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The effects of high caloric diets on hippocampus of juvenile rats- study of GABAergic system and adult hippocampal neurogenesis

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RESUMO

Apesar dos numerosos estudos sobre os impactos na saúde das dietas ricas em calorias, típicas das sociedades ocidentais, a informação sobre seus efeitos no cérebro, particularmente no hipocampo, ainda é escassa. Partindo de um estudo anterior, em que descobrimos que, em ratos juvenis, uma dieta de cafetaria induz danos na aprendizagem e memória espacial associado a diminuição da neurogénese, pretendemos perceber melhor os mecanismos subjacentes a tais alterações. Para tal, submetemos ratos de quatro semanas de idade a duas dietas altamente calóricas, uma rica em açúcar (solução líquida de sacarose a 30%) e outra baseada numa dieta de cafeteria, rica em açúcar refinado e gordura saturada, por 12 semanas. Após os tratamentos, focamos o estudo no sistema ácido gama-amino-butírico (GABA)érgico analisando as proteínas quelantes de cálcio parvalbumina (PV), calretinina (CR) e calbindina (CB) e os neuropeptídeos neuropeptídeo Y (NPY) e somatostatina (SS) no hipocampo, bem como o conteúdo global de GABA. Como a população GABAérgica é regulada pelo sistema colinérgico, também analisamos o número de varicosidades colinérgicas. Finalmente, tentando entender melhor as alterações na neurogénese, analisamos os efeitos dessas dietas na expressão relativa de mRNA do fator neurotrófico derivado do cérebro (BDNF), da reelina (RELN) e da quinase-5 dependente de ciclina (CDK-5).

Os nossos resultados mostram que a dieta de cafeteria provocou redução significativa dos neurónios PV positivos nas regiões hipocampais da camada granular, hilo e CA1, bem como dos neurónios NPY positivos no hilo, sem alterações relevantes nas outras subpopulações GABAérgicas e sem alterações nos níveis gerais de GABA. Além disso, descobrimos que a dieta de cafeteria reduziu a expressão relativa de mRNA da RELN sem alterações significativas dos níveis de BDNF e CDK5 no hipocampo. A dieta rica em açúcar apenas induziu pequenas alterações na PV em CA3 e da CB em CA1. Não foram observadas alterações nas varicosidades colinérgicas do hipocampo.

Esses dados mostram que, no que diz respeito ao sistema GABAérgico e à neurogénese, as dietas ricas em gorduras saturadas e açúcar são mais prejudiciais para os jovens do que aquelas com apenas alto teor de açúcar. Esses resultados também sugerem que a redução de PV, NPY e RELN no hipocampo pode explicar as alterações cognitivas dos jovens que consomem dietas de cafeteria. Juntos, estes resultados sugerem que uma dieta rica em açúcar e gordura pode ter efeitos mais deletérios do que as dietas ricas em açúcar.

Palavras-chave: adolescência; neurogénese no hipocampo adulto; sistema GABAérgico; hipocampo; obesidade; dietas ocidentais.

ABSTRACT

Despite numerous studies of the impacts in health of high caloric diets, typical of western societies, the information about their effects in the brain, particularly in the hippocampal formation, is still scarce. Starting from a previous study, where we found that, in juvenile rats, a cafeteria diet caused spatial learning and memory impairments associated with a decrease of the neurogenesis, we wanted to better understand the mechanisms underlying those alterations. To do so, we submitted 4 weeks old rats to two high-caloric diets, one rich in sugar (30% sucrose liquid solution) and of another based on a cafeteria diet, i.e., rich in refined sugar and saturated fat, for 12 weeks. After treatments we focused on the gamma-amino-butyric acid (GABA)ergic system, analysing the calcium binding proteins parvalbumin (PV), calretinin (CR) and calbindin (CB) and the neuropeptides neuropeptide Y (NPY) and somatostatin (SS) in the main regions of hippocampal formation, as well as the global content of GABA. Since the GABAergic population is regulated by the cholinergic system, we also analysed the number of cholinergic varicosities in the dentate hilus. Finally, trying to understand better the alterations in the neurogenesis, we analysed the effects of these diets in the brain derived neurotrophic factor (BDNF), reelin (RELN) and cyclin- dependent kinase-5 (CDK-5) mRNA relative expression levels.

Our results show that cafeteria diet significantly reduced PV positive neurons in hippocampal granular layer, hilus and CA1 regions as well of NPY positive neurons in dentate hilus, without relevant alterations in the other GABAergic subpopulations and without changes of GABA general levels. The high sugar diet only induced slight alterations in PV positive cells of CA3 and CB positive cells of CA1 region. No alterations were observed in hippocampal cholinergic varicosities. Furthermore, we found that cafeteria diet reduced the mRNA relative expression of RELN without significant changes of BDNF and CDK5 levels.

These data show that, concerning the GABAergic system and neurogenesis, the diets rich in saturated fats and sugar are more detrimental for juvenile hippocampal formation than high sugar diets. These findings also suggest that reduction of PV, NPY and RELN in hippocampus may be underlying the cognitive disturbances of juvenile that consume cafeteria diets. Together, these results suggest that a diet rich in both sugar and fat may be more deleterious than the effects of sugar alone.

Key-words: adolescence; adult hippocampal neurogenesis; GABAergic system; hippocampal formation; obesity; western diets.

ABREVIATIONS

AHN	Adult Hippocampal neurogenesis
BDNF	Brain derived neurotrophic factor
CAF	Cafeteria diet
CB	Calbindin
CBPs	Calcium binding proteins
CR	Calretinin
CNS	Central nervous system
CA	Cornu ammonis
CDK5	Cyclin- dependent kinase-5
DG	Dentate gyrus
DCX	Doublecortin
Fig.	Figure
GABA	Gamma-amino-butyric acid
GAD	Glutamate decarboxylase
GL	Granular cell layer
HS	High-sugar diet
H	Hilus
HF	Hippocampal formation
-IR	Immunoreactive
LTP	Long term potentiation
mRNA	Messenger Ribonucleic acid
Mol	Molecular layer
NPY	Neuropeptide Y
PV	Parvalbumin
PBS	Phosphate-buffered saline
RELN	Reelin
SS	Somatostatin
VAChT	Vesicular acetylcholine transporter

1. INTRODUCTION

Obesity and overweight are big problems of today's society. World Health Organization tells us that in 2016, around the world there were over 340 million children and adolescents (aged 5-19) that were obese or overweight. The prevalence of these conditions showed a big increase since 1975 from 4% to 18% (World Health Organization, 2018). The growth of obese population is attributed to environmental factors like increase of high caloric foods intake and reduced physical activity (Glendinnning *et al.*, 2011). Susceptibility to obesity could also be related to genetic factors, however it cannot explain the epidemic state found nowadays in developed countries but also in middle- and low-income countries. Also, if that would be the reason, the prevalence in the past should have been similar to current values (Levine *et al.*, 2005).

Diet is one of the main factors to the development of obesity. A diet based on large amounts of red meat, refined sugars, high fatty foods rich in saturated- and trans-fatty acids and refined grains is typical of western societies and because of that is known as "western diet". It is known that the consumption of western diets has severe consequences in general health like obesity, insulin resistance or cardiovascular complications (Winocur and Greenwood, 2005; Stallmann-Jorgensen *et al.*, 2007; Park *et al.*, 2010). In the central nervous system (CNS) we can also find several deleterious effects of western diets, including increased risk of dementia, impaired gluco-regulation, reduced levels of neurotrophins, neuroinflammation, blood-brain-barrier disruption (Kanoski and Davidson, 2011; Freeman *et al.*, 2014) and impairment of neurogenesis and cognition (Ferreira *et al.*, 2018). Interestingly, even short periods of western diet consumption may compromise cognitive functions (Murray *et al.*, 2009). Indeed, cognitive impairments, such as in working memory or spatial reference, may emerge prior to changes in body weight and obesity development (Lindqvist *et al.*, 2006; Murray *et al.*, 2009; Kanoski and Davidson, 2011).

A very important factor of the effects of the diets is the age period when the diet focus or/and start, particularly in the brain since crucial events of brain development, neurogenic processes and maturation occur during early life (Reichelt, 2016; Morin *et al.*, 2017). For example, high-caloric diets affect learning and memory performance in an age-dependent way, being more deleterious in young rats (Valladolid-Acebes *et al.*, 2013). Moreover, the early exposure to high-caloric diets impairs relational memory flexibility and learning and affects dentate gyrus neurogenesis (Boitard *et al.*, 2012;

Valladolid-Acebes *et al.*, 2013). Contributing to this area of research, a previous work from our group showed that juvenile rats treated with a high-caloric diet have impaired spatial learning and memory, increased anxiety and reduction of neurogenesis (Ferreira *et al.*, 2018) showing that brain is susceptible to potential deleterious effects of high-caloric diets, particularly during the juvenile period.

In the brain, the hippocampal formation (HF) is one of the most susceptible areas to nutritional imbalances, as shown by the impairment of spatial learning (Farr *et al.*, 2008; Stranahan *et al.*, 2008; Valladolid-Acebes *et al.*, 2011) and memory (Kosari *et al.*, 2012; Darling *et al.*, 2013; Sobesky *et al.*, 2014), and changes in anxiety (Maniam and Morris, 2010; Hu *et al.*, 2014; Leffa *et al.*, 2015) after high-caloric diets treatment.

Morphologically, HF is an elongated structure that lies, in rodents, beneath the neocortex and comprises the dentate gyrus (DG), the hippocampus proper and the subiculum complex (Witter *et al.*, 1989; Witter, 2012; Knierim, 2015). HF is critical for explicit memory acquisition and consolidation (Squire and Zola-Morgan, 1991; Squire, 1992; Eichenbaum, 1999) and the structural and functional integrity of HF is particularly important in spatial memory. In this regard, for instance, many hippocampal and subicular neurons act as place cells and head direction cells, establishing a neuronal representation of a given spatial environment. HF also plays an important role in working memory, since it establishes reciprocal connections with the dorsolateral prefrontal cortex. Moreover, HF, although of non-primordial mode, participates in the acquisition of emotional memory (Ledoux and Ledoux, 2000) and in regulation of hypothalamic stress response (Sapolsky, Krey and McEwen, 1984).

Briefly, hippocampus proper is subdivided into Ammon's horn areas (CA), we find CA3 emerging from the DG and CA1more distally (see Fig.1) (Witter *et al.*, 1989; Witter, 2012; Knierim, 2015). DG like the other components of HF can also be subdivided and is organized in layers. The outer one is the molecular layer and has low density of cells being occupied mainly by dendrites of granule cells. The molecular layer delimits the granular cell layer (GL) where we find densely packed granule cells. Granule cells originate unmyelinated axons, known as mossy fibres that will innervate cells in CA3 area and in the hilus. Hilus, or polymorphic cell layer, is the most inner part of the DG. In the hilus we find several neuronal cells and a big portion are neurons that use gamma-amino-butyric acid (GABA) as main neurotransmitter, known as GABAergic interneurons. GABA is known to be the major inhibitory neurotransmitter in the CNS, so

these cells are considered inhibitory neurons (Kosaka, Wu and Benoit, 1988; Witter, 2012).

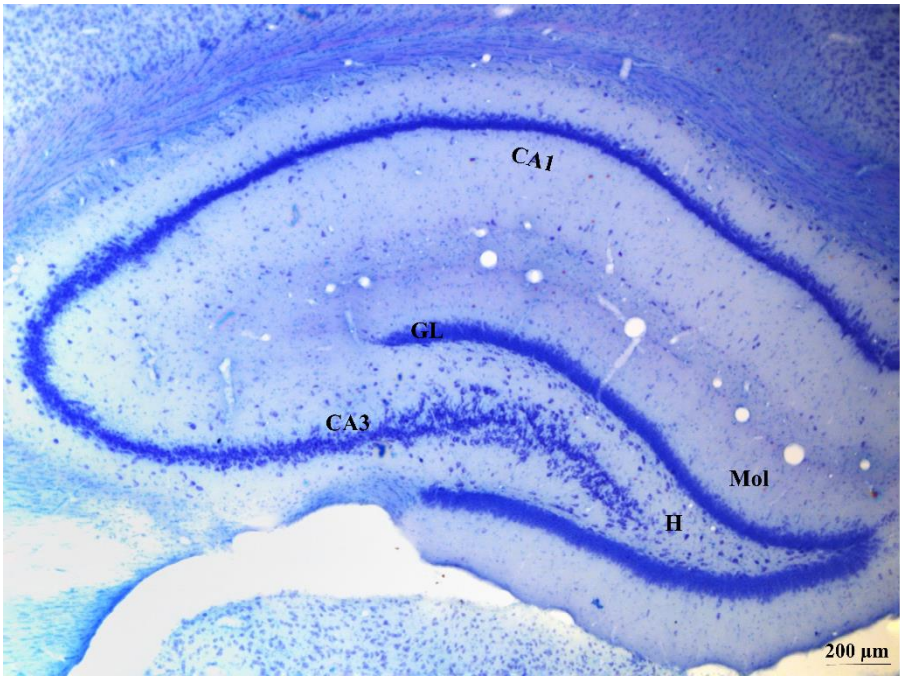

Fig.1.- Photomicrograph of coronal section of the HF from rat stained with giemsa. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

The major input to the HF comes from the entorhinal cortex, via the DG that is mainly composed by the excitatory granule cells and a smaller proportion of GABAergic interneurons that arborize preferentially in the same local and are mainly concentrated in the hilus. Although interneurons are a minority in terms of number, they have an important role in regulating the excitatory cells and are involved in the formation as well as regulation of brain oscillations (Myers and Scharfman, 2009; Andrews-Zwilling *et al.*, 2012; Lucas and Clem, 2018). However, the role of inhibitory interneurons is not yet completely elucidated. For instance, inhibition of GABAergic neurons in the hilus followed by activation of dentate granule neurons, leads to impairments in spatial learning and memory retrieval showing the implication and importance of GABAergic cells in spatial learning and memory formation process (Andrews-Zwilling *et al.*, 2012). So, learning seems to involve an increase in inhibitory synaptic plasticity and this mechanism is involved on the HF functioning (Ruediger *et al.*, 2011; Andrews-Zwilling *et al.*, 2012). Previous studies show the extreme importance of the GABAergic interneuronal population in the cognition, but the information about how high caloric diets affect the

GABAergic system is still scarce. In a previous study, we have found that a cafeteria (CAF) diet, rich in fat and sugar, caused impaired spatial learning and memory process in rats (Ferreira *et al.*, 2018). Taking this in mind and knowing, as cited above, that GABAergic neurons are fundamental to cognition, we decided to analyse the effects of the high-caloric diets in the expression of the GABAergic interneuronal population.

