Title Hepatic glycogen participates in the regulation of hypothalamic pAkt/Akt ratio in high-sugar/high-fat diet-induced obesity

Hepatic glycogen likely participates in the regulation of hypothalamic pAkt/Akt ratio in high-sugar/high-fat diet-induced obesity.

Breno P Casagrande [a], Allain A Bueno [b], Luciana P Pisani [a], Debora Estadella [a]*

[a] Biosciences Department, Institute of Health and Society, Federal University of São Paulo, 1015-020, Santos, São Paulo, Brazil

[b] College of Health, Life and Environmental Sciences, University of Worcester, Worcester, Henwick Grove, WR2 6AJ, United Kingdom

ORCID / Email addresses:

0000-0001-9478-8262 / brenopcasagrande@gmail.com

0000-0002-9456-8558 / a.bueno@worc.ac.uk

0000-0001-6579-6167 / lucianapisani@gmail.com

0000-0001-9853-3662 / estadella.debora@gmail.com*

*Corresponding author at Biosciences Department, Institute of Health and Society, Federal University of São Paulo, Campus Baixada Santista – UNIFESP/BS, Santos, 11015-020, SP, Brazil.

Abstract

The hypothalamus is a major integrating centre that controls energy homeostasis and plays a major role in hepatic glycogen (HGlyc) turnover. Not only hypothalamic and hepatic Akt levels influence glucose homeostasis and glycogen synthesis, exposure to high-sugar/high-fat diets (HSHF) can lead to hypothalamic inflammation and HGlyc accumulation. HSHF withdrawal overall restores energy and glucose homeostasis, but the actual relationship between hypothalamic inflammation and HGlyc after short-term HSHF withdrawal has not yet been fully elucidated. Here we investigated the short-term effects of HSHF withdrawal preceded by a 30-day HSHF intake on the liver-hypothalamus crosstalk and glucose homeostasis. Sixty-day old male Wistar rats were fed for 30 days a control chow (n=10) (Ct), or a HSHF diet (n=20). On the 30th day of dietary intervention a random HSHF subset (n=10) had their diets switched to control chow for 48 h (Hw) whilst the remaining HSHF rats remained in the HSHF diet (n=10) (Hd). All rats were anesthetized and euthanized at the end of the protocol. We quantified HGlyc, Akt phosphorylation, inflammation and glucose homeostasis biomarkers. We also assessed the effect of propensity to obesity on those biomarkers, as detailed previously. Hd rats showed impaired glucose homeostasis, higher HGlyc and hypothalamic inflammation, and lower pAkt/Akt. Increased HGlyc was significantly associated with HSHF intake on pAkt/Akt lowered levels. We also found that HGlyc breakdown may have prevented a further pAkt/Akt drop after HSHF withdrawal. Propensity to obesity showed no apparent effect on hypothalamic inflammation or glucose homeostasis. Our findings suggest a comprehensive role of HGlyc as structural and functional modulator of energy metabolism, and such roles may come into play relatively rapidly.

Keywords: Hepatic glycogen; hypothalamus; liver; withdrawal; obesogenic diet

1.0 Introduction

Unhealthy dietary habits associated with a modern lifestyle often include the intake of ultra-processed foods, with high content of simple carbohydrates and saturated fats, associated with low intake of complex carbohydrates (Hu 2002; Bortolin et al. 2018; Afshin et al. 2019). Increased body adiposity, resulting from chronically positive energy balance, is often explained by associations between energy dense diets and sedentary behaviours (Moore and Boesch 2019; WHO 2020). Dietary factors are associated with around one-fifth of deaths worldwide (Afshin et al. 2019), and on those grounds high-sugar/high-fat diets (HSHF) have been widely used in scientific research to induce metabolic disarrangements such as those manifested in conditions characterised by a mild chronic proinflammatory background, including obesity, type 2 diabetes, and fatty liver disease (Lutz 2018).

Hypothalamic regulatory pathways can be impaired in metabolic disarrangements and in inflammation (Morton et al. 2006; Cesar and Pisani 2017). Hypothalamic inflammation has been observed in rats fed high-fat diets for extended periods (De Souza et al. 2005). Interestingly, comparable findings have been observed rather rapidly, such as in mice fed a high-fat diet for one day only (Waise et al. 2015).

In addition to regulating food intake, the hypothalamus plays a major role in glucose homeostasis (Lundqvist et al. 2019). Glucose homeostasis is finely regulated to meet energy demands during alternatingly acute and chronic periods of scarcity and abundance of food. To this end, glucose is transiently stored as glycogen, mainly in the liver. In periods of energy deficit, glucose is synthesised via gluconeogenesis with precursors including pyruvate, lactate, glycerol, and some amino acids (Jitrapakdee 2012). Hepatic glycogen (HGlyc) mobilization is the major contributor to glycaemic normalization after hypoglycaemic episodes in healthy subjects (Kishore et al. 2006).

