anterior commissure. Scale bars: 250 and 50 μm in A and C, respectively and 300 and 100 μm in B and D, respectively.

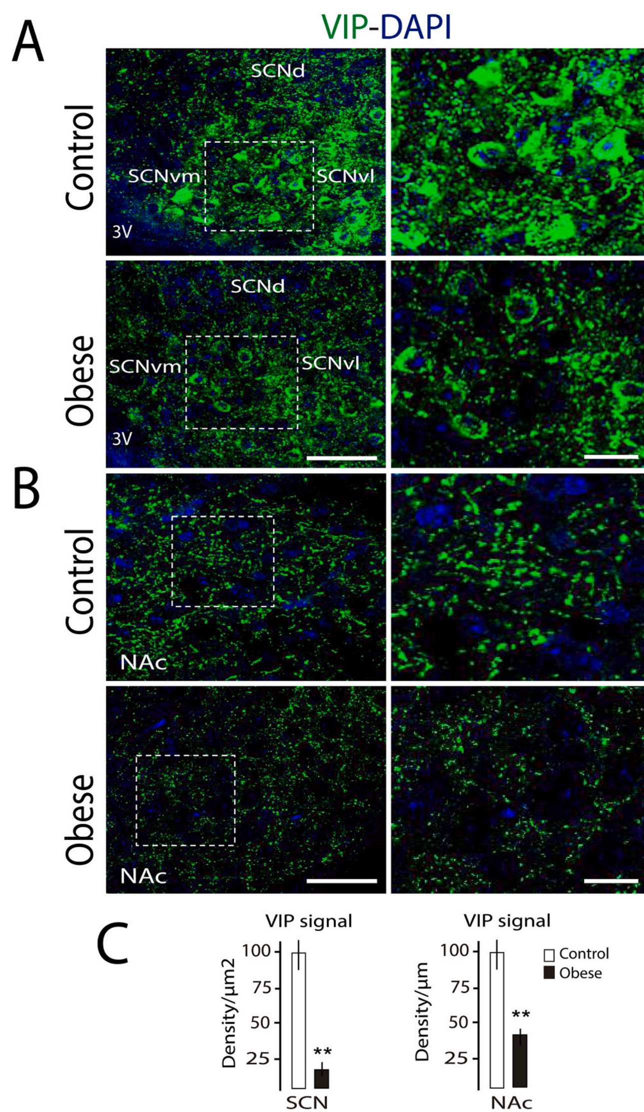

Fig. 2. A, Single immunofluorescence of VIP in the SCN of healthy and obese mice. Note the distribution and expression of VIP in the cell bodies and fibers of neurons located in the SCN between the different parts, with higher labelling in the ventromedial and ventrolateral parts in control mice compared to obese mice, whose intracytoplasmic signal in both regions is drastically reduced. B, Expression of VIP in fibres located in the NAc in healthy and obese mice, finding high immunoreactivity especially in the NAc in control mice compared to obese mice, whose signal decreases drastically. The dashed box represents the magnified area for morphological analysis of VIP expression. C, Densitometric analysis of the VIP expression in the soma of the SCN neurons and fibres located in the NAc in healthy and obese mice. This study shows a decrease in the intracytoplasmic signal in the SCN neurons ($p < 0.01$) and also in fibres located in the NAc ($p < 0.01$) in long-term HFD-fed mice compared to SD-fed mice. SCNd: suprachiasmatic nucleus, dorsal part; SCNvm: suprachiasmatic nucleus, ventromedial part; SCNvl: suprachiasmatic nucleus, ventrolateral part; NAc: nucleus accumbens. Scale bars: 100 and 20 μm, respectively.

(left side)) compared to control mice fed a SD (102.8 $\pm$ 6 (right side) and 97.3 $\pm$ 3 (left side); $p < 0.01$; Fig. 2B and C). In summary, when comparing the images of VIP expression in the SCN and fibres in the NAc between obese mice and control mice in both morphological studies (immunohistochemistry and immunofluorescence, see Figs. 1 and 2, respectively), we observe, at the macroscopic level, a loss of VIP expression in the HFD-treated animals compared to SD-treated mice control mice.