HF is composed by a wide range of GABAergic interneurons that can be classified through their neurochemical content using immunohistochemical markers such as calcium binding proteins (CBPs) or neuropeptides. Indeed, neuronal populations containing parvalbumin (PV), calretinin (CR), calbindin (CB), neuropeptide Y (NPY) and somatostatin (SS), although having some colocalization between themselves, are generally considered distinct GABAergic populations (Sloviter, 1989; Gulyás, Hájos and Freund, 1996). The CBPs and peptides considered for classification are highly soluble and so can be found throughout the soma, axons and dendrites of neurons (Houser, 2007; Kirkcaldie, 2012). Although variable according to the different regions of HF, the proportions of some of the main interneuronal subpopulations, identified accordingly to some marker proteins, on the hippocampus are generally 25% PV, 18% CR and 24% for SS (Besser *et al.*, 2015).

PV-immunoreactive (IR) interneurons have its cell bodies concentrated in the granule cell layer, but are also found in the hilus of the DG and in CA3 and CA1 regions (Kosaka *et al.*, 1987; Houser, 2007). PV interneurons form synapses mainly in the soma and proximal axons as well as dendrites of principal cells (Lucas and Clem, 2018). Since PV interneurons receive inputs from mossy fibres within the granule cell layer, they provide feedback inhibition to the granule cells. Although the function of PV is not completely clear, the presence of this CBP can be an adaptation to the high level of activity of these neurons and their fast-spiking activity. Moreover, this group of interneurons is associated to high levels of metabolic activity and is specialized in rapid recruitment to modulate excitatory neurons spiking activity (Houser, 2007; Lucas and Clem, 2018).

The CR-IR interneurons are observed in all layers and subfields of the hippocampus proper and DG, mainly in the hilus. In the rat we find spiny CR-expressing neurons that contact primarily with other interneurons and their axons form synapses with dendritic spines of granule cells (Gulyás *et al.*, 1992; Houser, 2007). CR was first identified in the retina and it is known to modulate neuronal excitability with its fast

calcium buffering effect (Kirkcaldie, 2012). Interestingly, CR-IR neurons target preferentially other subclasses of interneurons, they innervate CB expressing interneurons at the same time they avoid PV-IR neurons. The connectivity of CR-IR populations makes it crucial in the regulation of hippocampal activity by controlling other interneurons and principal cells (Gulyás, Hájos and Freund, 1996). Besides being used as an interneuron population marker, this protein is specifically expressed at the onset of the differentiation stage during adult neurogenesis, so it seems to have a role that contributes to differentiation and survival of progenitor cells pool as well as its maintenance (Todkar, Scotti and Schwaller, 2012).

The majority of CB-IR neurons are present in the CA1 and CA3 stratum radiatum and CB is also expressed in some "superficial" CA1 pyramidal cells, but they are relatively scarce in the hilus although CB is expressed in dentate GL (Von Bohlen Und Halbach, 2011; Virawudh Soontornniyomkij *et al.*, 2012). CB is also a CBP with rapid intracellular calcium buffering effect and CB-IR interneurons are associated burst firing (Kirkcaldie 2012).Outside the population of interneurons, we find CB being expressed by newly generated mature granule cells, so it can be used as marker to study newly formed neurons in adult neurogenesis (Todkar, Scotti and Schwaller, 2012)

SS-IR neurons are one of the largest groups when it comes to chemical classification of GABAergic neurons of the HF. This subgroup is particularly concentrated in the hilus and more than a half (approximately 55%) of hilar GABAergic neurons express somatostatin (Houser, 2007; Lucas and Clem, 2018). SS-IR neurons provide inhibition to pyramidal neurons on the CA1 area and receive cholinergic projections from the medial septum. Studies found that these interneurons have plasticity that allows the formation and elimination of axonal boutons and dendritic spines in the CA1 area, providing a mechanism for the formation of long term storage (Schmid *et al.*, 2016).

NPY-IR cells are also one of the largest group of interneurons and well expressed across the entire HF. In the DG, the NPY-IR neurons are preferentially concentrated in the hilus. There, it acts as inhibitory peptide to a subpopulation of interneurons, inhibiting inhibitory neurons. The inhibitory effect of this peptide acts on controlling glutamate release on CA1 area of the hippocampus (Sperk, Hamilton and Colmers, 2007). In the hypothalamus, NPY-IR interneurons are subjective to short term alterations and have a

role regulating energy homeostasis. Their function is involved in food intake, expenditure and storage (Levin, 1999).

Furthermore, recent works suggest that diets interfere with the cholinergic system in the HF (Andrade and Paula-Barbosa, 1996; Cardoso *et al.*, 2014; Pereira *et al.*, 2016), however the information about the specific effects of high-caloric diets in the cholinergic system is scarce (Kaizer *et al.*, 2004). Moreover, knowing also that at least in the cortex, the NPY- and SS-IR systems seem to be dependent of cholinergic system (Jolkkonen, Kähkönen and Pitkänen, 1997; Zhang *et al.*, 1998; Cardoso, Paula-Barbosa and Lukoyanov, 2006), another propose of the present study was to analyze the effects of high caloric diets in the cholinergic fibers by targeting the vesicular acetylcholine transporter (VAChT)- IR varicosities density and try to correlate it with potential alterations in the GABAergic system. Cholinergic fibres innervate interneurons participating in their recruitment and in the hilus we find connections of cholinergic neurons GABAergic neurons (Freund and Buzsáki, 1996; Lucas and Clem, 2018).

In our study we were looking if there were any changes in the GABAergic population of neurons in the HF. However, this is a very diverse group and there is not a morphological way that allows an overall quantification of interneurons population and also the immune-histological studies for this have shown to be unregular and even contradictory sometimes (Houser, 2007). Glutamate decarboxylase 1 (GAD 1) is an enzyme involved in the synthesis of GABA (Freund and Buzsáki, 1996). Although the diversity found in interneurons subpopulations, they have in common the use of GABA as neurotransmitter which makes GAD1 a good target to study changes in this population. Thus, we evaluated its mRNA levels to see if there were changes in the GABAergic population.

Finally, it is known that diets could induce alterations in the neurogenic process and high fat high sugar diets seems to induce decrease of neurogenesis in the HF(Lindqvist *et al.*, 2006; Reichelt, Morris and Westbrook, 2016; Ferreira *et al.*, 2018). However, many doubts remain about how the high-caloric diets affect the neurogenic process. Adult hippocampal neurogenesis (AHN) is a process composed by several steps that are represented in the schematic Fig.2. It consists in the generation of new functional neurons from a pool of adult neuronal precursors found in the borderline of the granular zone, usually called subgranular cell layer. These neuronal stem cells proliferate, and the daughter cells then migrate deeper into the granule cell layer where they mature to form

new granule cells. Newly formed granule cells get integrated in the circuit and project their axons to the CA3 region to form functional synapses as it occurs with the rest of granule cells (Kuhn, Dickinson-Anson and Gage, 1996; Sperk, Hamilton and Colmers, 2007; Jessberger *et al.*, 2009; Stangl and Thuret, 2009; Todkar, Scotti and Schwaller, 2012; Zainuddin and Thuret, 2012).

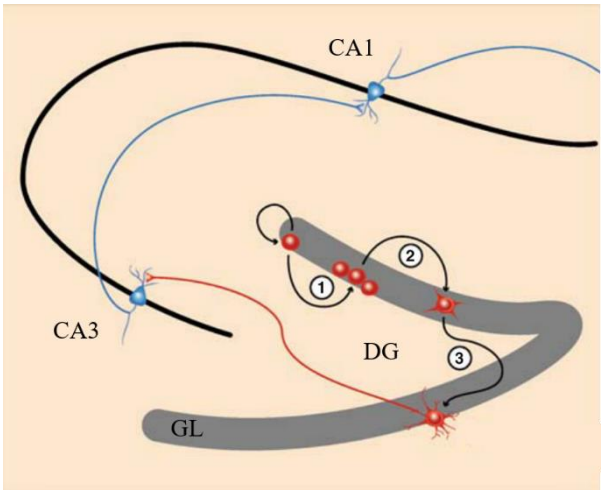

Fig.2.- Schematic representation of coronal view of rat hippocampal formation. The scheme shows (1) proliferation of adult neuronal precursor cells in the subgranular cell layer of the DG, (2) daughter cells migrate deeper into the granule cell layer and (3) mature to form new granule neurons that are integrated in the hippocampal circuitry. Adapted from (Stangl and Thuret, 2009)

Evidence suggest that AHN is involved in DG plasticity necessary for the encoding of new memories and the recalling of old ones and also plays a role in learning (Todkar, Scotti and Schwaller, 2012; Cho *et al.*, 2015). So, insults to this process, that can occur at any of its stages, proliferation, migration or integration, may induce alterations in DG normal functioning. A recent study in human patients revealed the persistence of hippocampal neurogenesis throughout life, not just in aged but also in diseased brain (Tobin *et al.*, 2019).

Indeed, there are several factors that regulate or could interfere with AHN, such as exercise, long term potentiation (LTP), stress and molecular factors like NPY or BDNF (Sperk, Hamilton and Colmers, 2007). Generally, AHN seems to have beneficial effects, however under certain circumstances, such as epileptic seizures, it can result in aberrant hippocampal neurogenesis that forms ectopic cells (Cho *et al.*, 2015). Ectopic cells are new granule cells that migrated wrongly and are found in the hilus (Scharfman *et al.*, 2005). It was previously found that caloric diets decrease hippocampal neurogenesis

(Lindqvist *et al.*, 2006; Ferreira *et al.*, 2018). However, neurogenesis is a complex process composed by several steps occurring in sequence, so it is of interest to do a study that explores its different stages. For that, we made a selection of genes that encode proteins related to different stages of neurogenesis, including brain derived neurotrophic factor (BDNF), Reelin (RELN) and cyclin-dependent kinase-5 (CDK5).

BDNF is an enhancer of hippocampal neurogenesis (Park *et al.*, 2010). One indirect action of BDNF is its induction of NPY which in turn enhances the proliferation of granule cells of the DG (Scharfman *et al.*, 2005). A work by Scharfman showed that infusion of BDNF increases neurogenesis in the DG. However, that infusion also resulted in formation of ectopic granule cells in the hilus. This study also shows that it does not need to occur an insult for ectopic migration to occur (Scharfman *et al.*, 2005).While a diet rich in fat leads to decreased levels of BDNF (Park *et al.*, 2010), a diet restriction increases BDNF levels and neurogenesis (Lee *et al.*, 2008). BDNF is also involved in learning since it was found that increased levels of this factor are associated with better performance in a spatial learning task (Molteni *et al.*, 2002). These characteristics make this component a relevant parameter to our study.

RELN is a glycoprotein important to CNS development and functioning being involved in cell migration and is an important component in the coordination of cortical a subcortical layers morphogenesis. The expression peak of this protein is shortly after birth, but during adulthood it is found it being expressed by GABAergic interneurons. It also seems to be involved in maturation of neurons, their synaptic formation and neuronal plasticity during AHN (Reichelt, Maniam, *et al.*, 2015; Armstrong, Anderson and McDermott, 2019). Studying the effects of CAF diets in rats, an association of impairments in hippocampal-dependent memory with a decrease in RELN was found (Reichelt, Maniam, *et al.*, 2015).

Cyclin-dependent kinases are a family of kinases involved in cell cycle regulation and CDK5 is the most common in the brain being highly expressed by neurons. CDK5 has some distinct functions. Although having a role regulating several proteins involved in the cell cycle, on the contrary of the other CDKs, CDK5 does not seem to have a direct role in cell cycle regulation. Evidence suggest that instead it acts suppressing cell cycle re-entry in postmitotic neurons (Jessberger *et al.*, 2009; Crews *et al.*, 2011). Aside than that, CDK5 acts in synaptic plasticity, dendritic growth morphological maturation and migration of neurons (Jessberger *et al.*, 2008, 2009; Crews *et al.*, 2011). The mechanism

of CDK5 action is not clearly understood but it is thought to be involved in microtubule dynamics (Jessberger *et al.*, 2009). Jessberger and colleagues showed that knockdown of CDK5 lead to aberrant growth of dendritic processes which in turn will influence the migration of new-born cells leading to formation of ectopic synapses in neurons in the hilar region (Jessberger *et al.*, 2008). Studies on Alzheimer disease found an association between abnormal activation of CDK5 and irregular maturation of neuronal progenitor cells (Crews *et al.*, 2011).