The hypothalamus regulates the synthesis and breakdown of HGlyc by ‘slow’ and ‘fast’ mechanisms of action. Briefly, slow actions involve hypothalamic influence upon pancreatic actions by the release of insulin, or lack of thereof. Insulin in the liver triggers the bioconversion of glucose delivered from the small intestine into glycogen (Uyama et al. 2004) by activation of the PI3K/Akt/GSK3/GS pathway (Huang et al. 2018). Alternatively, autonomic actions modulated by the hypothalamus dictate fast glycaemic control, for example via adrenergic activation of adipose tissue and resulting lipolysis independent of serum glucose or insulin/glucagon levels (Izumida et al. 2013). Sympathetic and parasympathetic activation are classically known to control glycaemia (Uyama et al. 2004), and HGlyc levels have been shown to influence hepato-vagal nerve activation, regulating the liver-brain-adipose tissue axis (Izumida et al. 2013; López-Soldado et al. 2017).

It is known that HSHF induces not only hypothalamic inflammation (De Souza et al. 2005; Fuente-Martín et al. 2013; Waise et al. 2015) but also HGlyc accumulation (Lambert et al. 2003; Lozano et al. 2016). It is also well

understood that fasting reduces HGlyc (Izumida et al. 2013). More recently, it was observed that the overnight withdrawal of a HSHF diet decreased inflammation-associated markers in the hypothalamus of Wistar rats (Belegri et al. 2018). The relationship between hypothalamic inflammation and HGlyc after HSHF diet withdrawal is not yet fully understood, nor is the interplay between HGlyc and Akt in hypothalamus and liver in a HSHF dietary intervention model.

The withdrawal of HSHF diets, with its subsequent replacement to diets featuring better nutritional composition, is known in the long-term to ameliorate metabolic disturbances, and overall decrease in total calorie intake has also been documented (Hazarika et al. 2016, 2017). Our group has recently identified adaptations in hepatic and adipose biomarkers of inflammation (Casagrande et al. 2020), and on AMPK phosphorylation in the colon-liver-hypothalamus axis (Casagrande et al. 2021), after HSHF acute withdrawal. Nonetheless, the knowledge around the liver-hypothalamus crosstalk in models of dietary withdrawal (WTD) appears to be very scarce.

Similarly to other obesogenic diets, HSHF diet intake induces adiposity accumulation, although it is also know that not all rodents become obese after feeding exclusively on such diets (Giles et al. 2016). Rodents can be characterised as obesity-prone, based on their body mass gain as compared to counterparts that do not show similar propensity (Pagliassotti et al. 1994). We have recently shown that obese-prone male Wistar rats, identified as featuring higher body mass gain in comparison to those that did not show the same gain under the same experimental conditions, presented higher hypothalamic AMPK phosphorylation compared to non-obesity-prone ones, (Casagrande et al. 2021). As a key factor for the development of obesity, hypothalamic inflammation may also be influenced by propensity to obesity.

The main aim of this study was to investigate in Wistar rats the acute effects of HSHF diet withdrawal preceded by a 30-day HSHF intake on the liver-hypothalamus crosstalk. For that purpose, we evaluated Akt and its phosphorylation (Ser473) in liver and hypothalamus, alongside quantification of HGlyc and circulating glucose and insulin. Additionally, considering the hypothalamus as a homeostatic regulator, we investigated some of its inflammatory biomarkers and their relationship with calorie intake. Lastly, we assessed the effects of obesity-propensity (OP) on the parameters analysed.

2.0 Methods

2.1 Ethics

Ethical approval for this experiment was granted by the Sao Paulo Federal University Ethics Committee on Welfare and Use of Animal Models for Experimental Research (CEUA application 4641210318, certifiable at http://ceua.sirpp.unifesp.br/). Institutional, national, and international guidelines for the care of animals used in this study have been strictly followed throughout. The ARRIVE checklist (Kilkenny et al. 2010) was referred to for the preparation of this manuscript.

2.2 Experimental design

Sixty-day old male Wistar rats (*Rattus norvegicus*) were randomly allocated to a standard rat chow (n=10) (Nuvilab CR1, Quimtia, Colombo, Brazil) or a high-sugar high-fat (HSHF) diet (n=20), employed as described previously (Casagrande et al. 2020). The average body weight for all rats at the beginning of the study was approximately 294 g and there were no significant differences amongst groups. All rats were fed their respective diets *ad libitum* for 30 days.

The HSHF obesogenic diet consisted of ground chow (Nuvilab CR1) enriched with sweetened condensed milk (Nestle), lard (Sadia, Sao Paulo, Brazil), and sucrose (Camil, Sao Paulo, Brazil). A vitamin and mineral supplement (RHOSTER, Araçoiaba da Serra, Brazil) was added to the mixture, and so was soybean oil (Bunge, Gaspar, Brazil) as a source of essential fatty acids, and casein (Labsynth, Diadema, Brazil) as a source of protein, aiming at the AIN-93 nutritional recommendations (Reeves et al. 1993). Energy availability within macronutrients in the control diet was 63% from carbohydrates, 26% from protein, and 11% from fat, while the HSHF diet featured 53% of energy from carbohydrates (out of which 66% was sucrose), 16% from protein, and 31% from lipids (out of which 61% were saturated fatty acids).