3.2. Presence of NPY- fibres around the suprachiasmatic nucleus and reduction of their signal in obese mice treated with an obesogenic diet

NPY is a peptide identified in 1982 that quickly emerged as a key element in appetite regulation. In human and rodent brains, NPY is highly expressed in regions such as the septum, basal ganglia, ARC and paraventricular nuclei (PVN), cortex, amygdala, hippocampus, periaqueductal gray matter, locus coeruleus, NAc and cortical areas, particularly layers V and VI (Adrian et al., 1983; Tang et al., 2020; Tanaka et al., 2021) and project to various hypothalamic areas involved in the control of food intake (Sahu et al., 1988). Given that NPY is one of the most potent orexigenic peptides affecting adiposity and has been linked to obesity risk (Tang et al., 2020), in this work we wanted to investigate whether there was co-localisation with VIP neurons in the SCN (Fig. 3). In this context, we not observe co-expression of NPY and VIP in SCN neurons, but we found NPY positive fibres reaching VIP neurons within the SCN in normal non-obese mice SD-treated (Fig. 3A). We also observed a significant decline in NPY signal in the fibres

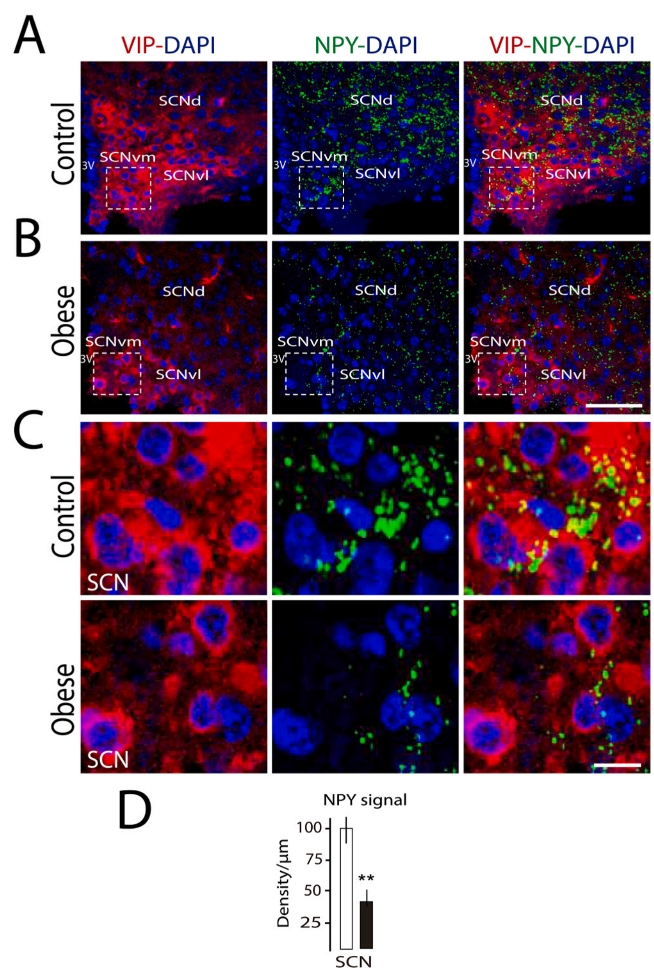

Fig. 3. A-D, Double immunofluorescence of VIP combined with NPY along the SCN in healthy mice (A) and obese mice (B). The morphological study revealed the presence of NPY-positive fibres (in green) close to VIP neurons (in red) distributed throughout the SCN. C, Magnified images of double immunofluorescence for VIP/NPY between the ventrolateral and ventromedial regions of the SCN, obtained in A and B. The dashed box represents the magnified area for morphological analysis of VIP expression. D, Densitometric analysis of immunofluorescence for NPY fibres in the SCN. Note the decreased signal of NPY in the SCN in obese versus control mice ($p < 0.01$). SCN: suprachiasmatic nucleus; SCNd: suprachiasmatic nucleus, dorsal part; SCNvm: suprachiasmatic nucleus, ventromedial part; SCNvl: suprachiasmatic nucleus, ventrolateral part; 3 v: third ventricle. Scale bars: 100 and 20 μm, respectively.