Despite the numerous studies that verse in the effects of high-caloric diets in the general health, information about the effects of different high-caloric diets in the brain, particularly on HF, are still insufficient to have a clear idea about how the different hypercaloric diets affect the brain. Indeed, the mechanisms behind cognitive impairments found in CAF diet treated rats are still unclear. Most studies of diet induced obesity have focused on high fat diets or high sugar diets separately, but as these components are commonly mixed in the western diets (Levine *et al.*, 2005) it is important to investigate them together and compare them. Thus, the present study is innovative because it evaluates the effect of these two factors simultaneously. With the increase of obesity and its impacts, it gets more important to study and understand its pathogenesis as well as its consequences. Starting from a previous study where we found that a CAF diet induced alterations in the cognition, particularly in the spatial learning and memory, associated with a decrease in the neurogenesis, we wanted to go further and try to understand possible mechanisms underlying those alterations. To do that, in the present work, we compared the effects of two different high-caloric diets, one diet rich in sugar (30% sucrose liquid solution) and of another inspired on a CAF diet, i.e., rich in refined sugar and saturated fat that were administrated for 12 weeks. We then focused on the GABAergic system, analysing the CBPs PV, CR and CB and the neuropeptides NPY and SS in the main regions of HF as well as the global content of GABA in the HF. Since the expression of neuropeptides could be dependent of the cholinergic system, we also analysed the levels of VAChT. Finally, trying to understand better the alterations in the neurogenesis, we analysed the effects of these diets in the mRNA levels of BDNF, RELN and CDK-5.

2. MATERIAL AND METHODS

2.1. Animals and diets

Male Wistar rats obtained from the colony of the Institute for Molecular and Cell Biology (Porto, Portugal) were kept under standard laboratory conditions (20–22 °C and a 12h light/dark cycle) with free access to food and water. Rats were housed 2 per cage to avoid social isolation and allow daily quantification of liquid and food consumption. They were weighted weekly, and bedding was changed at the same time to minimize stress caused by handling. At 4 weeks of age, rats were randomly assigned to one of the following 3 groups: control group (n = 10) had free access to tap water and were fed with standard rat chow (Mucedola, Italy) containing proteins (17%) supplemented with lysine (0.7%), methionine (0.3%) and cysteine (0.5%), carbohydrates (57%), fat (4%) and salts (7%). The standard chow provided approximately 3.9 Kcal/g, 20% of energy as protein, 12% as fat and 68% as carbohydrates. The high-sugar (HS)-treated group (n = 10) drank a solution of 30% sucrose (Sigma-Aldrich Company Ltd., Madrid, Spain; 1.2 Kcal/ml), instead of water, and were fed with standard laboratory chow. The CAF-treated rats (n = 10) drank a solution of 15% sucrose (Sigma; 0.6 Kcal/ml) and were fed with assorted food composed of standard rat chow, chocolate cake, biscuits, dog roll and a high-fat rat chow (40% fat). This diet provided an average of 4.5 Kcal/g, approximately 12% of energy as protein, 45% as fat and 43% as carbohydrates, in addition to that provided by the standard chow and the sucrose solution. All diets were supplemented with diet vitamin fortification mixture (MP Biomedicals, USA). Food and liquids were available *ad libitum* throughout the entire experimental period (12 weeks) and were replaced daily. The handling and care of the animals followed the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and the European Communities Council Guidelines in Animal Research (86/609/UE). Possible alternatives of refinement, reduction, and replacement were all taken into consideration in the present study and, as such, all efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation

After being submitted to the diets for 12 weeks, rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body weight). For immunohistochemical studies, 6 animals of each group were randomly selected and transcardially perfused with 150 ml of 0.1 M phosphate buffer, followed by a fixative

solution containing 4% paraformaldehyde in phosphate buffer at pH=7.6. For mRNA studies, the remaining animals of each group were decapitated and then the brains were quickly removed in a cold base and stored at -80ºC until usage.

2.3. Immunohistochemical studies

Brains were collected, codded for blind processing, separated by a midsagittal cut into right and left hemispheres. The frontal and occipital poles were removed and the blocks containing the HF were separated and processed for immunohistochemistry. Because there is evidence that the HF of rodents displays right/left asymmetries (Slomianka and West, 1987), the blocks were alternately sampled from the right and left hemispheres. The same blocks were immersed for 1 hour in the same fixative and maintained overnight in a solution of 10% sucrose in phosphate buffer, at 4ºC. Using a vibratome, the brains were serially sectioned in the coronal plane at 40 μm through the HF. The sections were collected in phosphate-buffered saline (PBS).

Vibratome sections of each animal containing the HF were selected, using a systematic random sampling procedure in a proportion of 1:12. Sections were washed twice in PBS, treated with 3% H_2O_2 solution for 7 min to inactivate endogenous peroxidase and then were incubated at 4ºC for 72h with primary antibodies against either PV (Swant, 1:5000 dilution in PBS 0.5% T), CR (Swant, 1:5000 dilution in PBS 0.5% T), CB (Swant, 1:5000 dilution in PBS 0.5% T), VAChT (Millipore, 1:2000 dilution in PBS 0.5% T), or overnight also at 4ºC for SS (Peninsula Laboratories, 1:10000 dilution in PBS 0.5% T) and NPY (Peninsula Laboratories, 1:10000 dilution in PBS 0.5% T). After this, the sections were washed three times in PBS 0.5 %T and incubated with the respective biotinylated secondary antibody. Sections were then treated with avidin–biotin–peroxidase complex (Vectas-tain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS 0.5% T). These last two incubations were carried out at room temperature for 1 hour. After the treatment with the peroxidase complex, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma-Aldrich) to which 30μl of 0.01% H_2O_2 solution was added. Sections were rinsed with PBS for at least 15minutes between steps. To increase tissue penetration, 0.5%Triton X-100 was added to the PBS that was used in all immunoreactions and washing steps. Specificity of the immune reactions was controlled by omitting the incubation step with primary antiserum. All immunohistochemical reactions and washings were carried out in 12-well tissue culture plates with 8-9 sections in each well, to ensure that staining of the sections from all groups

was performed in parallel and under identical conditions. The sections were mounted on gelatin-coated slides, air dried, dehydrated and cover-slipped using Histomount (National Diagnostics, USA).

2.3.1. Morphometric analysis

Immunostained brain sections were analysed using a light microscope equipped with a camera lucida at final magnification of ×160. Using a camera lucida all sections were drawn. The layers boundaries of either the DG, CA3 and CA1 were consistently defined at all levels along the septotemporal axis of the HF based on cytoarchitectonic criteria. Neurons belonging to the CA2 hippocampal field were included in the CA3 region. Immunoreactive neurons were identified as darkly stained perikarya. The number of neurons in each layer of the DG or hippocampus proper were counted from the drawings. The same camera lucida drawings were used to calculate the areas of the layers. For that, a transparent sheet bearing a test system composed by a set of regularly spaced points was overlaid on the drawings and the number of points that fell within the limits of molecular layer, GL, hilus, CA3 and CA1 were counted. The area of each layer was then estimated by multiplying the number of points that fell on within the limits by the value of the area per point of the test system ($0.0096 mm^2$). The cell numbers obtained were divided by the values of the corresponding laminar areas to yield the values of the areal densities (number of cells/mm^2).

2.4. RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Hippocampal formation was dissected from one of the brain hemispheres, this tissue was homogenized, and the total RNA was isolated using NZYOL reagent (NZYTech, Portugal) followed by chloroform extraction and isopropanol precipitation. Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Fisher Scientific, Portugal) and quality controlled using the 2100 Bioanalyzer Instrument (Agilent, USA).

Prior to reverse transcription, total RNA was DNase-treated with RQ1 DNase (Promega, USA.) in order to remove contaminating genomic DNA. Reverse transcription was carried out using the NZY First-strand cDNA Synthesis Kit (combined oligo-dT and random hexamers) (NZYTech, Portugal). Quantitative real time PCR (RT-qPCR) was performed in a StepOne Plus qPCR system (Applied Biosystems) using the SensiFAST

SYBR Hi-Rox Kit (Bioline, UK) with the standard curve method. The samples were assayed in triplicate. qPCR reaction efficiencies for all sets of primers ranged from 92% to 100%. Gene expression was normalized against the expression levels of two endogenous house-keeping genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin.

Gene-specific primers used are presented in table 1.

Gene	Forward	Reverse	Annealing temperature
GAD1	CCTAAAGTACGGGGTTCGCA	CAGCCATTCGCCAGCTAAAC	60 ºC
BDNF	GGCCCAACGAAGAAAACCAT	TTCCTCCAGCAGAAAGAGCA	60 ºC
RELN	TCAAAGACGCCTTAGCCCAG	TTCAGCGAGGTGCGAGTAAG	60 ºC
CDK5	GTGACCTGGACCCTGAGATTG	ACGTTACGGCTGTGACAGAA	57 ºC

Table.1.- Specific forward and reverse primers for the genes studied are presented. Alongside we have the annealing temperature for each pair of primers.

2.5. Statistical analysis

One-way ANOVA was used to analyse areal densities as well as relative expression of mRNAs for each studied marker. Whenever appropriate, ANOVAs were followed by Tukey highest signification difference (HSD) post-hoc comparisons. Differences were considered to be statistically significant when $p < 0.05$.

3. RESULTS

3.1. Caloric consumption, body weight and fat mass

Relative to caloric consumption, it was observed that intake per cage (2 rats per cage) during the whole treatment (12 weeks) was 14,453 (220.5) for control rats, 15,223 (155.8) for HS rats and 20,120 (60.8) for CAF rats, expressed in Kcal (SEM). Statistically, we found a significant effect ($F(2,12) = 370.94$, $p < 0.001$), since HS rats consumed more calories than controls ($p < 0.05$) and CAF rats more calories than control and HS rats ($p < 0.001$). Body weights of the animals across the experiment are shown in Fig.3. Body weights before diet intervention expressed in g (SEM), were 114.3 (2.45) for control group, 111.7 (2.90) for HS group and 116.1 (1.41) for CAF group, having no significant differences between groups ($F(2,27) = 0.9$, n.s.). After treatments with the different diets during 12 weeks, the body weights increased to 430.1 (8.51) for control rats, 410.2 (14.44) for HS rats and 456.7 (13.48) for CAF rats. Indeed, as revealed by ANOVA analysis, the dietary treatments had a significant effect on body weights ($F(2,27)=3.53$, $p < 0.05$). CAF treated rats were, on average, 46.5 g heavier than HS rats ($p < 0.05$); although we have found a slight reduction in the weight of HS-treated rats, when compared to controls, this was not statistically significant. Relative to fat mass of body (% body weight (SEM)), the results were 4.12 (0.24) in controls, 6.28 (0.55) in HS and 8.54 (0.31) in CAF animals. As shown by ANOVA, diets significantly changed total adipose tissue ($F(2,27) = 31.58$, $p < 0.001$). Indeed, CAF fed animals had more adipose tissue than control and HS-treated rats ($p < 0.001$) and HS fed rats had more adipose than controls ($p < 0.01$).

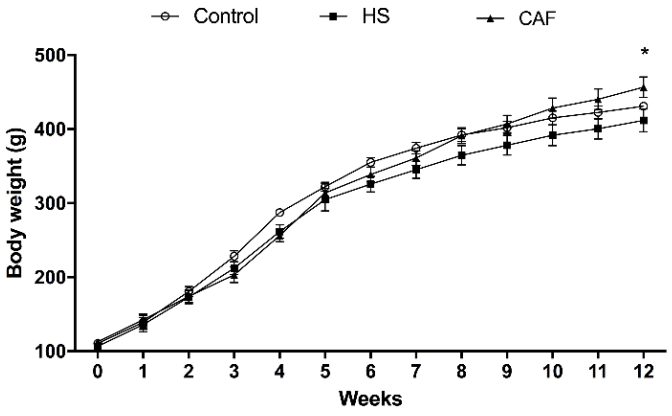

Fig.3.- Graphic representation of the body weight increase, in grams per week (mean ± SEM) with n=10 animals per group, of control, HS and CAF groups across the entire experiment. *p <0.05 CAF versus HS-treated rats.

3.2. Neuronal density studies

Histological studies were made to characterize the GABAergic neuronal population in the HF.

3.2.1. PV areal density in the DG

The estimates of the areal density of PV-IR neurons in the subregions of DG are shown in Fig.4. ANOVA statistical analysis revealed that there was a significant effect of treatment in the areal density of PV-IR cells in the GL ($F(2,15)=4.153$, $p < 0.05$) and hilar region ($F(2,15)=11.91$, $p < 0.001$) but not in the molecular layer ($F(2,15)=0.4300$, n.s.). In the GL, the number of PV-IR neurons was significantly decreased in rats fed with the CAF diet when compared to controls and HS-treated rats ($p < 0.05$) but no differences were found between HS and controls. Moreover, it was also observed that the CAF-treated animals show a significant decrease in PV-IR neurons in hilar region when compared to both control and HS groups ($p < 0.01$), but no differences were found between HS and control groups.