On the 30th day of dietary intervention, 10 randomly picked HSHF-fed rats had their diets replaced with the standard chow (withdrawal group, Hw) for 48 h whilst the remaining 10 rats were kept on the HSHF diet. The experimental design therefore consisted of three groups (Ct, Hd, Hw) with 10 rats in each group. Throughout the study all rats were kept in collective cages (40 cm × 35 cm × 15 cm) and received food and drinking water *ad libitum*. The room temperature was set at 22 ±2 ºC in a 12 h /12 h light-dark cycle. Body mass was measured at the beginning and end of the study. The approximate energy intake was determined by the difference between the food offered and consumed by all rats in the cage and divided by the number of rats.

At the end of the experimental protocol, all rats were fasted for 6 hours, anaesthetized and euthanized. One of the Hd rats showed complications during the study and was removed from all subsequent analyses. Liver, hypothalamus, and serum were collected and stored at -80 ºC until analyses. A trained researcher performed the hypothalamus dissection to ensure precision.

2.3 Serum analysis and hepatic glycogen

Serum glucose was assessed colorimetrically using a commercially available kit (#133, Labtest, Lagoa Santa, Brazil). Hepatic glycogen content was quantified based on the micro-method proposed by Balmain et al. (1956) and standardised by our group (Casagrande et al. 2019). Serum insulin was quantified by a chemiluminescent enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (ALPCO, Salem, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated by multiplying the serum glucose and insulin concentrations and dividing by 22.5.

2.4 ELISA and western blotting

Tissue protein content analyses were performed by immunodetection. Dissected tissues were weighted and homogenised in ice-cold lysis buffer for 15 s in 2 mL centrifuge tubes in the approximate proportions of hypothalamus at 30 mg in 600 μL and liver at 150 mg in 1 mL, as standardised previously (Casagrande et al. 2020). Tubes were subsequently centrifuged at 20,000 g for 40 min at 4 °C. Supernatant protein was quantified using Bradford reagent (Bradford 1976). The hypothalamic content of interleukins (IL6, IL10, IL1β) and tumour necrosis factor alpha (TNFα) in the supernatant of each sample was quantified by ELISA following the manufacturer's instructions (R&D Systems, USA). The hypothalamic protein relative content of phosphorylated nuclear factor-kappa B RelA/p65 (pNFκBp65) (1:5,000. #3033 Cell Signaling Technology, Danvers MA, USA, henceforth defined as CST) and myeloid differentiation factor 88 (MyD88) (1:5,000. #4283S, CST) were determined by western blotting (WB). The hypothalamic and hepatic protein relative content of serine 473 phosphorylated Akt (pAkt-Ser473, 1:5,000. #9271S, CST) and total Akt (1:5,000. #9272, CST) was determined by WB. β-actin was the housekeeping protein chosen for hypothalamus (1:10,000. #3700. CST), and heat shock protein 90 (HSP90, 1:10,000. ab13495, Abcam, USA) for liver. Secondary antibodies anti-mouse (1:20,000. #7076, CST) and anti-rabbit (1:20,000. #7074, CST) were employed.

Briefly, 50 μg of protein from each sample were resuspended in Laemmli buffer 5× (1:4), separated by electrophoresis on SDS-PAGE (10%) and transferred to nitrocellulose membranes. The membranes were rinsed and blocked for nonspecific bindings with 1-3% bovine serum albumin for 1 h. After incubations with primary antibody, rinse and secondary antibody, the membranes were exposed to ECL Pierce™ substrate (ThermoFisher Scientific, USA), and the images obtained using the chemiluminescence intensifier scanner UVITEC (Cambridge, UK). Quantification was performed with Scion Image (NIH Image).

2.5 Obesity propensity

Obesity propensity was investigated in this study as previously employed by our group (Casagrande et al. 2021). Briefly, HSHF-fed rats were characterised as obesity-prone (OP) when their body mass gained in the first week of HSHF intake was in the upper tertile of its group (>66th percentile) (Pagliassotti et al. 1994; Sun et al. 2017; Casagrande et al. 2021). The rats in the middle and lower tertile were defined as "not-obesity-prone" (NP).

2.6 Statistical analysis

The sample size was calculated with G*Power software (v3.1.9.4), and JASP v0.14.1 software was used for statistical analyses. Data was assessed for normality and homoscedasticity. Group parameters were tested using one-way analysis of variance (ANOVA) with Tukey's post-hoc or Kruskal-Wallis with Dunn's post-hoc, depending on data distribution. We employed two-way ANOVA with independent effects (obesity-propensity and withdrawal) to investigate the effect of obesity-propensity on the parameters analysed.

Pearson's or Spearman's test were used to investigate correlations, which supported multiple linear regressions and the mediation models. The term 'mediation' in this manuscript refers to the results of the mediation models. Mediation in statistical analysis enables a distinction to be made among effects observed, breaking them down into direct and indirect components, which in turn facilitates the individual analysis of the strength and significance of each predictor/factor on the observed final effect. Additionally, it helps us characterise the effect's pathway. The parameters that carry the effect in the model and stand in between the independent and the dependent variables are called mediators. The mediation analysis, or mediation models, permits the identification of the contribution of the independent variable and of the mediator to the observed effect on the dependent variable.