reaching VIP neurons of the SCN in both hemispheres (45.7 ± 6 (right side) and 44.6 ± 3 (left side)) in HFD-treated obese mice compared to SD-treated control mice (103.2 ± 5 (right side) and 98.3 ± 2 (left side); $p < 0.01$; Fig. 3A-D).

To the best of our knowledge, this is the first time that the NPY fibres close to SCN neurons are being described, along with the decrease in the signal of these NPY fibres in animals treated with a HFD.

3.3. Neurochemical characterisation of VIP neurons in the mouse suprachiasmatic nucleus

Double immunofluorescence for VIP and either GAD67 or SST in the SCN of healthy mice (Fig. 4, white arrows) confirms that VIP-expressing neurons co-localise with GAD67 (Fig. 4A) and SST (Fig. 4B) and are mainly located in the ventrolateral and ventromedial parts of the SCN.

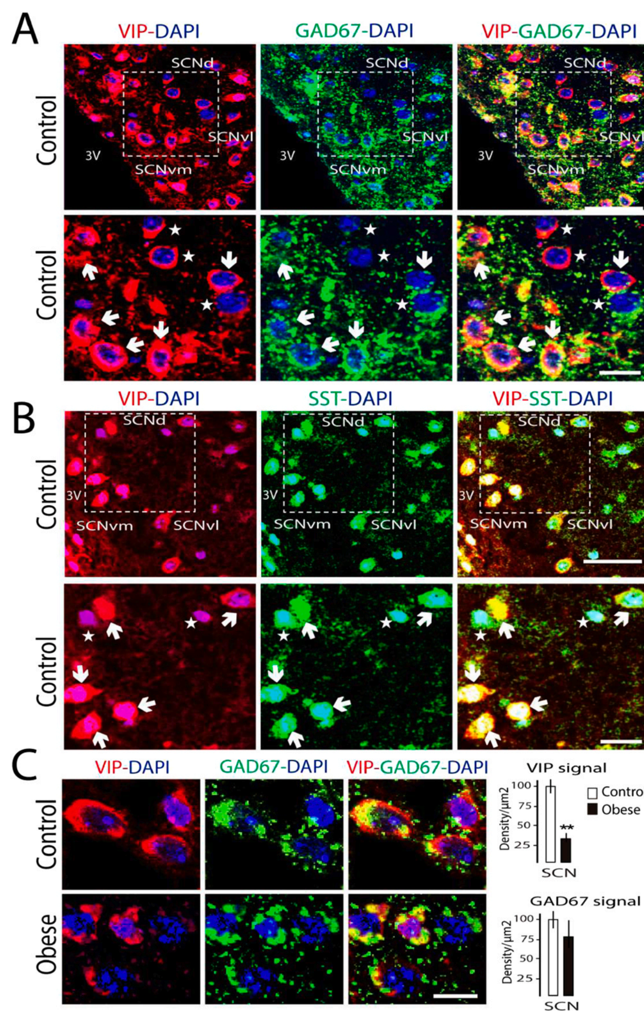

Fig. 4. A-B, Double immunofluorescence for VIP (in red) combined with GAD67 (in green) (A), or SST (in green) (B) along the SCN in healthy mice. The colocalisation of VIP with GAD67 and SST in SCN neurons, mostly between SCNvm and SNCvl, was in the range of 95.6 ± 5% and 90.5 ± 2% for both markers, respectively (*see white arrows*). However, we found a small number of neurons that did not colocalise with any marker (*white stars*). (C) Densitometric analysis of the immunofluorescence signal for GAD67 and VIP in neurons of the SCN. Note that there is no significant change in cytoplasmic signal for GAD67 in VIP-positive neurons, although we observed a reduction in signal for VIP in these same SCN neurons in obese versus control mice ($p < 0.01$). The dashed box represents the magnified area for morphological analysis of VIP expression in the SCN. SCNd: suprachiasmatic nucleus, dorsal part; SCNvm: suprachiasmatic nucleus, ventromedial part; SCNvl: suprachiasmatic nucleus, ventrolateral part; 3 v: third ventricle. Scale bars: 100 and 20 μm, respectively.

The majority of VIP neurons were co-labelled with GAD67 (95.6 ± 5%; 124 of 130 cells) and SST (90.5 ± 2%; 112 of 124 cells) (Fig. 4A and B). These results provide further support to the findings previously reported by other authors (Tanaka et al., 1997; Hogenboom et al., 2019; Todd et al., 2020). We know that this hypothalamic nucleus contains primarily two populations of peptidergic neurons, based on the literature on the neurochemical profile of the SCN (Cayetanot et al., 2005). On one side, there are neurons expressing arginine vasopressin (AVP), mainly located in the dorsal part of the SCN. On the other side, neurons expressing VIP, whose cells are mainly intrinsic to the ventral part of the SCN (Cayetanot et al., 2005).