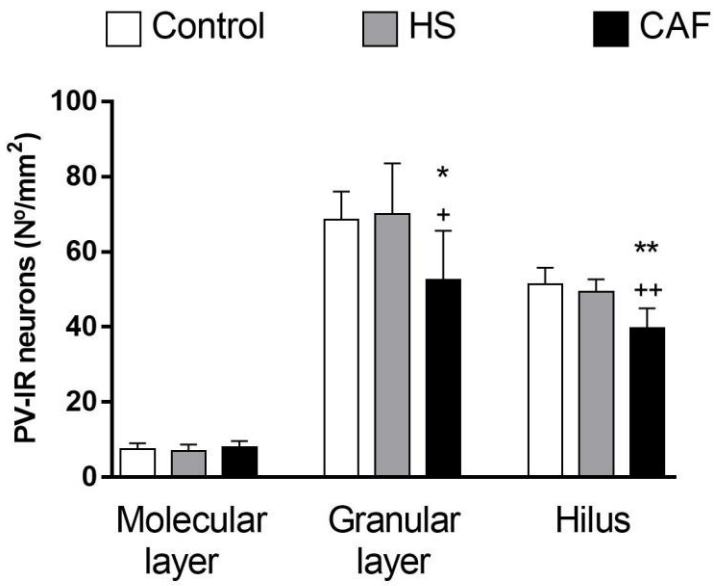

Fig.4.-The histogram shows the mean + SD areal density of PV-IR cells in the regions of the DG with n=6 animals per group. Note that there is a significant reduction in the number of PV-IR cells in the GL in the CAF rats when compared to controls and HS-treated rats, and also a significant reduction in hilus of CAF treated animals when compared to controls and HS-treated rats. *$p < 0.05$ versus controls; +$p < 0.05$ versus HS; **$p < 0.01$ versus controls; ++$p < 0.01$ versus HS.

3.2.2. PV density in CA3 and CA1 regions

The estimates of the total number of PV-IR neurons in the CA3 and CA1 regions of the HF are shown in Fig.5. ANOVA statistical analysis revealed a significant effect of treatment in the areal density of PV-IR neurons in the CA3 ($F(2,15)=4.938$, $p < 0.05$) and CA1 areas ($F(2,15)=5.272$, $p < 0.05$). In CA3 region, the number of PV-IR neurons was significantly decreased in rats fed with the HS diet when compared to control animals ($p < 0.05$). No significant differences were found between control group and CAF group nor between HS and CAF treatments. In CA1 region, the number of PV-IR cells was significantly decreased in rats fed with the CAF diet when compared to HS fed animals ($p< 0.05$), but not compared with controls. No differences were found between controls and HS animals.

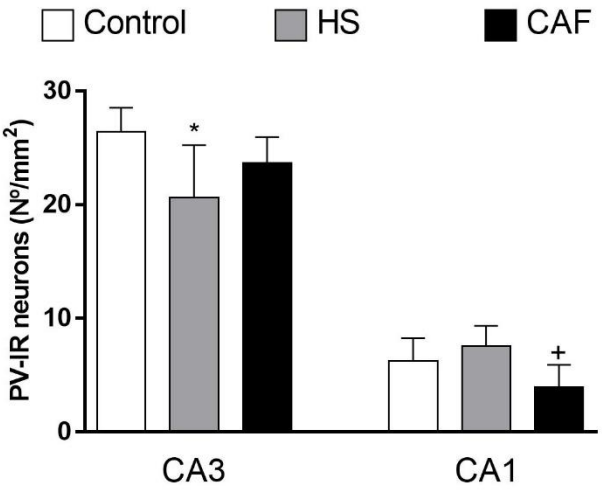

Fig.5.-The histogram shows the mean + SD areal density of PV-IR cells in the CA3 and CA1 regions of the HF with n=6 animals per group. Note that there was a significant reduction in areal density of PV-IR cells in the CA3 region in the HS rats when compared to controls. There was also a significant reduction of the density of PV-IR cells in the CA1 region in the CAF-treated rats when compared to HS animals. *$p < 0.05$ versus controls, +$p < 0.05$ versus HS.

In Fig.6 we show representative photomicrographs of the HF region immunostained for PV.

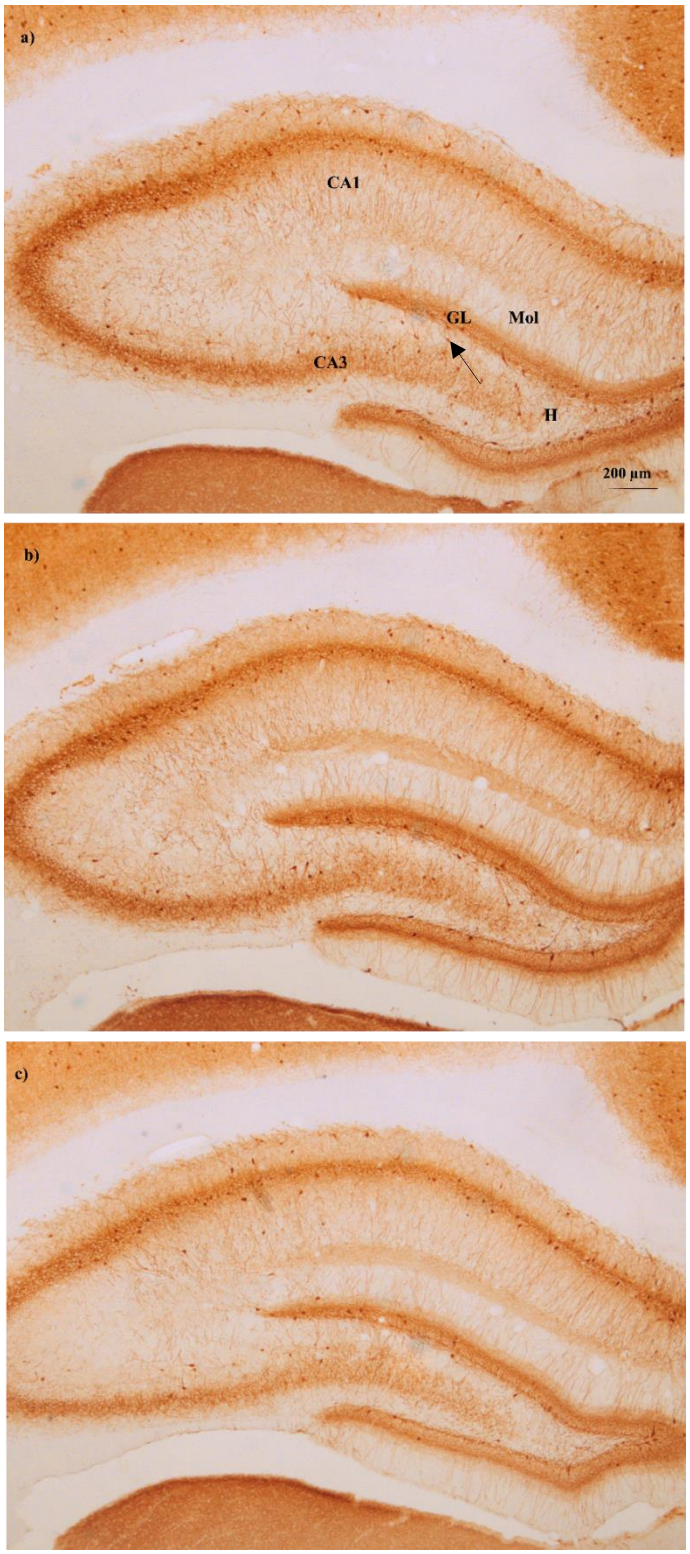

Fig.6.- (a-c) Representative photomicrographs of level- matched coronal sections of the HF from control (a), HS (b) and CAF (c) treated rats immunostained for PV. CAF diet animals display decreased areal density of PV-IR neurons in the hilus and GL hippocampal fields. Arrow in a) shows PV-IR cell in the hilus. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

3.2.3. CR areal density in the DG

The estimates of the areal density of CR-IR neurons in the DG are shown in Fig.7. ANOVA revealed that there was no significant effect of diets in molecular layer ($F(2, 15) = 0.02174$, n.s.), nor in the GL ($F(2, 15) = 1.164$, n.s.), nor in hilar region ($F(2, 15) = 0.6365$, n.s.).

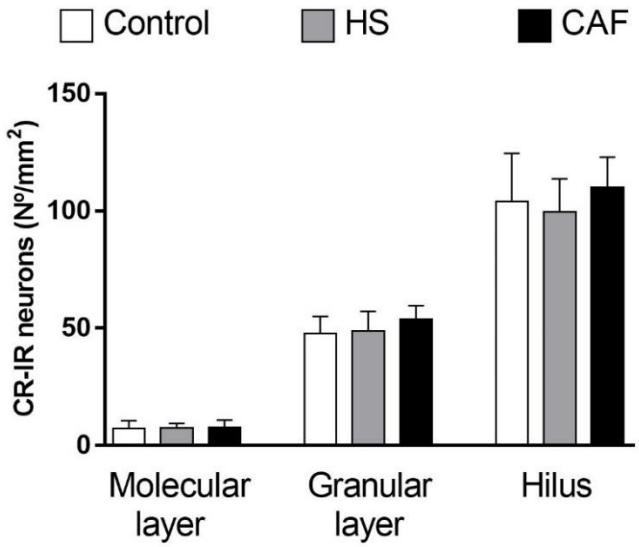

Fig.7.-The histogram shows the mean + SD areal density of CR-IR cells in the regions of the DG with n=6 animals per group. There are no significant changes between groups.

3.2.4. CR areal density in CA3 and CA1 regions

The estimates of the areal density of CR-IR neurons in the CA3 and CA1 region of the HF are shown in Fig.8. ANOVA analysis showed that there were no significant effects of diets in the number of CR-IR cells in the CA3 region ($F(2,15)=0.2229$, n.s) nor in CA1 region ($F(2,15)=1.734$, n.s).

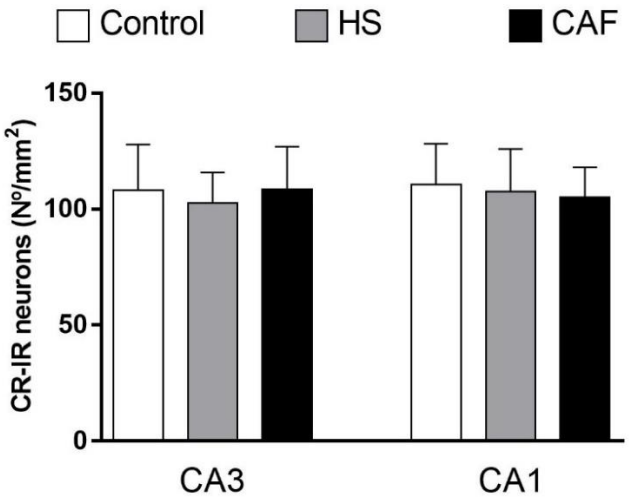

Fig.8.-The histogram shows the mean + SD areal density of CR-IR cells in the CA3 and CA1 regions with n=6 animals per group. There were no significant effects of diet in any of these regions.

In Fig.9 we show representative photomicrographs of the HF region immunostained for CR.

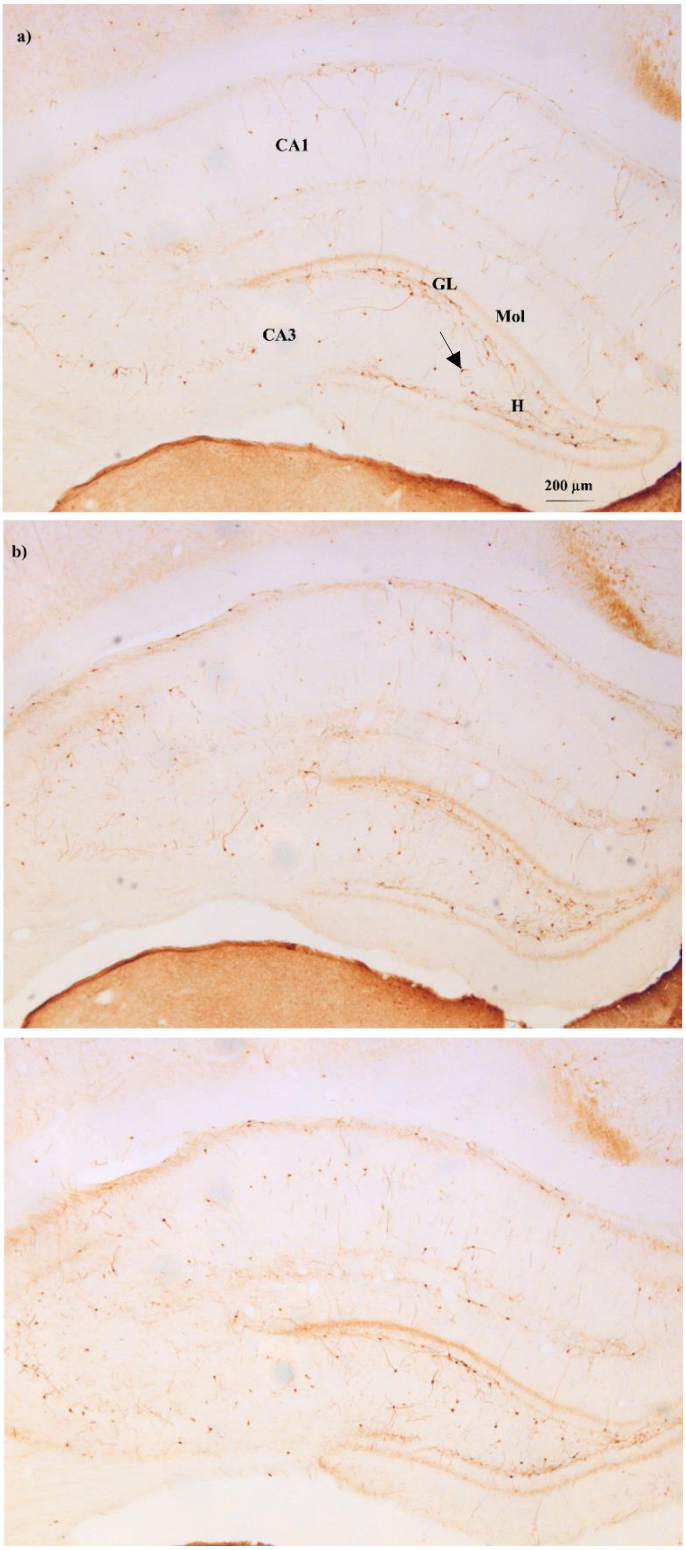

Fig.9.- Representative photomicrographs of level-matched coronal sections of the HF from control (a), HS (b) and CAF (c) treated rats immunostained for CR. Arrow in a) shows CR-IR cell in the hilus. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

3.2.5. CB areal density in the DG

The estimates of the areal density of CB-IR neurons in the regions of DG are presented in Fig.10. ANOVA showed that there was no significant effect of diets in the density of CB-IR cells in molecular layer ($F(2,15)=0.4540$, n.s.), GL ($F(2,15)=6.945$, n.s.), nor in hilus ($F(2,15)=1.138$, n.s.).