Statistical significance was set at 5% for all analyses. For effect size, Cohen's *f* was reported for among-group comparisons, while Hedges' *g* was reported for between-group comparisons.

3.0 Results

3.1 Energy intake, body weight, and metabolic parameters

Total calorie intake was higher in the HSHF-fed groups (Hd and Hw) as compared to Ct during the 30 days dietary intervention. Calorie intake in the final 48 h withdrawal period was lower in Hw compared to Hd (Table 1). The percentage of body mass gained (%BMG) was higher in both HSHF-fed groups (Table 1). The hypothalamic absolute and relative masses were lower in Hd compared to Ct, but in Hw it was similar to Ct and higher than Hd (Table 1, Figure 1.A and Supplementary table 1), whilst the exact opposite effect was observed in the liver absolute and relative masses (Table 1, Figure 1.B and Supplementary table 1). Serum glucose and HGlyc were elevated in Hd compared to Ct, reduced in Hw compared to Hd, and similar between Hw and Ct (Figure 1.C, 1.D and Supplementary table 1).

Insulin and HOMA-IR index were elevated in Hd as compared to Ct, but similar between Hw and Ct (Figure 1.E, 1.F and Supplementary table 1).

3.2 Hypothalamic and hepatic Akt

In the hypothalamus, pAkt was similar between Hd and Hw, and both were elevated in comparison to Ct. Akt was higher in Hd compared to Ct. However, the Akt phosphorylation ratio (pAkt/Akt) was reduced in both Hd and Hw (Figure 2.A and Supplementary table 1) as compared to Ct.

In the liver, HSHF intake had no significant effect on pAkt, Akt, or pAkt/Akt as compared to Ct. However, Hw showed reduced pAkt compared to Hd, as well as reduced pAkt/Akt ratio compared to Ct (Figure 2.B, Table 1, Supplementary table 1). Hepatic and hypothalamic pAkt/Akt were positively correlated with each other (r=0.614, p=0.015).

3.3 Hypothalamic inflammation markers

Hypothalamic MyD88 was higher in Hd compared to Ct, whilst in Hw it was around halfway between Hd and Ct (Figure 2.A and Supplementary table 1). pNFκBp65 did not show significant changes amongst the three groups. Hypothalamic IL10 and IL1β were higher in Hd compared to Ct and Hw, but similar between Hw and Ct. Whilst no differences were found in TNFα levels amongst groups, IL6 was higher in Hw compared to Ct (Figure 2.C and Supplementary table 1).

Positive correlations between hypothalamic IL6, IL10 and TNFα with hypothalamic mass were identified in Hd, but not in Hw (Table 2). MyD88 was positively correlated with IL10 (r=0.568, p=0.014), IL1β (r=0.649, p=0.006) and TNFα (r=0.513, p=0.042) and partially mediated the effect of HSHF upon IL1β increase (Figure 3.A and Supplementary table 2). IL6 was a positive independent predictor of hypothalamic pAkt (β=0.742, p=0.025, 95% CI 0.117 to 1.367). Conversely, MyD88 (r=-0.709, p=0.007) and IL1β (r=-0.533, p=0.041) were negatively correlated with pAkt/Akt ratio.

3.4 Energy intake, hypothalamus, and liver

Total energy intake

Calorie intake was positively correlated with liver relative mass (r=0.929, p<0.001) and negatively correlated with hypothalamic relative mass (r=-0.762, p=0.017). The hypothalamic parameters MyD88 (r=0.740. p=0.023), TNFα (r=0.740. p=0.036), pAkt (r=0.728, p=0.026) and total Akt (r=0.875, p=0.002) were all positively correlated with

calorie intake. Hepatic total Akt was positively correlated with calorie intake (r=0.785, p=0.012), whilst the pAkt/Akt ratio was negatively correlated (r=-0.762, p=0.017).

Final 48 h energy intake

The HSHF-diet withdrawal induced a range of metabolic manifestations within a relatively short period, which led us to investigate correlations around energy intake during the final 48 hours of the experiment. Calorie intake in the final 48 h was negatively correlated with hypothalamic absolute (r=-0.801, p=0.009) and relative masses (r=-0.733, p=0.025). We also found positive correlations between calorie intake in the final 48 h and HGlyc (r=0.908, p=0.002), hepatic pAkt (r=0.759, p=0.018), as well as hypothalamic IL10 (r=0.762, p=0.028) and IL1β (r=0.789, p=0.020).

3.5 Hepatic glycogen

HGlyc, hypothalamic and hepatic masses

Hypothalamic and hepatic absolute (r=-0.544, p=0.005) and relative masses (r=-0.617, p=0.001) were negatively correlated with each other. Hypothalamic mass was negatively correlated with %BMG (r=-0.592, p<0.001), serum glucose (r=-0.432. p=0.028), HGlyc (r=-0.592 p=0.004), and hypothalamic IL1β (r=-0.456. p=0.029). In the opposite direction, liver mass was positively correlated with serum glucose (r=0.428, p=0.033), HGlyc (r=0.727, p<0.001), and hypothalamic IL10 (r=0.462, p=0.020). HGlyc was a negative predictor of hypothalamic mass (β=-0.399, p=0.016, 95% CI -0.710 to -0.089), depending on HSHF intake (β=-3.665, p=0.049, 95% CI -7.311 to -0.019). Additionally, HGlyc mediated the positive effect of HSHF intake and the negative effect of its withdrawal on hepatic mass (Figure 4.A and Supplementary table 2).