In an attempt to confirm if the effect of the obesogenic diet affects only VIP expression in SCN neurons, we aimed to perform a densitometric analysis of the GAD67 immunofluorescent signal in these same neurons (Fig. 4C). In this case, we did not observe significant changes in GAD67 expression in VIP-positive neurons of the SCN in obese mice, although we observed the reduction of VIP signal in these same neurons (27.3 ± 5) compared to control mice (100.2 ± 5; $p < 0.01$; Fig. 4C).

4. Discussion

Previous studies have consistently demonstrated the predominant expression of VIP in the hypothalamus, particularly in the SCN, in different species, including humans (Klein et al., 1991; Zhou et al., 1995; Romijn et al., 1999; Kudo et al., 2013; Kingsbury and Wilson, 2016; Todd et al., 2020). VIP has also been shown to play a key role in the regulation of appetite and energy homeostasis (Mounien et al., 2009; Báez-Ruiz et al., 2014). Therefore, the aim of our study was to investigate the neurochemical phenotype and distribution of VIP in the SCN neurons. Additionally, we sought to investigate possible morphological changes, specifically in the expression of this neuropeptide in the SCN, following prolonged treatment with a HFD in C57BL/6 J mice.

First, we found that VIP-expressing neurons in the SCN have a spindle-shaped, oval morphology and are predominantly labelled at the cell soma and fibers. VIP-positive neurons are mainly located in the ventral-medial (SCNm) and ventral-lateral (SCNl) parts of the SCN. However, we observed a limited number of VIP neurons in the dorsal region of the same hypothalamic nucleus.

These VIP neurons extend rostrocaudally (345 ± 7 vs 337 ± 8, respectively), similar to previous studies in humans (Hofman et al., 1996). Based on their size and morphology, we assumed that these neurons were primarily GABAergic. This assumption was further supported by the results from double labelling between VIP and GAD67, or in combination with SST, which were consistent with previous studies (Tanaka et al., 1997; Fan et al., 2015; Todd et al., 2020).