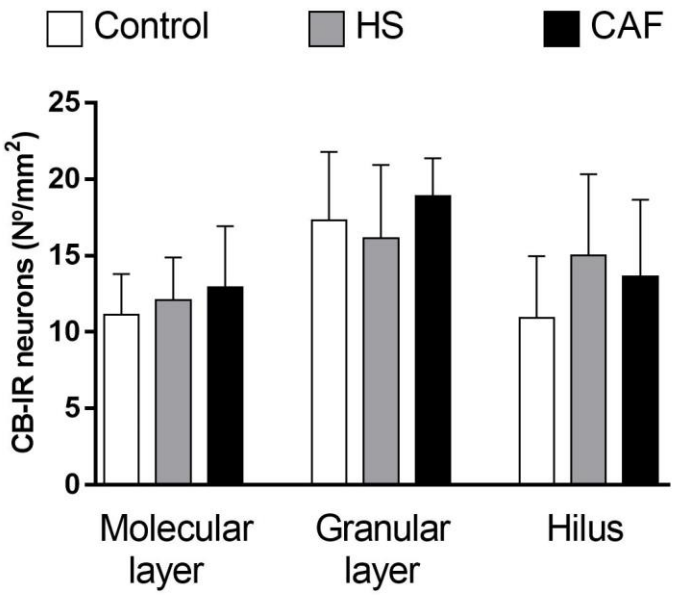

Fig.10.-The histogram shows the mean + SD areal density of CB-IR cells in the regions of DG with n=6 animals per group. There are no significant changes in the density of CB-IR cells.

3.2.6. CB areal density in the CA3 and CA1 regions

The estimates of the areal density of CB-IR neurons in the CA3 and CA1 regions of the HF are shown in Fig.11. The ANOVA analysis for the CA3 region showed that there was no effect of treatment in the number of CB-IR cells ($F(2,15)=2.679$, n.s.). Conversely, in the CA1 region ANOVA analysis showed a significant effect of treatment in the number of CB-IR cells ($F(2,15)=8.973$, $p < 0.05$). The number of CB-IR cells in CA1 region was significantly increased in rats fed with the HS diet when compared to control animals ($p < 0.05$) and when compared to CAF treated animals ($p < 0.01$). No significant differences were found between controls and CAF animals.

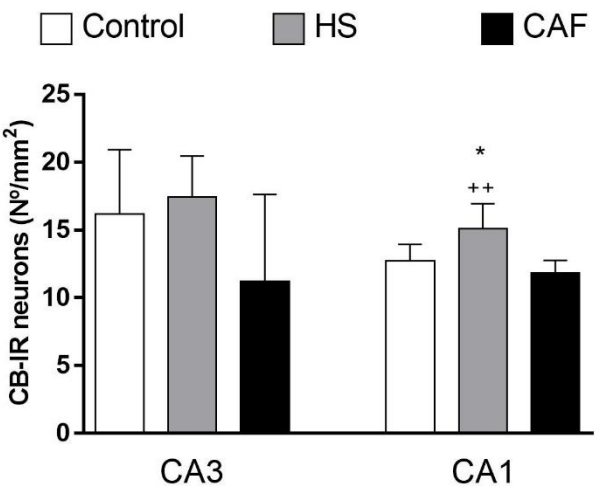

Fig.11.-The histogram shows the mean + SD areal density of CB-IR cells in the CA3 and CA1 regions of the HF with n=6 animals per group. Note that there is a significant increase in the number of CB-IR cells in the CA1 region in HS rats when compared to control animals and also when compared to CAF animals. There were no significant changes in the number of CB-IR cells in the CA3 region of the HF. *$p < 0.05$ versus controls; ++$p<0.01$ versus CAF.

In Fig.12. we show representative photomicrographs of the HF immunostained for CB.

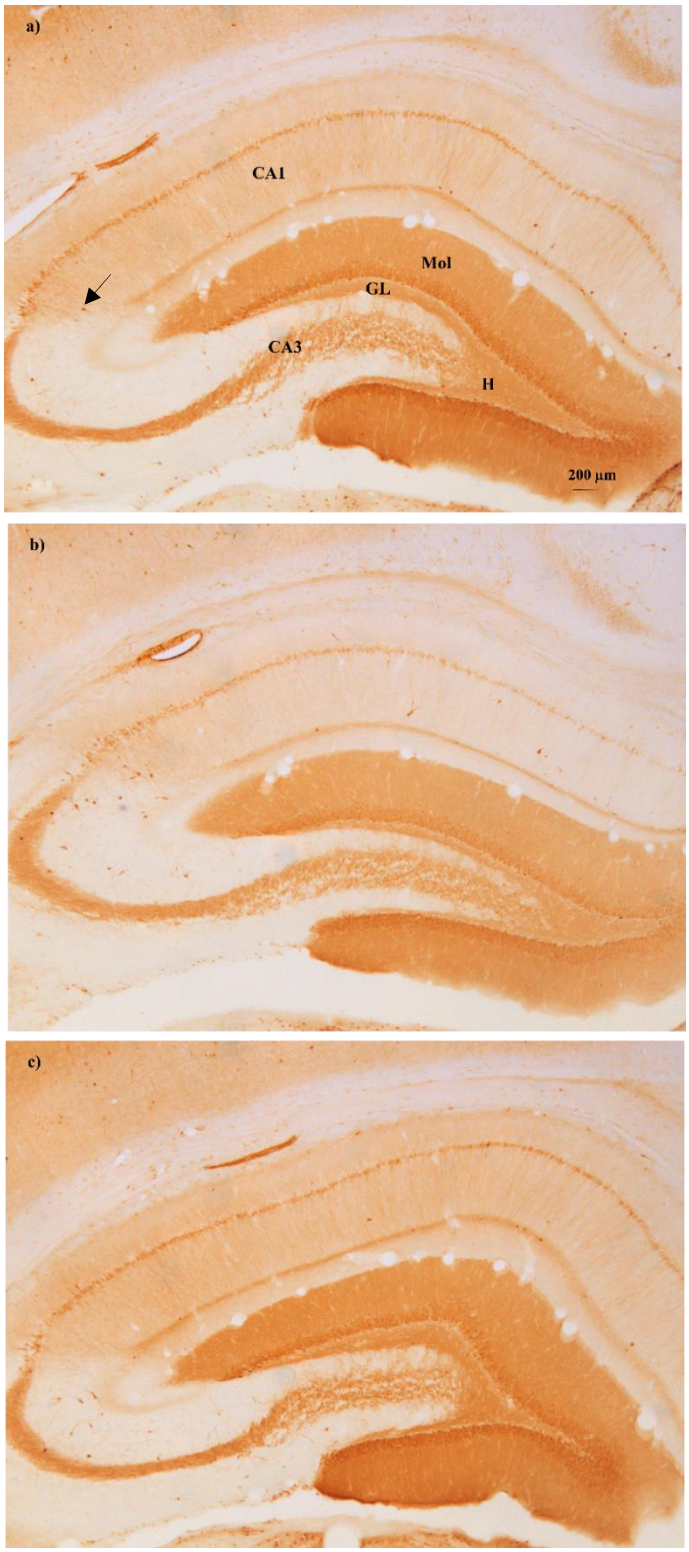

Fig.12.- High sugar diet treated animals display increased CB-IR neurons in the CA1 region when compared to controls and CAF animals. (a-c) Representative photomicrographs of level- matched coronal sections of the HF from control (a), HS (b) and CAF (c) treated rats immunostained for CB. Arrow in a) shows CB-IR cell in CA1 region. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

3.2.7. SS areal density in the DG

The estimates of the total number of SS-IR neurons in the DG are shown in Fig.13. ANOVA showed no significant effects of treatment in the areal density of SS-IR cells in molecular layer (F(2,15)=0.5565, n.s.), GL (F(2,15)=1.230, n.s.), nor in hilus (F(2,15)=0.2832,n.s.).

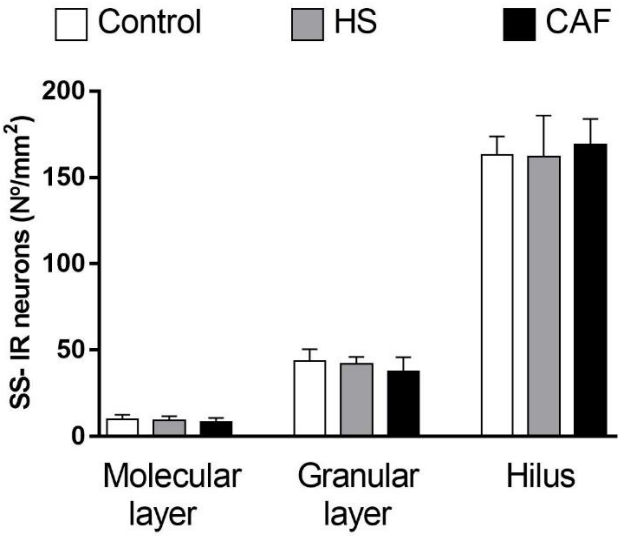

Fig.13.- The histogram shows the mean + SD areal density of SS-IR cells in the regions of DG, n=6 animals per group. There is not any significant change in the number of SS-IR cells.

3.2.8. SS areal density in CA3 and CA1

The estimates of the areal density of SS-IR neurons in the CA3 and CA1 region of the HF are shown in Fig.14. ANOVA analysis showed that there were no significant effects of diets in the CA3 region (F(2,15)=0.6988, n.s) nor in CA1 region (F(2,15)=0.7248, n.s).

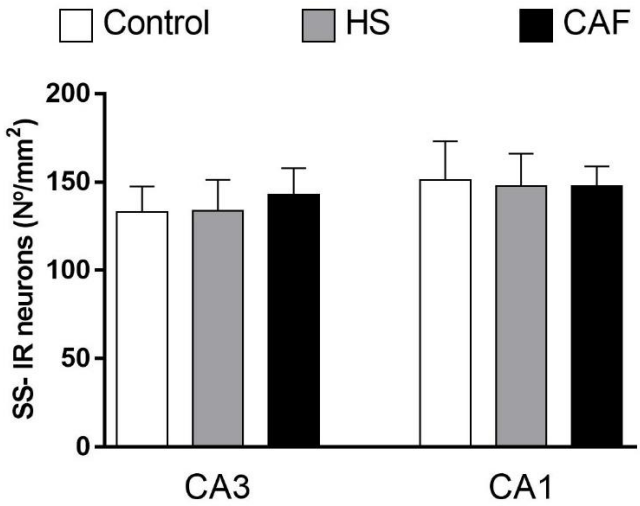

Fig.14.- The histogram shows the mean + SD areal density of SS-IR cells in the CA3 and CA1 regions of the HF, n=6 animals per group. There were no significant effects in SS-IR cells.

In Fig.15 we show representative photomicrographs of HF immunostained for SS.