HGlyc, glycemia, and hypothalamic parameters

HGlyc was predicted by hyperglycaemia (β=0.153, p=0.015, 95% CI 0.033 to 0.273) and mediated its positive effect on IL1β (Figure 4.B and Supplementary table 2). Additionally, HGlyc mediated the positive effect of HSHF intake and the negative effect of its withdrawal upon IL1β (Figure 3.B and Supplementary table 2). Despite being positively correlated (r=0.609, p=0.12), HGlyc and MyD88 did not mediate each other's effect on IL1β (HGlyc on MyD88 effect, β=0.008, p=0.873; MyD88 on HGlyc effect, β=0.227, p=0.215), suggesting independent effects.

HGlyc mediated the negative effect of HSHF intake on hypothalamic pAkt/Akt (Figure 4.C and Supplementary table 2). We further analysed the significant effect of HGlyc on pAkt/Akt following HSHF withdrawal (Supplementary table 2). Separately, HGlyc presented a significant effect on hypothalamic pAkt/Akt controlling for

withdrawal. However, it was opposite to the significant direct effect of withdrawal on hypothalamic pAkt/Akt, resulting in a non-significant total effect of withdrawal on the pAkt/Akt ratio in the hypothalamus (Figure 4.D and Supplementary table 2).

Hepatic glycogen and serum insulin

HGlyc exerts roles in glucose homeostasis that are insulin-dependent, but insulin-independent actions have been identified. Here we attempted to examine whether the effects of HGlyc described above could be attributed to insulin.

As described in the previous section, HGlyc influenced hypothalamic IL1β and pAkt/Akt ratio, as well as hepatic mass. Insulin was not a predictor of hypothalamic IL1β (β=-0.181, p=0.516, 95% CI -0.781 to 0.419), hypothalamic pAkt/Akt ratio (β=-0.340, p=0.186, 95% CI -0.873 to 0.194), nor hepatic mass (β=0.189, p=0.487, 95% CI -0.383 to 0.761). Therefore, insulin could not have mediated the effects of HSHF or its withdrawal upon those variables, since predicting the dependant variable is a requirement for performing mediation analyses.

Interestingly, insulin was not correlated with serum glucose (r=0.29, p=0.266) nor HGlyc (r=0.39, p=0.125), not even within groups (Glucose, Ct r=-0.04, p=0.951; Hd r=-0.06, p=0.914; Hw r=-0.13, p=0.806; HGlyc, Ct r=0.57, p=0.238; Hd r=-0.16, p=0.754; Hw r=0.134, p=0.830). Additionally, the HOMA-IR index was not correlated with HGlyc (r=0.44, p=0.065), not even within groups (Ct r=0.678, p=0.139; Hd r=-0.37, p=0.470; Hw r=0.234, p=0.656). Similar to insulin, the HOMA-IR index was not a predictor of hypothalamic IL1β (β=-0.203, p=0.447, 95% CI -0.769 to 0.363), hypothalamic pAkt/Akt ratio (β=-0.334, p=0.239, 95% CI -0.929 to 0.261), nor hepatic mass (β=0.141, p=0.597, 95% CI -0.417 to 0.698). Hence, it could not have mediated the effect of HSHF intake nor its withdrawal.

3.6 Obesity propensity

OP rats presented higher overall body mass gain (F=12.93, p=0.003, f=0.91), but obesity propensity exerted no influence on hypothalamic inflammatory cytokines (IL6, F=0.49, p=0.502; IL10, F=0.15, p=0.627; IL1β, F=2.15, p=0.168; TNFα, F=0.004, p=0.950), nor on glucose homeostasis parameters (glycaemia, F=0.64, p=0.436; serum insulin, F=2.01, p=0.181; HOMA-IR index, F=1.19, p=0.301; hepatic glycogen, F=0.02, p=0.902).

4.0 Discussion

In the present study, male Wistar rats received either a control chow or a HSHF diet, which is known to induce metabolic disarrangements leading to obesity and metabolic syndrome. In the final 48 hours of dietary intervention, half

of the HSHF rats had their diet switched to the control chow, which in comparable terms features a better nutritional composition and lower energy density. The HSHF rats showed significantly increased %BMG. We have reported previously (Casagrande et al. 2020) that HSHF rats showed higher body mass and adiposity, hepatic and adipose tissue inflammation, and disturbances in serum and hepatic lipids. The results of the present investigation add to the body of evidence that the restoration of an euglycemic state in obesity is reliant upon the period of exposure to high energy diets, but maybe more importantly, the period of withdrawal from it (Casagrande and Estadella 2020).

