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Effects of the combination of intermittent fasting and physical exercise on autophagic and leptin pathway in the hypothalamus of obese mice

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Oliveira, Luciana da Costa

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DEDICATION

I am grateful to God, who gave me the gift of life and for had the opportunity to do my doctor's degree in this great university, the University of São Paulo, and I dedicate this work to Him. I thank my family for all the love and support they gave through the way until here. I thank my husband, Gustavo, to be with me and give me support in all I needed. I also thank professor Adelino for the orientations, advice and help me to grow in my knowledge and my academic life, and professor Rodrigo, to listen me and also support me in this journey. Thanks to my friends from the laboratory, who helped me every time I need. I am fortunate to be surrounded by such good people. I thank to São Paulo Research Foundation (processes: 2017/25492-4) for the financial support.

RESUMO

OLIVEIRA, LC. **Efeitos da combinação do jejum intermitente com o exercício físico sobre a via autofágica e via da leptina no hipotálamo de camundongos obesos**. 2022. Tese (doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2022.

A obesidade induzida pela dieta é associada a inflamação de baixo grau persistente no hipotálamo a qual promove o desenvolvimento da resistência central a leptina. Esse processo inflamatório envolve a desregulação da via autofágica hipotalâmica comprometendo a dinâmica da ativação/inibição dos neurônios orexígenos AgRP (associados a sensação de fome) e anorexígenos POMC (associados a sensação de saciedade), desregulando a homesostase energética e predispondo ao ganho de peso. Estudos apontam que tanto o jejum intermitente (JI) quanto o exercício físico (EF) são capazes de modular proteínas inflamatórias hipotalâmicas e atuar na ativação/inibição de neuropeptídios; nesse sentido, é possível hipotetizar que a combinação dos protocolos (JI e EF) traria efeitos mais expressivos no reestabelecimento da sensibilidade hipotalâmica a leptina. Objetivo: investigar o efeito da associação do JI com o EF combinado nas vias inflamatória, da autofagia e da leptina no hipotálamo de camundongos alimentados com dieta hiperlipídica. Metodologia: os camundongos foram divididos em 5 grupos: sendo dois controles: dieta padrão (DP) e dieta hiperlipídica (DH); e 3 grupos de intervenção: dieta hiperlipídica-treinados (DH-T), dieta hiperlipídica-jejum intermitente (DH-JI), e dieta hiperlipídica-jejum intermitente e treinados (DH-JIT). Os métodos utilizados foram: imuno-histoquímica, Immunoblotting, RTqPCR, ELISA e ITT, além das análises da massa corporal e ingestão alimentar. Principais resultados: comparado ao grupo DH, apenas os grupos que realizaram o JI apresentaram redução nas proteínas inflamatórias hipotalâmicas avaliadas e aumento na expressão de RNAm de POMC. Após estimulação intraperitoneal com leptina o grupo DH-JIT apresentou aumento da pSTAT3 no ARQ e VMH em comparação com os grupos DH, DH-T e DH-JI. As três intervenções apresentaram menor ingestão calórica e massa corporal quando comparadas ao grupo DH, sugerindo melhora no comportamento hiperfágico e resistência ao ganho de peso induzido pela dieta. Conclusão: nossos dados indicam que os efeitos benéficos da combinação do JI e EF na homeostase energética pode estar associada a melhora da sensibilidade à leptina no ARC e VMH, provavelmente se deve parcialmente à redução da SOCS3 hipotalâmica e melhora na resposta autofágica.

Palavras-chave: exercício físico; jejum intermitente; hipotálamo; obesidade.

ABSTRACT

OLIVEIRA, LC. **Effects of the combination of intermittent fasting and physical exercise on autophagic and leptin pathway in the hypothalamus of obese mice**. 2022. Thesis (doctor) – Ribeirão Preto Medical School of Ribeirão Preto, University of São Paulo, Brazil, 2022.

Diet-induced obesity is associated with persistent low-grade inflammation in the hypothalamus which promotes the development of central leptin resistance. This inflammatory process involves dysregulation of the hypothalamic autophagic pathway, compromising the dynamics of activation/inhibition of the orexigenic AgRP neurons (associated with the sensation of hunger) and the anorectic POMC (associated with the sensation of satiety), deregulating energy homeostasis and predisposing to weight gain. Studies point out that fasting (IF) and physical exercise (E) are capable of modulating hypothalamic inflammatory proteins and activate/inhibit neuropeptides; in this sense, we hypothesized that there is possible that the combination of the protocols (IF and execise) would bring more expressive effects in the reestablishment of hypothalamic sensitivity to leptin. Objective: to investigate the effect of the association of IF with combined E on inflammatory, autophagy and leptin pathways in the hypothalamus of high-fat diet fed mice. Methodology: mice were divided into 5 groups: two controls: standard diet (C) and high fat diet (HF); and 3 intervention groups: high fat diet-trained (HF-T), high fat diet-intermittent fasting (HF-IF), and high fat diet-intermittent fasting and trained (HF-IFT). The methods used were: immunohistochemistry, Immunoblotting, RT-PCR, ELISA and ITT, in addition to the analysis of body mass and food intake. Mains Results: compared to the HF group, only the groups that performed the IF showed a reduction in the evaluated hypothalamic inflammatory proteins and an increase in POMC mRNA expression. After intraperitoneal stimulation with leptin, the HF-IFT group showed an increase in pSTAT3 in ARQ and VMH compared to the HF, HF-T and HF-IF groups. The three interventions showed lower caloric intake and body mass compared to the HF group, suggesting a correction in hyperphagic behavior and resistance to diet-induced weight gain. Conclusion: these data indicate that the beneficial effects of the combination of IF and physical exercise on energy homeostasis may be associated with improved leptin sensitivity in ARC and VMH, probably due, at least in part, to the reduction of hypothalamic SOCS3 and improved autophagic response.