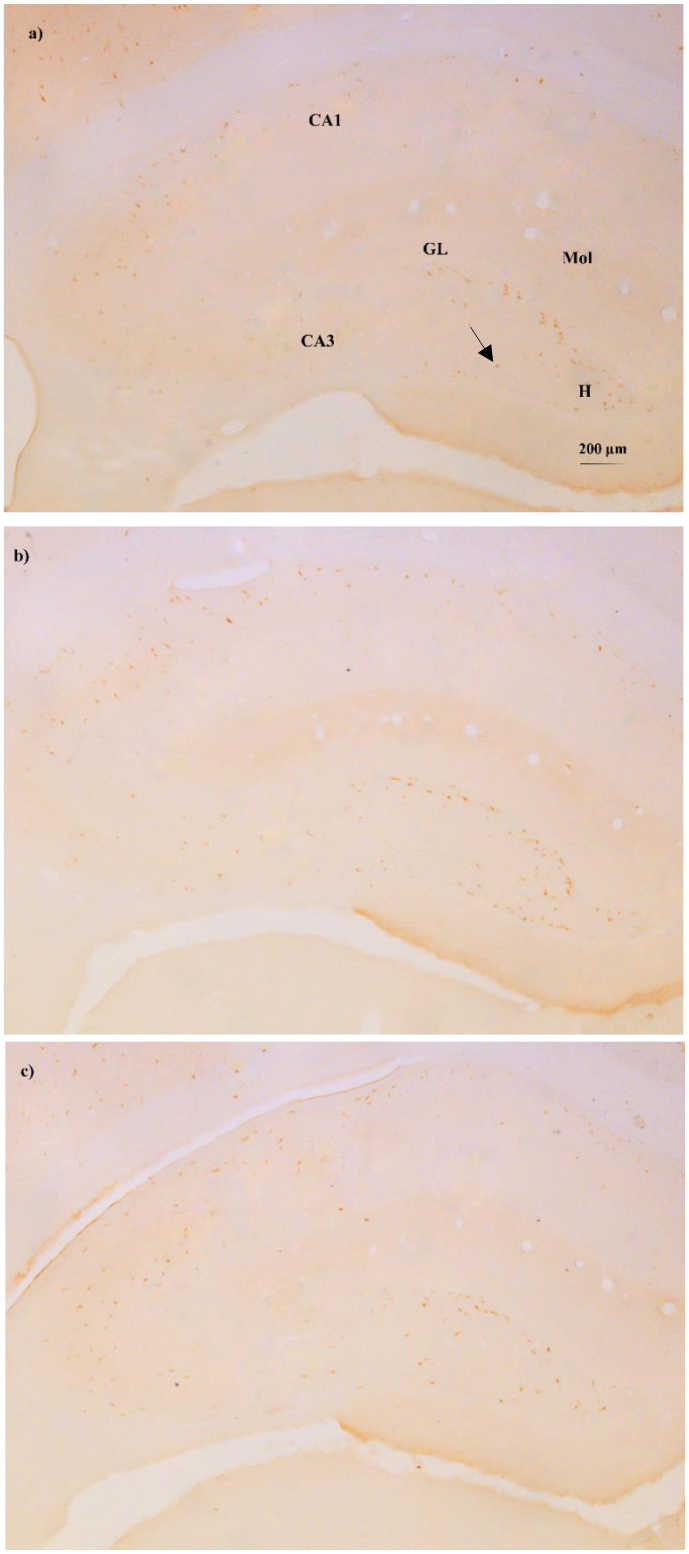

Fig.15.- Representative photomicrographs of level-matched coronal sections of the HF from control (a), HS (b) and CAF (c) treated rats immunostained for SS. Arrow in a) shows SS-IR cell in the hilus. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

3.2.9. NPY areal density in the DG

The estimates of the areal of NPY-IR neurons in regions of dentate gyrus are shown in Fig.16. ANOVA showed a significant effect of treatment in the hilus ($F(2,15)=4.456$, $p < 0.05$) but not in molecular layer ($F(2,15)=1.519$, n.s.) nor in the GL ($F(2,15)=0.4215$, n.s.). In the hilus, CAF diet causes a significant reduction of the number of NPY-IR neurons ($p< 0.05$) when compared to controls. No significant effects were found between HS and controls.

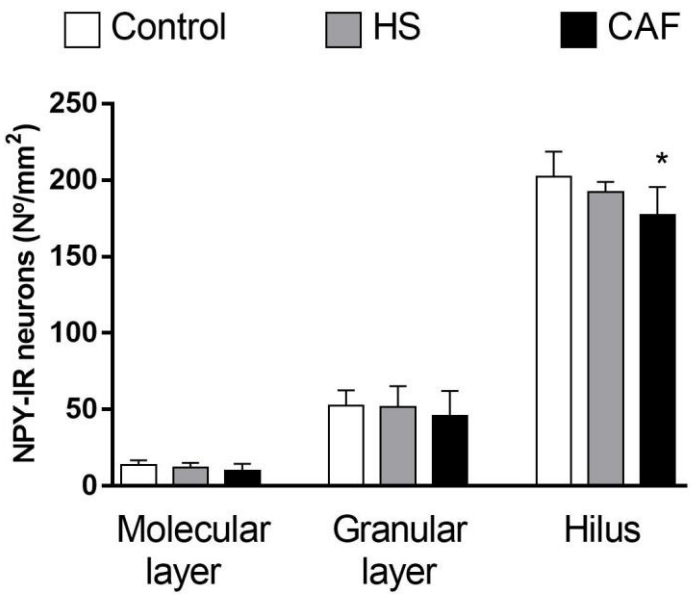

Fig.16.- The histogram shows the mean + SD areal density of NPY-IR cells in the regions of dentate gyrus with n=6 animals per group. Note that there is a significant reduction in the number of NPY-IR cells in the hilus in CAF group when compared to control animals. There were no other significant effects. *$p < 0.05$ versus controls.

3.2.10. NPY areal density in CA3 and CA1 regions

The estimates of the areal density of NPY-IR neurons in the CA3 and CA1 region of the HF are shown in Fig.17. ANOVA analysis showed that there were no significant effects of diets in the CA3 region ($F(2,15)=0.8707$, n.s) nor in CA1 region ($F(2,15)=3.506$, n.s).

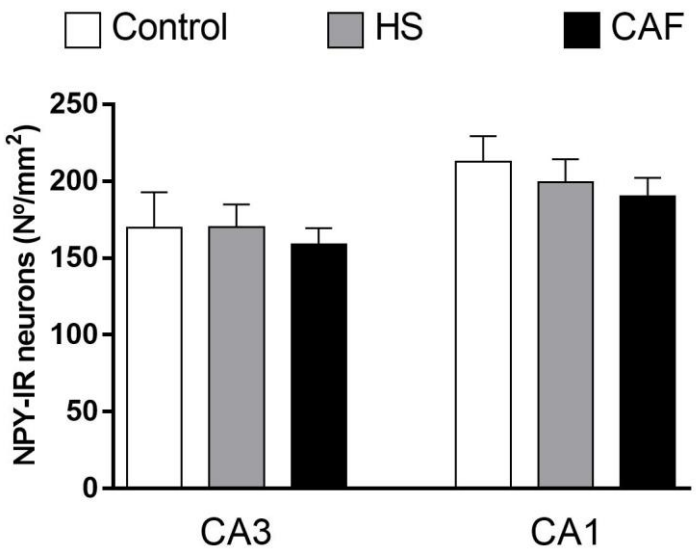

Fig.17.- The histogram shows the mean + SD areal density of NPY-IR cells in the CA3 and CA1 regions of the HF with n=6 animals per group. There were no significant effects of diets in any of the CA3 and CA1 regions.

In Fig.18. we show representative photomicrographs of the HF immunostained for NPY.

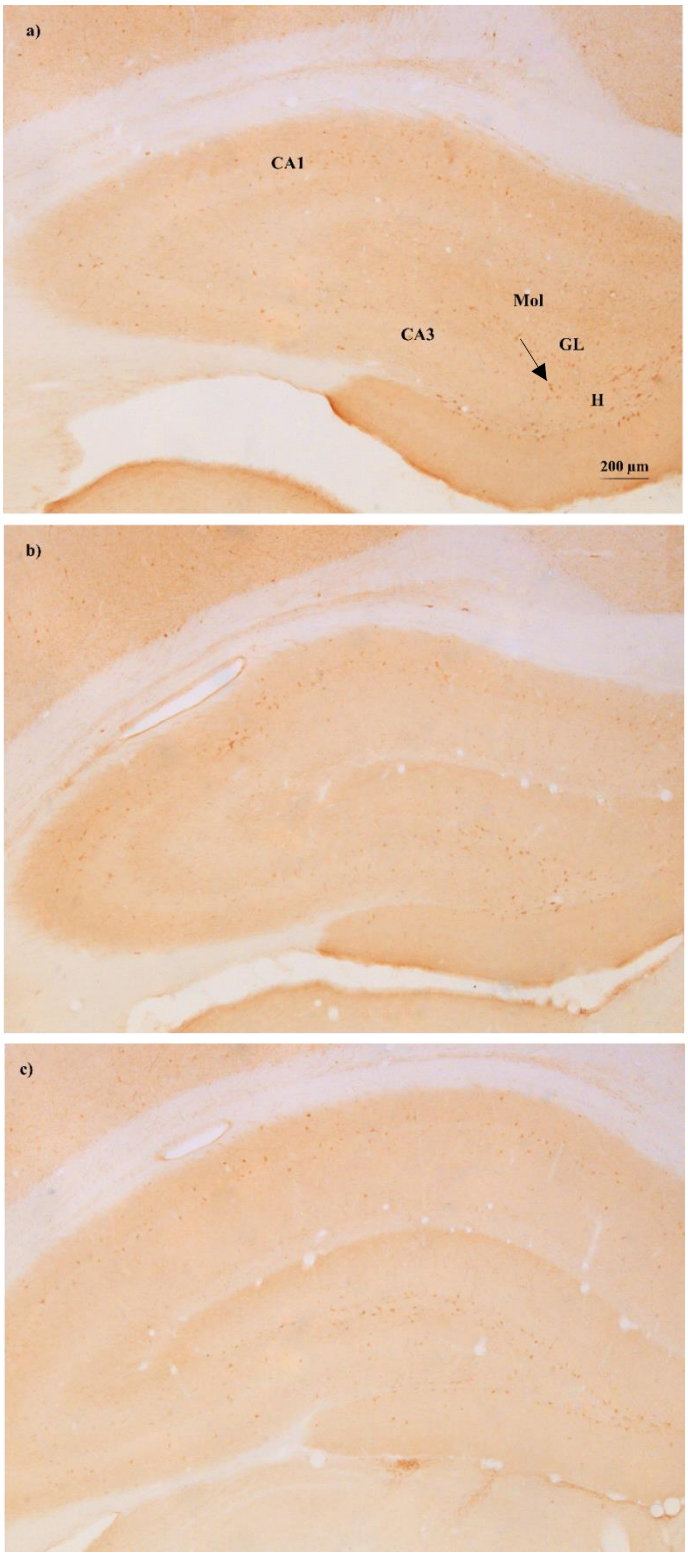

Fig.18.- (a-c) Representative photomicrographs of level- matched coronal sections of the HF from control (a), HS (b) and CAF (c) treated rats immunostained for NPY. CAF diet treated animals display decreased NPY-IR neurons in the hilus. Arrow in a) shows NPY-IR cell in the hilus. Mol, molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field, CA1, pyramidal cell layer of CA1 hippocampal field.

3.2.11. VAChT areal density in the hilus

The estimates of the areal density of VAChT in the hilar region of the HF are shown in Fig.19. ANOVA analysis showed that there was no significant effects of treatment in the density of cholinergic varicosities marked by VAChT ($F(2,15)=2.053$, n.s.).

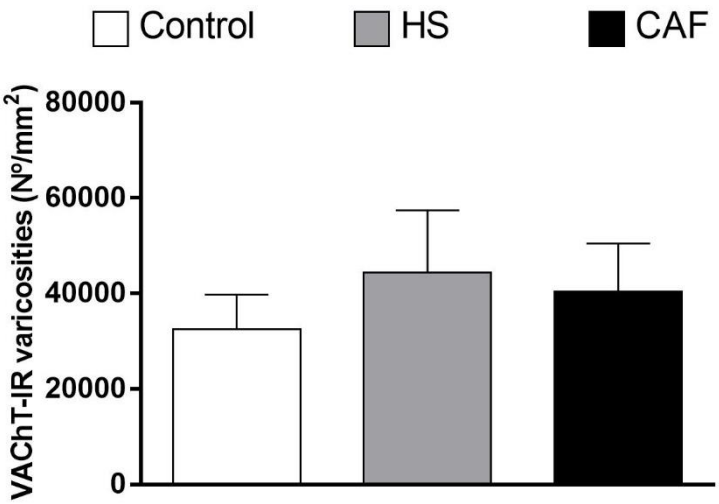

Fig.19.- The histogram shows the mean + SD areal density of VAChT-IR varicosities in the hilar region of the HF with n=6 animals per group. There is not significant changes in the density of VAChT-IR varicosities.

In Fig.20 we show representative photomicrographs of the hilus region immunostained for VAChT where it is visible the varicosities.