The intake of high-sucrose (Lambert et al. 2003) and high-fat (Lozano et al. 2016) diets increase HGlyc in the short-term, while sucrose intake was shown to prevent HGlyc decline induced by chronic stress (Corona-Pérez et al. 2017). It has also been observed that high-fat feeding and high-sugar/high-fat feeding in the long-term decrease HGlyc, a manifestation observed in type 2 diabetes (Irimia et al. 2017; Tu et al. 2019; Casagrande et al. 2019; Liu et al. 2020). Therefore, the HGlyc overload observed in short duration protocols evolves to a state of HGlyc depletion in the long-term. Thus, a study protocol that withdraws HSHF intake after a longer exposure is likely to produce a distinct response. The literature on diet withdrawal and HGlyc, and how both relate to glucose and energy homeostasis, appears to be scarce.

The literature so far available confirms changes in hepatic mass induced by an obesogenic diet followed by its withdrawal (Lalanza et al. 2014). However, the status of hypothalamic mass after exposure to HSHF diet and its subsequent withdrawal is a topic yet to be fully described. Changes in hypothalamic mass could be attributed to increased neuronal apoptosis, once higher microglial activation in the hypothalamus was seen after 24 h on a high-fat diet (Waise et al. 2015), an observation that is consistent with neurodegeneration in the long term (Marinho et al. 2018).

In the present study, we identified group-dependent correlations between inflammatory parameters with hypothalamic mass. In the Hd group, IL6, IL1β, and TNFα were all negatively correlated with hypothalamic mass. Additionally, hypothalamic inflammation is a key factor in obesity and a major component of either insulin or leptin resistance, leading to unbalanced energy homeostasis (Tran et al. 2016; Cesar and Pisani 2017; Samodien et al. 2019). In our study, hypothalamic mass was negatively correlated with %BMG, energy intake in the final 48 h, and total energy intake. Moreover, energy intake in the final 48 h was positively correlated with hypothalamic IL10 and IL1β, while the total energy intake was positively correlated with MyD88 and TNFα. The overall associations found amongst hypothalamic inflammation, tissue mass, food intake, and %BMG suggest an exacerbated inflammatory state that likely leads to poor homeostatic control, as identified previously (De Souza et al. 2005; Fuente-Martín et al. 2013; Waise et al. 2015). The elevated levels of hypothalamic IL10 and IL1β in the Hd group – alongside higher levels of IL1β – could be associated with its regulatory role upon inflammation (Moore et al. 2001), further confirming its functional capacity of

the hypothalamus to respond to biochemical insults. The ratio IL1β/IL10 was not different amongst groups (p=0.953, data not shown).

Belegri and colleagues (2018) reported that HSHF-diet overnight withdrawal prevented higher inflammation in the hypothalamus (Belegri et al. 2018). In our study, HSHF withdrawal significantly reduced IL10 and IL1β, returning their levels down to those found in the Ct group (Figure 2.C). Although IL6 was higher in Hw compared to Ct, it was not higher in Hw compared to Hd. Not only a period of 48 h may be insufficient to allow for changes in hypothalamic IL6 levels between Hw and Hd, the roles of hypothalamic IL6 are yet to be fully elucidated, as it appears to feature interchangeable roles as a pro- and anti-inflammatory interleukin, depending on the local and systemic biochemical environment (Carey et al. 2006; Ropelle et al. 2010).

Moreover, by engaging the same pathways as leptin, IL6 may exert a beneficial effect on the hypothalamus by contributing to glucose tolerance and suppressing feeding in baseline conditions and in an obese and leptin-resistant state (Timper et al. 2017). Accordingly, we found decreased levels of MyD88 (Figure 2.A), IL10 and IL1β (Figure 2.C) in Hw when compared to Hd. We found no relationship between IL6 and any of the other inflammatory markers investigated. Instead, IL6 was a positive independent predictor of hypothalamic pAkt, suggesting that its increase in HSHF is more related to its PI3K/Akt-dependent pro-trophic activity (Leibinger et al. 2013).

In our study, HGlyc mediated the effect of both HSHF intake and its withdrawal on hepatic mass. In fed states, approximately one-tenth of the hepatic mass consists of glycogen (Stone et al. 2020), and experimental models of glycogenolysis have demonstrated a positive relationship between glycogen content and hepatic mass (Roseman et al. 2018). Although lipids are also major contributors to this parameter, our group has previously found no evidence of lipid accumulation or hepatic triacylglycerol abnormalities for this model (Casagrande et al. 2020). Our results suggest that a continued surplus of serum glucose facilitated by HSHF intake could be responsible for HGlyc accumulation with consequent alteration in hepatic and hypothalamic masses. Hyperglycaemia is well known for its role in increased accumulation of hepatic glycogen (Kruszynska et al. 1986).

HGlyc in our study was found to mediate the effects of HSHF and its withdrawal upon IL1β and pAkt/Akt ratio in the hypothalamus, linking HGlyc to both trophic and inflammatory factors. The HGlyc increase mediated the effect of HSHF intake over pAkt/Akt decrease, while its decrease appeared to have prevented a further drop in pAkt/Akt ratio in the hypothalamus. The dual role of HGlyc – firstly upon HSHF exposure then its subsequent withdrawal – may suggest either a two-way route or a floor effect upon hypothalamic Akt phosphorylation.