Secondly, our work revealed that obese mice treated with HFD had a weight gain (x2) compared to normal non-obese mice treated with SD. In addition, we found changes in VIP expression in the SCN and positive fibres in the NAc in those mice treated long-term with HFD. A significant decrease in VIP expression in the SCN and positive fibres in the NAc (obese mice vs. control mice) was identified as the most relevant finding. The drastic reduction in VIP expression we observe is in line with previous studies in models of obesity, which have shown that serum VIP levels are lower in obese patients compared to non-obese patients and that they normalise after body weight reduction (Baranowska, 1991), and even VIP levels are decreased in the antrum of morbidly obese individuals (Atas et al., 2021). These results were later confirmed by the same authors who found a decrease in plasma VIP levels together with an increase in SST in obese women compared to controls (Baranowska et al., 2000). However, these authors found the opposite effect in women with anorexia nervosa, with increased VIP plasma levels and decreased SST (Baranowska et al., 2000). Significantly, a human post-mortem study in the hypothalamus of 28 individuals with type 2 diabetes showed a loss of VIP neurons in the SCN compared to healthy individuals (Hogenboom et al., 2019), confirming our findings in obese HFD-treated mice. Furthermore, previous studies have found a reduced number of

VIP neurons in individuals with hypertension, suggesting that the reduction in VIP neurons might be related to insulin resistance rather than high blood pressure (Goncharuk et al., 2001; Hogenboom et al., 2019). However, these findings contrasted with other studies in VIP-deficient mice, which found a reduction in body weight and fat mass along with an increase in lean mass as the mice aged (Vu et al., 2015b). These authors suggest that VIP signalling is critical for the modulation of appetite/satiety (Vu et al., 2015b) and VIP pathway plays an important role in the development of obesity (Báez-Ruiz et al., 2014). In the same line, another study carried out on VIP-deficient mice (VIP-/- mice) revealed significant changes in bacterial composition, biodiversity, and weight loss from VIP-/- mice, irrespective of sex.

The gut bacteria compositional changes observed in VIP-/- mice was consistent with gut microbial structure changes reported for certain inflammatory and autoimmune disorders (Bains et al., 2019). Other authors have shown that VIP plasma concentrations increase after a carbohydrate meal or water intake (Pedersen-Bjergaard et al., 1996; Vu et al., 2015b), suggesting a possible role for VIP in the modulation of appetite and food intake. Similarly, another study showed that there is a significant increase in plasma concentrations of VIP after a breakfast rich in fat and protein (Hill et al., 1986).

Another interesting finding is that plasma VIP has also been found to increase after 12 h starvation in obese subjects (Andrews et al., 1981). This is supported by studies demonstrating that VIP plays a significant role in the regulation of pancreatic endocrine function by stimulating insulin and glucagon secretion (Inagaki et al., 1996; Persson-Sjögren et al., 2006) and therefore involved in energy metabolism and body fat storage (Persson-Sjögren et al., 2006; Vu et al., 2015b). Other evidence suggests that VIP may influence energy metabolism and obesity through motor control in mammals. Indeed, it is known that intracerebroventricular and intraperitoneal administration of VIP induced a significant decrease in locomotor activity, which in turn may result in increased fat deposition (Matsuda et al., 2006). However, other studies indicate that central administration of VIP reduces food intake in vertebrates (Tachibana et al., 2003; Vu et al., 2015b; Montégut et al., 2021). Indeed, the activation of VIP neurons in the prefrontal cortex of mice produces a reduction in high-calorie palatable food intake that is independent of food novelty (Newmyer et al., 2019). Furthermore, it is worth highlighting that our work shows the presence of VIP fibres within the motivation and reward system, aligning with previous studies conducted in rats (Chang and Tian, 1991), without knowing their origin. Current literature on SCN and NAc/VTA clearly shows that there is a relationship between both neuronal groups (Becker-Krail et al., 2022). The SCN sends indirect connections to the VTA via the medial preoptic area (mPOA) of the hypothalamus and the lateral habenula (Luo and Aston-Jones, 2009; Becker-Krail et al., 2022), and the VTA in turn sends direct connections with a dopaminergic profile to the SCN, participating in the circadian clock, thus suggesting that both circuits are involved in motivational behaviour (Grippo et al., 2017; Becker-Krail et al., 2022). As with the VTA, the direct projection from the SCN to the NAc remains unspecified. Although it has been shown that the NAc receives circadian information indirectly from the SCN via the PVN nucleus, it has not been possible to specify the direct projection from the SCN to the NAc (Becker-Krail et al., 2022). Based on these data, we suggest that VIP may play an important role in uncontrolled food intake and thus in addiction to certain food. Another notable aspect of our work is the detection of NPY fibres around the SCN. In this case, we observed a decrease in the number of NPY fibres in the SCN of obese mice treated with HFD compared to non-obese SD-treated mice.