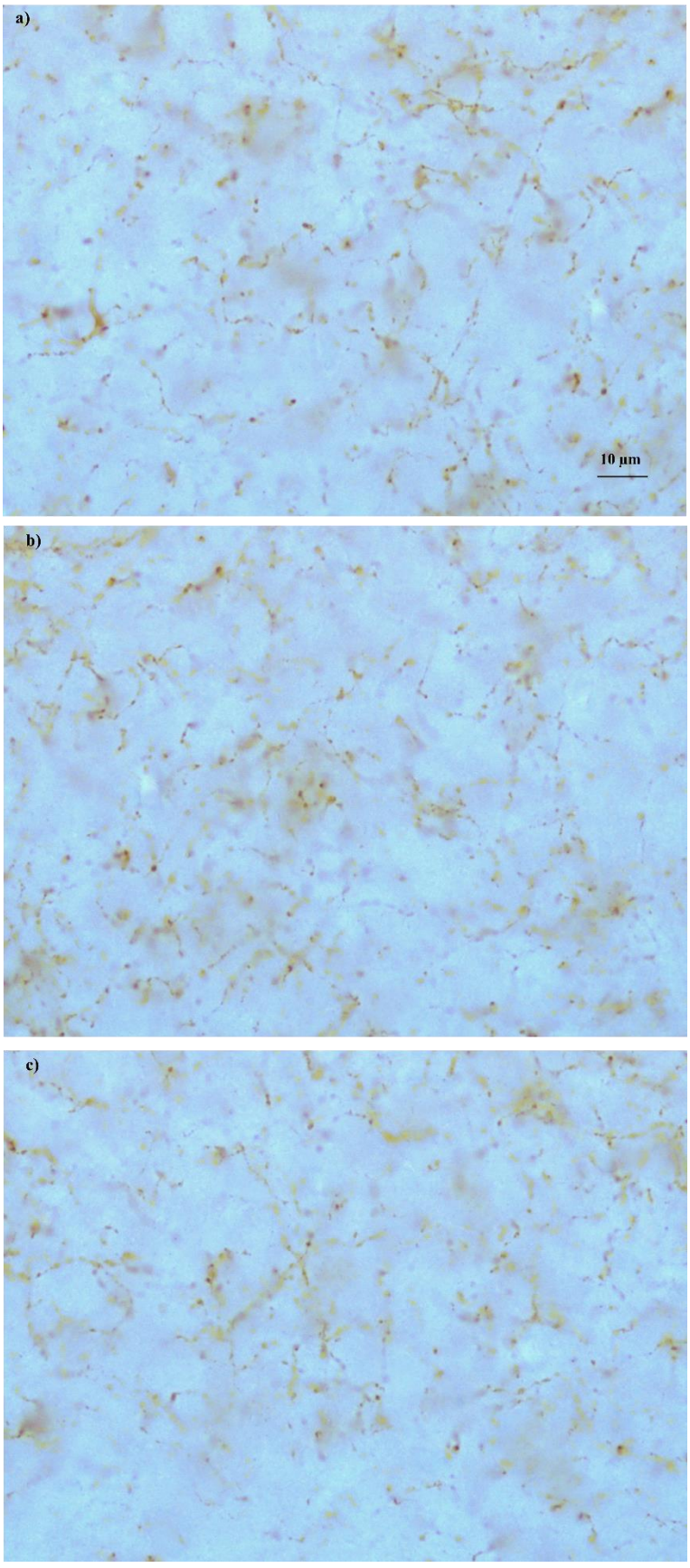

Fig.20.- Representative photomicrographs of level- matched coronal sections of the hilus from control (a), HS (b) and CAF(c) treated rats immunostained for VAChT varicosities.

3.3. mRNA relative expressions in HF

3.3.1. GAD1

In Fig.21 we present the mRNA relative expression of GAD1 in HF. ANOVA statistical analysis showed that there is no significant effect of treatment (F(2,9)=0.111, n.s.) in the GAD1 levels on HF.

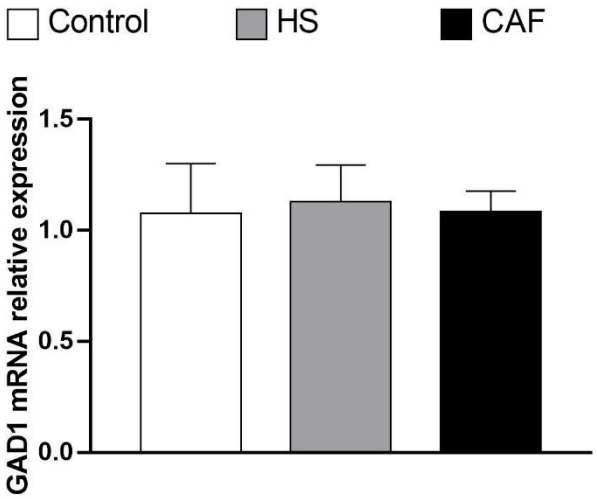

Fig.21.- Hippocampal GAD1 mRNA expression relative to the mean of housekeeping genes GAPDH/β-Actin. Data expressed as mean + SEM with n=4 animals per group.

As it was previously found that CAF diets caused a decrease in neurogenesis (Ferreira *et al.*, 2018), we used qRT-PCRs to study the expression of some genes related to neurogenesis in order to infer what might be impaired in this process.

3.3.2 Brain derived neurotrophic factor

The estimates of mRNA relative expression of BDNF, a gene related to proliferation, in HF are shown in Fig.22. ANOVA analysis showed no significant effect of treatment in the expression of this gene (F(2,9)=0.880, n.s.).

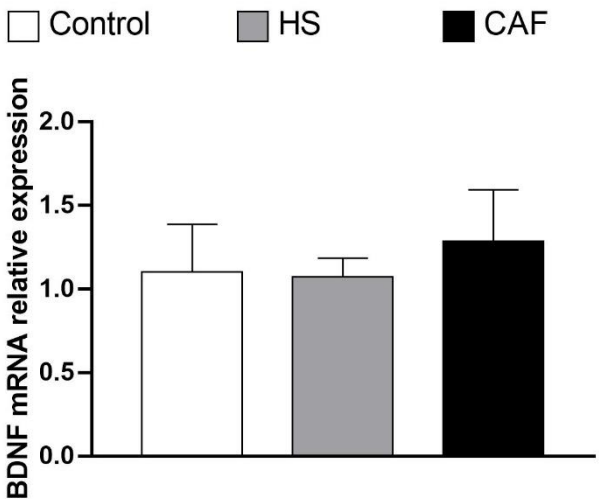

Fig.22.- Hippocampal BDNF mRNA expression relative to the mean of housekeeping genes GAPDH/β-Actin. Data expressed as mean + SEM with n=4 animals per group.

3.3.3 RELN

The estimates of mRNA relative expression of RELN in HF are shown in Fig.23. ANOVA analysis showed a significant effect of treatment in the expression RELN in the HF ($F(2,9)=5.159$, $p < 0.05$). It was observed that animals fed with CAF diet have a significant decrease in the RELN mRNA relative expression when compared to control animals ($p < 0.05$). There were no significant differences between HS and control rats nor between HS and CAF treatments.

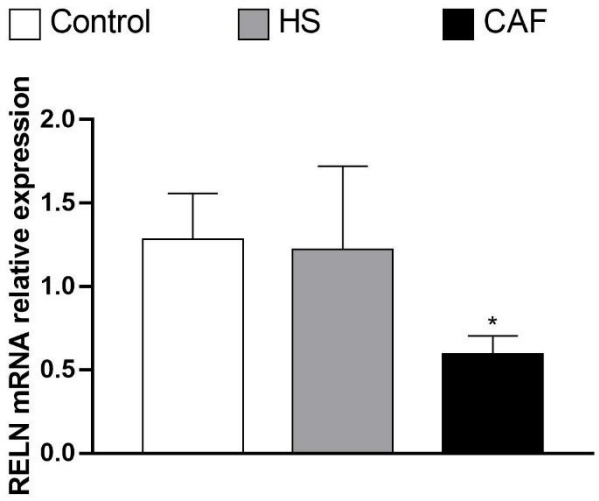

Fig.23.- Hippocampal RELN mRNA expression relative to the mean of housekeeping genes GAPDH/β-Actin. Data expressed as mean + SEM with n=4 animals per group. *$p < 0.05$ versus controls.

3.3.4 CDK5

The estimates of the mRNA relative expression of CDK5 in HF are shown in Fig.24. An ANOVA analysis showed no significant effect of diets in the expression of CDK5 ($F(2,9)=2.368$, n.s.).

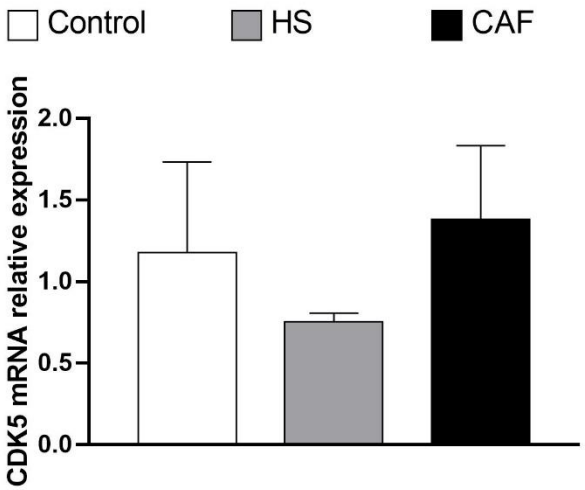

Fig.24.- Hippocampal CDK5 mRNA expression relative to the mean of housekeeping genes GAPDH/β-Actin. Data expressed as mean + SEM with n=4 animals per group.

4. DISCUSSION

Our work aimed to find the impairments that could underlie and contribute to the behavioural changes found in rats treated with high caloric diets. We have previously found that rats treated with a CAF diet, rich in both fat and sugar, show impaired spatial learning and memory as well as increased anxiety that was associated with a decrease of neurogenesis (Ferreira *et al.*, 2018). As the HF is involved in spatial learning and memory formation, we hypothesized that this structure could be affected by these treatments. In this study we compared two distinct high-caloric dietary regimens, HS and CAF diets, fed to juvenile rats and did a histological study focused in the GABAergic population of the HF as well as a genetic study of neurogenesis, a process that in adulthood occurs almost exclusively in the subventricular zone and in the DG. We have found that CAF diet induced a specific and significant reduction of the number of PV-IR neurons in the hilus and GL of the DG and CA1, accompanied by a significant reduction of NPY-IR neurons in the dentate hilus without significant alteration of the overall GABAergic population in the HF. Moreover, CAF diet induced a significant reduction of RELN levels in the HF. HS only showed a significant effect on CB population of CA1 region where we had an increase of these cells when compared to controls and CAF. No significant other alterations were seen in the number of CR-, CB-, SS-IR neurons and VAChT-IR varicosities in the HF and there was also no alterations in the levels of BDNF nor CDK5 in the HF.

When we analyse the weight changes during the 12 weeks experimental period, we see that there are no significant effects between the two high caloric diets and control animals, although there is a statistically significant decrease in weight of HS animals when compared to CAF animals. However, inspecting the graph of body weight it is possible to see that there is a gradual separation of the weight curves of the different groups in the last weeks, so it is plausible to assume that maintaining this progression it would be possible to have significant alterations, if we had maintained the diets during more time. Indeed, it was already verified that high-caloric diets do not forcibly induce weight gain in juveniles (Lindqvist *et al.*, 2006). Nevertheless, it is important to note that there was an increase in the body fat mass in both CAF and HS fed animals, which is in accord with previous works (Toida *et al.*, 1996; Martinez *et al.*, 2010; La Fleur *et al.*, 2014). We need to take these data in mind when evaluating the histological results because morphological and functional impairment of diets could occur even before

significant weight changes or obesity development. Our results, showing that CAF fed rats have significant alterations in the GABAergic system and in the neurogenesis, as we will discuss next, even without significant increase of body weight, suggests that this CAF diet is capable to impair neuronal systems, even before the onset of overt obesity, as already suggested by previous works (Valladolid-Acebes *et al.*, 2011; Beilharz, Maniam and Morris, 2014; Ferreira *et al.*, 2018).

As mentioned previously, GABAergic interneuronal population is very heterogeneous and widespread throughout the HF, thus we opted to study these populations in all the main subregions of HF, DG, CA3 and CA1. It is known that interneurons have several subpopulations, however the role of each interneurons subtype is not yet well established. Their activation pattern may diverge during behavioural states (Cobb *et al.*, 1995), so understanding and finding this differences between subpopulations would be important to understand the functioning of HF and its role on behaviour. However, before we start to analyse the different GABAergic subpopulations, and to have an idea of the global effect of high caloric diets in the GABAergic system, we measured, through GAD1 mRNA levels, the effects on GABA population in the HF and we found no significant alterations between groups. Although we did not find alterations in the GABA levels, a previous study using a high fat diet found a decrease in GABA concentration in the HF (Sandoval-Salazar *et al.*, 2016; Lizarbe *et al.*, 2019).

Although there was no significant alteration of the levels of GABA and knowing that as high caloric diets could interfere with activity of their subpopulations, we moved to the quantification of it. Interestingly, we have found that CAF diet induced a significant reduction of the number of PV-IR neurons in the hilus and GL of DG, and in regions CA3 and CA1, although achieved significant level only in CA1, when compared to control animals. Conversely, HS diet was not capable to induce such massive alteration in the number of PV-IR neurons as seen in CAF diet, since HS diet do not induce significant alterations in the number of PV-IR neurons in the HF regions, except a decrease of PV-IR cells in the CA3 region. These results show that PV-IR neurons are a GABAergic population vulnerable to high caloric diets, but clearly CAF diet had more impact in PV-IR neurons than HS diet. The reduction of PV-IR cells could be due to cell death, decrease of activity or alteration of protein content. However, at the present we cannot answer this. Future studies, using nutritional rehabilitation will be needed. At best of our knowledge, this is the first work showing that CAF diets induce reduction of the number of PV-IR

neurons in the HF. Our results are according to a previous study about the impact of high sugar consumption in juvenile rats, where it was shown a significant PV-IR reduction in DG, CA3 and CA1 regions of HF after daily sucrose consumption (Reichelt, Killcross, *et al.*, 2015). Reichelt and collaborators have recently found that high fat high sugar diets induced significant reduction of PV-IR neurons in the prefrontal cortex (Reichelt *et al.*, 2019) showing, together with our results, that PV-IR neurons are especially vulnerable to high caloric diets, at least during the juvenile period. Indeed, the activity of PV-IR cells is more susceptible to changes as Hashimoto and colleagues verified in a study of prefrontal cortex levels of PV and CR in human schizophrenic brains (Hashimoto *et al.*, 2003).