Hypothalamic control of HGlyc content has been hypothesized as one of the central mechanisms of homeostatic control (Shimazu and Fukuda 1965). More recently, HGlyc levels were found to stimulate vagal afferent

fibres, contributing to hypothalamic modulation of appetite and glucose homeostasis (López-Soldado et al. 2017). In our study, a positive correlation between calorie intake in the final 48 h and HGlyc was found. Increased glycaemia, HGlyc and hypothalamic Akt observed in Hd, as compared to Ct, was followed with restorations of such parameters in Hw. Hence, HGlyc accumulation, which appears to result from a hyperglycaemic state, could have altered hypothalamic Akt phosphorylation. Reduced pAkt/Akt ratio in an indicator of reduced protein synthesis (Zhao et al. 2017) and has been associated with lowered neuronal migration and differentiation (Fornasari et al. 2016). Considering the literature presently available, our results may explain the influence of HGlyc in predicting hypothalamic mass.

In an experimental study of melatonin intracerebroventricular administration, increased hypothalamic Akt phosphorylation was associated with decreased hepatic gluconeogenesis (Faria et al. 2013). A review study further discussed hypothalamic PI3K/Akt activation in the suppression of hepatic gluconeogenesis and food intake in metabolically normal conditions (Dridi and Taouis 2009; Huang et al. 2018). In our study, the hypothalamic pAkt/Akt ratio was reduced in both Hd and Hw as compared to Ct, but at the same time, glycaemia was higher in Hd, but not in Hw, as compared to Ct. We believe that hyperglycaemia in Hd could be associated with a loss of hypothalamic control and that HGlyc might have played a role in this process. A possible explanation is that, in a hyperglycaemic state, HGlyc accumulation in Hd leads to decreased pAkt/Akt ratio, whilst in Hw, with restoration of a normoglycemic state, HGlyc reduction prevented further pAkt/Akt ratio decrease.

Previous studies have shown that hepatic gluconeogenesis and glycogenolysis are modulated by insulin signalling both in the liver and hypothalamus, and also that hypothalamic insulin injection increases local Akt phosphorylation (Sandoval et al. 2009; Jitrapakdee 2012). However, despite the well documented hypothalamic-hepatic glucose homeostasis crosstalk, an integrated communication system between HGlyc and hypothalamic Akt phosphorylation has not yet been clearly identified. Interestingly, in our study insulin did not appear to exert significant modulatory effects upon our findings, but on the contrary, we found that insulin did not significantly mediate the effects of HSHF and its withdrawal upon glucose, HGlyc, and hypothalamic Akt phosphorylation. We hypothesise that the effects of HGlyc upon hypothalamic pAkt/Akt ratio were triggered possibly by pathways not investigated in the present study, for example via the hepatic branch of the vagus nerve.

In the present study we categorised rats as either obese prone (OP) or not-obese prone (NP), once the HSHF effects may be more impactful in OP rats. OP could be associated with hypothalamic AMPK phosphorylation, as discussed by our group in further details previously (Casagrande et al. 2021).Although increased body adiposity is associated with hypothalamic inflammation, insulin resistance and hyperglycaemia, we could not find in the present study evidence that obesity propensity affects those parameters in a 30-day obesogenic diet intake protocol. However, it

is likely that longer feeding protocols may trigger a different set of results, considering that more time under an obesogenic stimulus will lead to a more exacerbated metabolic damage, hypothalamic inflammation, impaired insulin signalling and glucose homeostasis (Darbre 2017; Sweeney et al. 2017; Konstantynowicz-Nowicka et al. 2019; Seong et al. 2019).

In summary, the present study showed that exposure to a 30-day HSHF diet induced damage to the hypothalamic glucose homeostatic control in Wistar rats, but also that this damage appeared to be at least in part reversible, as evidenced by the findings in rats whose HSHF diet were replaced with a control diet for the final 48 h. Hypothalamic Akt, MyD88, IL10 and IL1β were increased in HSHF but decreased after its acute withdrawal. Peripherally, HSHF induced hyperglycaemia, elevated HGlyc levels and increased hepatic mass, which were all reverted to control levels after withdrawal. Previous studies agree with our suggestion that very short-term dietary improvements are substantial enough to initiate a cascade of inflammation attenuation. A new point of view gathered from the present study however is that HGlyc may play a role that is more significant than previously thought upon hypothalamic control of glucose homeostasis. Our findings contribute to further elucidation of the liver-hypothalamus axis and its role in regulating glucose metabolism, but the question as to how the autonomic nervous system exactly controls it remains unanswered.

Statements and Declarations

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Conflict of interest

The authors have no conflict of interest to declare.

Data availability

Following publication, the data will be available at DOI: 10.17632/dcy22r53ns

Authors' contributions

BPC: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, visualization, writing – original draft, writing – review & editing; AAB: Visualization, writing – review & editing; LPP: Conceptualization, funding acquisition, methodology, resources, visualization, writing – review & editing.; DE: Conceptualization, funding acquisition, investigation, methodology, resources, visualization, writing – original draft, writing – review & editing.