These results are consistent with previous studies showing that prolonged exposure to a palatable high-fat diet leads to the development of adiposity and this is associated with a decrease in hypothalamic NPY expression, consistent with a counter-regulatory mechanism to reduce energy intake and limit the development of obesity (Beck, 2006).

Such findings are relevant because the NPY is one of the most potent orexigenic peptides in the brain, capable of stimulating food intake with a preferential effect on carbohydrate intake, and it is abundantly synthesised by neurons of the ARC nucleus (Chronwall, 1985; Beck, 2006).

NPY is also sensitive to dietary composition, with variable effects observed in response to different carbohydrate and fat contents. Indeed, genetic models of obesity developed in rodents, are characterised by a reduced expression of NPY in the ARC nucleus (Beck, 2006). However, other studies in models of obesity suggest that increased NPY expression in the central nervous system leads to overeating and obesity (Aguliar et al., 2005; Rojas et al., 2012). Indeed, the intracerebroventricular administration of NPY leads to increased food intake in satiated animals (Aguliar et al., 2005).

Based on current literature, obesity is a heterogeneous disease, yet sex/gender is rarely taken into account in the prevention or clinical care of this disease. Recent findings show that obesity is more prevalent in women than in men in most countries (Cooper et al., 2021). Recent studies show that women with obesity report nearly double the rates of food addiction compared to men and increased food cravings and heightened reactivity to food cues, leading to a greater incidence of hedonic eating behaviours (Bhatt et al., 2023). Sex differences have also been observed in individuals with obesity regarding attention, memory and impulsivity domains (Bhatt et al., 2023). Overall, preliminary findings suggest that women with obesity are sensitive to food cravings, particularly during menstrual phases, which renders it difficult to successfully diet or lose weight (Bhatt et al., 2023). For all these reasons, these findings in obese female mice treated with an obesogenic diet may help to better understand this disease and the search for new and more effective treatments in the future.

Although the literature on VIP and obesity is sparse, evidence to date indicates that this neuropeptide is intimately linked to metabolic homeostasis and that dysregulation may contribute to diseases such as obesity (Basolo et al., 2021). The action of VIP in these functions is mainly mediated by its binding to VPAC1 and VPAC2 receptors, whose affinity is high (Harmar et al., 2012). Indeed, VIP plays an important role as a mediator of signalling in adipocytes, through its receptors (Vu et al., 2015a). Based on the current literature on the function of VIP and its receptors, we can suggest that VIP may play a regulatory role in appetite, food intake and metabolism.

In this work, our results conclude that VIP plays a crucial role in the regulation of body weight, as we found a scarce expression in obese mice subjected to an obesogenic diet, which is supported by other studies conducted in models of obesity.

Further studies are required to improve our understanding of the modulation of VIP and its receptors through which this anorexigenic neuropeptide acts in different regions of the nervous system associated with appetite regulation. These findings will enable the development of new and more effective therapies for the treatment of obesity.

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CRediT authorship contribution statement

PBCH and DAO designed the study. AERR and STT are responsible for obesity model. PBCH, DAO and LSC performed the experiments. LSC, ALM, ICM and MGG analysed the data. PBCH wrote the article. DAO provided useful comments and feedback on the drafting of this manuscript. All authors participated in and approved the final version of the

manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that has been used is confidential.

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