The majority of PV-IR interneurons in the HF belong to the GABAergic perisomatic inhibitory neuronal group and are positioned for the fine tuning and control of principal neurons efferents of HF (Sauer and Bartos, 2010). Taking this in consideration, the present results, where we found a reduction of PV-IR in the HF, suggest that the perisomatic inhibition and fine tuning of principal neurons of HF can be compromised leading, thus, to impairment of the functions dependent of the HF, like spatial learning and memory. Knowing this, it is plausible to suggest that the reduction of the number of PV-IR in the HF could be one of the mechanisms that underlie the spatial learning and memory impairment that we have found previously after CAF diet (Ferreira *et al.*, 2018). According to this, previous studies have shown that PV in the hippocampus seems to be important for cognition. Indeed, alterations in PV expression are associated with cognitive impairments and changes in dopamine signalling (Reichelt, Killcross, *et al.*, 2015). Also, PV role has been shown to have effects at network and behaviour levels, being involved in episodic memory (Fuchs *et al.*, 2007). Experiments in which mice had their PV-IR neurons inactivated, occurred an imprecise spiking activity of fast-spiking cells, impaired spatial working memory, impaired spatial learning as well as impaired memory retrieval (Fuchs *et al.*, 2007; Andrews-Zwilling *et al.*, 2012). Moreover, since this protein is involved events that involve calcium, alterations in PV levels can lead to impairments in processes such as neuronal excitation and synaptic transmission (Pauls, Cox and Berchtold, 1996; Hashimoto *et al.*, 2003). A dysfunction in this population may then be related to impaired rhythmic oscillations which in turn can lead to cognitive impairments. Therefore, the decrease that we have found in PV-IR interneurons in HF may be related to cognitive impairments found in CAF treated animals.

Moreover, this reduction of PV-IR neurons in the HF could be related to the increase of anxiety observed in CAF diet of our previous work (Ferreira *et al.*, 2018) since it was already verified that the reduction of PV levels are associated to an increase of anxiety levels (Godavarthi, Sharma and Jana, 2014).

Another thing that is important to say is that PV, being a CBP, confers neuroprotection (Van Den Bosch *et al.*, 2002) and its reduction, as seen in the present work, could lead to changes in the calcium homeostasis and consequently to an eventual cell death. However, since the present high caloric diets did not induce significant alterations in the other calcium binding proteins, CR and CB, as we will discuss next, the global calcium homeostasis could be preserved, although we cannot exclude an impairment due to the reduction of PV.

Conversely to PV, we did not find significant changes in the density of CR-IR cells in the HF. Indeed, looking to the results of GAD1 levels, where there were no differences between groups, it was expected that the majority of the GABAergic interneurons would be spared in these high caloric diets, and the CR-IR interneurons are one of them. So, we can postulate that interneuronal subpopulations, although interconnected, have different susceptibilities to high caloric diets. There are very few works that analysed the effects of high caloric diets in the CR expression (Niculescu and Lupu, 2009) and this is one of the firsts that look to the CR in the HF. It is important to say that PV-IR neurons are basically chandelier and basket cells that inhibit principal neurons at the cell body and axon initial segment (Freund, 2003; Lister *et al.*, 2011), opposite to CR-IR cells that are mainly dendrite-targeting, morphologically different and with no overlap at all with the PV-IR interneurons (Freund and Buzsáki, 1996; DeFelipe, 1997). Furthermore, whereas PV-IR neurons acts primordially in principal neurons, the CR-IR neurons are a special GABAergic population because it targets almost exclusively other GABAergic cells, including vasoactive intestinal polypeptide-, CB-, SS- and other CR-IR neurons, but avoid PV-IR neurons (Gulyás, Hájos and Freund, 1996; Barinka and Druga, 2010). Thus, the present results show that none of the present high caloric diets impairs the fine tune synchronization of the inhibitory drive upon principal neurons made by CR-IR interneurons.

In our work we found that none of the present high caloric diets induced significant reduction in the number of CB-IR neurons in the HF. This was not completely unexpected since there is some co-localization between CB and CR and because levels of

GAD1 in HF were unchanged. Indeed, we found a slight increase of CB-IR neurons that was only observed in CA1 region.

Relative to neuropeptides, in the present study we have found that CAF diet induced a significant decrease in the number of NPY-IR neurons in the HF, but only in the dentate hilus. Interestingly, this effect of the CAF diet seems to be specific to NPY, since the number of interneurons immunoreactive to SS, a neuropeptide known to be co-expressed by most hilar GABAergic interneurons including those that produce NPY, was not changed. This reduction of NPY could be related to cell death, decrease of activity or alteration of protein content. Future studies, using nutritional rehabilitation will be needed. However, knowing that NPY-IR neurons resist to several treatments and that they partially co-localize with SS we can speculate that this reduction is probably not due to cell death. Previous studies have already found a decrease of the expression of NPY in the HF in high fat diet (Hassan *et al.*, 2018), but our work is the first showing that a CAF diet induces reduction of neuropeptides in HF, specifically the NPY-IR and not SS-IR interneurons, and that this reduction occurs only in the dentate hilus. Indeed, NPY-IR cells are dispersed throughout the DG but are more concentrated in the hilus (Sperk, Hamilton and Colmers, 2007). Knowing that NPY-IR subpopulation could also inhibit other subpopulations of interneurons (Sperk, Hamilton and Colmers, 2007), we could think that a decrease of it may interfere with the activity of the other local interneuronal populations, which in turn would lead or contribute to the spatial learning and memory impairment in CAF fed animals that we have seen in our previous study (Ferreira *et al.*, 2018).

Taking in mind that NPY- and SS-ergic subpopulations could be related to the cholinergic innervation in the cerebral cortex (Zhang *et al.*, 1998; Cardoso, Paula-Barbosa and Lukoyanov, 2006) and that HF interneurons have a direct relation to cholinergic system since this system is involved in the recruitment of interneurons (Lucas and Clem, 2018), we decided to analyse the effects of these high caloric diets upon the cholinergic system of the HF. Interestingly, we did not find significant changes in cholinergic varicosities in the dentate hilus, which indicates that cholinergic system in the HF is not affected by these high caloric diets and that the reduction of the number of NPY-IR neurons in dentate hilus observed in CAF fed animals is probably not due to alterations in the cholinergic system. Furthermore, the cognitive impairment that we have previously found in CAF (Ferreira *et al.*, 2018) is also probably not directly related to the cholinergic

system. It is important to note that a previous study found a decrease of acetylcholinesterase in the HF of rats after treatment with high caloric diets (Kaizer *et al.*, 2004), however they followed the treatment during 6 months, which could justify the discrepancy to our results.

Until now we have been discussing the effects of high caloric diets in GABAergic populations. Next, we will focus on the effects of caloric diets on AHN, a process fundamental to hippocampal plasticity and learning and memory formation. In a previous work, our group has found that the CAF induced a decrease of doublecortin (DCX) in the HF that was associated to impairment of spatial learning and memory (Ferreira *et al.*, 2018). DCX is a brain specific microtubule-associated protein that is expressed by proliferating cells and immature neurons during AHN (Von Bohlen Und Halbach, 2011; Todkar, Scotti and Schwaller, 2012). Since neurogenesis is a mechanism that probably underlies the observed cognitive alterations in CAF, we tried to understand better how the high caloric diets affected this process. To do that, we studied mRNA expression levels of genes related to neurogenesis using qRT-PCR, including BDNF, RELN and CDK-5.

We choose BDNF because besides being involved in proliferation and/or survival of cells (Stangl and Thuret, 2009), is a neurotrophin essential for neurite out-growth and synaptic strengthening (Lu, Pang and Woo, 2005). Moreover, BDNF plays a role as regulator in the expression of PV and other CBPs (Marty, Da and Berninger, 1997). Interestingly we have found that none of the high caloric diets tested in the present study was capable to induce significant alterations in the levels of BDNF mRNA in the HF. This result was unexpected, because a previous work using high caloric diets showed reduction of BDNF expression correlated with memory deficits (Molteni *et al.*, 2002) however, the diet periods used in this study range from 2 months to 2 years. Indeed, our results are in agreement with other studies in which high caloric diets did not induced alterations in BDNF levels (Beilharz, Maniam and Morris, 2016; Arcego *et al.*, 2018) even when there is cognitive impairment (Heyward *et al.*, 2012; Beilharz, Maniam and Morris, 2014; Reichelt, Maniam, *et al.*, 2015). However, looking to the work of Molteni and collaborators (2002) it is plausible to assume that maintaining the high caloric regimen during longer periods it can cause effects on the levels of BDNF. Nevertheless, the present results show that spatial learning and memory alterations and decrease of DCX

that we found previously (Ferreira *et al.*, 2018) is probably not directly related to alteration in BDNF levels in HF.

Interestingly, we have found that both CAF and HS diets did not induce significant alterations in levels of CDK5 in the HF. This is the first work that focuses in CDK5 using a CAF diet but a previous work has also found absence of alterations of the levels of CDK5 in HF in a high fat diet (Ettcheto *et al.*, 2016). CDK5 is the main CDK of the brain, being particularly expressed in neurons, and has an essential role in synaptic plasticity, dendritic growth (Jessberger *et al.*, 2009) and neuronal development (Cicero and Herrup, 2005) in mature neurons. A work by Lagace and colleagues found that ablation of CDK5 in neuronal progenitor cells of the hippocampus lead to a decrease in DCX-IR neurons (Lagace *et al.*, 2008). Moreover, it acts upon DCX during adult neurogenesis and when CDK5 is inactivated it results in aberrant formation of new-born cells (Jessberger *et al.*, 2008). However, it seems that the migration of new neurons in the hippocampus was not overtly modified with the ablation of CDK5 and that it has an essential role in the survival, but not proliferation, of adult-generated hippocampal neurons. Tacking this all together, it seems that the reduction of DCX that we observed in CAF (Ferreira *et al.*, 2018) could be more related to alterations in the proliferation or migration of progenitor cells and not to survival, maturation, albeit we cannot exclude it because other factors, that we did not analysed, could be involved. Moreover, reduction of DCX is probably not directly related to any change of the CDK5 levels in the HF.

CDK5 is essential for adult neurogenesis (Lagace *et al.*, 2008) and in adults, both neurogenesis (Zhao, Deng and Gage, 2008) and CDK5 (Hawasli *et al.*, 2007) are important in learning and memory process. Knowing this, the present results, where we did not find significant alterations in CDK5 levels in both high caloric diets, seems to indicate that the spatial learning and memory that we have previously found (Ferreira *et al.*, 2018) is not related to any change of the levels of CDK5 in the HF.

Finally, in this work we found that mRNA expression levels of RELN are significantly decreased in the hippocampus of rats submitted to CAF. This is in agreement with a similar previous study (Reichelt, Maniam, *et al.*, 2015). RELN is an extracellular matrix protein that is crucial for neuronal migration during the development of brain regions (Tissir and Goffinet, 2003; Teixeira *et al.*, 2012) and its expression is present during adulthood in hippocampal and cortical interneurons (Pesold *et al.*, 1998; Teixeira *et al.*, 2012). Interestingly, it was demonstrated that the inactivation of the RELN

signalling pathway, specifically in adult neuroprogenitor cells, induced aberrant migration, formation of ectopic dendrites in the dentate hilus, establishment of aberrant circuits and decreased dendrite development (Teixeira *et al.*, 2012). Furthermore, it was previously shown that in adult brain, RELN regulates the neurogenesis and migration, as well as the structural and functional properties of synapses (Pujadas *et al.*, 2010). This information is very important, observing that CAF induced a reduction of DCX (Ferreira *et al.*, 2018) associated to a significant reduction on the levels of RELN but without alterations in CDK5 in HF, this suggests that the reduction of DCX in CAF could be underlined by the reduction of RELN levels in HF and that could also underlie the spatial learning and memory alterations observed in our previous work (Ferreira *et al.*, 2018). At last, because RELN is also fundamental for modulation of the structural and functional plastic properties of adult synapses, including induction and maintenance of LTP (Pujadas *et al.*, 2010) it is plausible to assume that learning and memory impairment in CAF could be underlined by potential alterations in synapses of HF induced by reduction on RELN levels.

AHN is a complex process that involves not just the progenitor cells and resulting new neurons, but is important to consider the vasculature and astrocytes that also play a relevant role in this process (Stangl and Thuret, 2009). Studies that take this in consideration and evaluate the relation between neurogenesis and these surrounding factors simultaneously can be useful in deeper understanding changes or effects of diets in AHN and are needed in future studies.

5. CONCLUSION

Our results show that CAF treatment in juveniles impacts neurogenesis and GABAergic system in the HF which was not observed in HS treatment. We have found that CAF reduces the number of PV- and NPY-IR neurons, without significant alteration on the overall GABAergic population. Moreover, CAF treatment induced a significant reduction of RELN mRNA levels in the HF with no changes on BDNF nor on CDK5. These findings also suggest that reduction of PV, NPY and RELN in hippocampus may explain the cognitive impairments of juveniles that consume typical cafeteria diets. These data also consolidate the evidence that early life is an extremely vulnerable period to dietary challenges and emphasize the importance of identifying the subtle molecular mechanisms that mediate the effects of diets rich in saturated fats and refined sugar on neurogenesis, GABAergic system and cognition in the maturing brain.

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