Ethics approval

The present study was approved by the institutional ethics committee.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Fig 1 Graphical representation (median, min to max) of (A) hypothalamic relative mass; (B) hepatic relative mass; (C) serum glucose; (D) hepatic glycogen (HGlyc); (E) Serum insulin; (F) Homeostasis model assessment of insulin resistance (HOMA-IR) index. All data was analysed with one-way ANOVA with Tukey's posthoc. Sample size: (A, B) 9-10, (C, D) 7-10, (E, F) 6-7 animals per group. Ct, control group; Hd, HSHF-fed group; Hw, HSHF-fed group + 48h withdrawal. * $p<0.05$ compared to the Ct group; & $p<0.05$ compared to the Hd group.

Fig 2 Graphical representation (median, min to max) of protein content determined by Western Blotting in the (A) hypothalamus (the dashed line separates the graph into two, the right y axis represents the values on the right side of the dashed line, the left y axis represents the values on the left side of the dashed line); and (B) liver; (C) protein levels of hypothalamic cytokines determined by ELISA; values were standardized by the z score to allow for better visual graphics. Hypothalamic MyD88 and hepatic pAkt were analysed by Kruskal-Wallis with Dunn's posthoc, the remaining data was analysed with one-way ANOVA with Tukey's posthoc. The sample size was 5-8 (A-B) and 6-10 (C) animals per group. Ct, control group; Hd, HSHF-fed group; Hw, HSHF-fed group + 48h withdrawal. * $p<0.05$ compared to the Ct group; &$p<0.05$ compared to the Hd group.

Fig 3 Graphical representation of mediation models for HSHF intake and WTD effects on IL1β. (A) Model for MyD88 mediation of HSHF intake and WTD effects on hypothalamic IL1β; (B) Model for hepatic glycogen (HGlyc) mediation of HSHF intake and WTD effects on hypothalamic IL1β. (a) Effect of the predictor (X) on the mediator (M); (b) effect of the mediator (M) on the outcome (Y) controlling for the predictor (X); (c') direct effect of the predictor (X) on the outcome (Y); (continuous arrow) significant effect; (dashed arrow) non-significant effect.

Fig 4 Graphical representation of mediation models for hepatic glycogen. (A) Model for hepatic glycogen (HGlyc) mediation of HSHF intake and WTD effects on hepatic mass; (B) Model for hepatic glycogen (HGlyc) mediation of serum glucose effect on hypothalamic IL1β; (C) Model for hepatic glycogen (HGlyc) mediation of HSHF intake and WTD effects on hypothalamic pAkt/Akt; (D) Model for hepatic glycogen (HGlyc) interference on isolated WTD effect on hypothalamic pAkt/Akt. (a) Effect of the predictor (X) on the mediator (M); (b) effect of the mediator (M) on the outcome (Y) controlling for the predictor (X); (c') direct effect of the predictor (X) on the outcome (Y); (continuous arrow) significant effect; (dashed arrow) non-significant effect.

Supplementary table 1. Statistical significance and effect size for table 1 and figures 1 and 2.

Supplementary table 2. Effect size (standardized β), z value, and significance of the mediation models' coefficients.

Table 1. Calorie intake, body and tissue parameters

	Ct (mean ±SEM (n))	**Hd (mean ±SEM (n))**	**Hw (mean ±SEM (n))**
Calorie intake (Kcal)	1787 ±34.89 (5)	**2437 ±92.06 (5) ***	**2404 ±51.36 (5) ***
Last-48h intake (Kcal)	63.33 ±0.44 (5)	**75.91 ±1.51 (5) ***	**63.41 ±1.59 (5) &**
Body mass gain (%)	33.96 ±1.76 (10)	**48.53 ±2.25 (9) ***	**44.71 ±2.28 (10) ***
Hypothalamus (mg)	34.84 ±1.31 (9)	**26.04 ±1.09 (9) ***	**32.77 ±1.18 (10) &**
Liver (g)	13.66 ±0.45 (10)	**15.64 ±0.36 (7) ***	**13.53 ±0.55 (10) &**

(Ct) control group; (Hd) HSHF-fed group; (Hw) HSHF-fed group + 48 h withdrawal; (SEM) standard error of the mean; (n) number of animals per group; * $p<0.05$ compared to Ct group; & $p<0.05$ compared to the Hd group.

Table 2. Correlations between hypothalamic mass and cytokines

	Ct group			Hd group			Hw group		
	r	r^2	p value	r	r^2	p value	r	r^2	p value
IL6	0.409	0.167	0.421	-0.844	0.712	**0.035**	-0.306	0.094	0.556
IL10	-0.043	0.002	0.913	0.370	0.137	0.414	0.209	0.044	0.561
IL1β	0.489	0.239	0.218	-0.796	0.634	**0.032**	0.600	0.360	0.116
TNFα	0.853	0.728	**0.010**	-0.817	0.667	**0.047**	0.157	0.025	0.710

(Ct) control group; (Hd) HSHF-fed group; (Hw) HSHF-fed group + 48h withdrawal; (IL6) interleukin 6; (IL10) interleukin 10; (IL1β) interleukin 1 beta: (TNFα) tumour necrosis factor alpha.