Keywords: physical exercise; intermittent fasting; hypothalamus; obesity.

SUMÁRIO

1. INTRODUCTION

The neuronal circuits controlling food intake and the hormonal mechanisms involved in this complex modulation network have been widely investigated to clarify the factors associated with the regulation of energy homeostasis. The hypothalamus is considered the central point of this regulatory system. Therefore, impairment of the hypothalamic response, generated by signaling disruption in crucial signaling molecules has been associated with the development of morbid obesity, highlighting the importance of controlling the hypothalamic function for health [1; 2; 3].

It is known that obesity is associated with the chronic low-grade inflammatory process, not only peripheral but also central, highlighted by increased expression of several inflammatory proteins related to impairments in the hypothalamic signaling of leptin and insulin, such as the suppressor of insulin signaling cytokine 3 (SOCS3) [4; 5; 6]. The high levels of leptin and insulin in the cerebrospinal fluid of obese individuals indicate a chronic state of resistance to the actions of these hormones in the CNS [7; 8].

The autophagic pathway also plays a important role in the development of obesity, since it is associated to the control of the activation of the agouti-related peptide (AgRP), an orexigenic neuron, and the pro-opium melanocortin (POMC), an anorectic neuron [9; 10; 11] Summarazing, the dysregulation of hypothalamic inflammatory and autophagic pathway compromises the feeling of satiety, thus leading to hyperphagia and weight gains.

Non-pharmacological strategies such as intermittent fasting/IF [12; 13] and physical exercise [13; 14] can improve peripheral sensitivity to insulin and leptin. Recently, Cho and coworkers [12] showed the efficiency of IF protocols in reducing serum insulin and leptin levels, as well as adiposity in adults. Although the data related to the effects of IF on the central inflammatory response are still contradictory, it was reported that IF protocol can modulate the expression of SOCS3 in the hypothalamus [14; 15] and also reduce TLR4 mRNA in the hippocampus of rats treated with lipopolysaccharide [16], suggesting that IF can be used to alleviate hypothalamic inflammation in obese animals.

Regarding physical exercise, a recent review demonstrates that a 2-week protocol can decrease body fat mass associated with lower levels of circulating leptin [17]. In addition, the endurance exercise is also able to reduce hypothalamic TLR4 total content and increase phosphorylation of the JAK/STAT3 pathway, inducing higher POMC mRNA expression compared

to sedentary obese mice [18]. However, to the best of our knowledge, there are no data examining the effects of the combination of IF and physical exercise on the impairment of the hypothalamic molecular pathways caused by the chronic ingestion of a high-fat diet. Therefore, we investigated whether the combination of IF and physical exercise would be more effective in alleviating hypothalamic dysfunctions in high-fat diet mice in comparison with these interventions applied alone.

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2. MANUSCRIPT I:

Original Article: Using Intermittent Fasting as a non-pharmacological Strategy to Alleviate Obesity-Induced Hypothalamic Molecular Pathway Disruption.

Using intermittent fasting as a non-pharmacological strategy to alleviate obesity-induced hypothalamic molecular pathway disruption

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ABSTRACT

Intermittent fasting (IF) is a popular intervention used to fight overweight/obesity. This condition is accompanied by hypothalamic inflammation, limiting the proper signaling of molecular pathways, with consequent dysregulation of food intake and energy homeostasis. This mini-review explored the therapeutic modulation potential of IF regarding the disruption of these molecular pathways. IF seems to modulate inflammatory pathways in the brain, which may also be correlated with the brain-microbiota axis, improving hypothalamic signaling of leptin and insulin, and inducing the autophagic pathway in hypothalamic neurons, contributing to weight loss in obesity. Evidence also suggests that when an IF protocol is performed without respecting the circadian cycle, it can lead to dysregulation in the expression of circadian cycle regulatory genes, with potential health damage. In conclusion, IF may have the potential to be an adjuvant treatment to improve the reestablishment of hypothalamic responses in obesity.

INTRODUCTION

The neuronal circuits controlling food intake and the endocrine mechanisms involved in this complex modulation network have been widely investigated to clarify the factors associated with the regulation of energy homeostasis. The hypothalamus is considered the central point of this regulatory system. Therefore, impairment of the hypothalamic response, generated by signaling disruption in crucial signaling molecules has been associated with the development of morbid obesity, highlighting the importance of controlling the hypothalamic function for health [1-3].

Different central nervous system regions mediate the regulation of food intake, body weight, and energy homeostasis. In this context, the mid-basal portion of the hypothalamus, where the arcuate nucleus is located, is composed of different subpopulations, including the orexigenic neurons, which are directly involved in the hunger stimulus, and also anorectic neurons, which are mainly involved in the response to satiety signals [4]. The agouti-related peptide (AgRP) orexigenic neuron and the pro-opium melanocortin anorectic neuron (POMC) are two essential components of energy expenditure, hunger, and satiety control neurocircuits, integrating central and peripheral energy status with metabolic signals [5]. It is essential to highlight that the hypothalamus contains other neuronal groups involved in controlling food intake and energy expenditure [6], which are not the focus of this review.

AgRP orexigenic neurons co-express the messenger ribonucleic acid (RNA) for the neuropeptide Y (NPY) and the neurotransmitter gamma-aminobutyric acid (GABA). Studies reveal that the intracerebroventricular administration of AgRP [7] or its overexpression is associated with increased food intake [8]. In contrast, POMC anorectic neurons are co-located with those expressing the cocaine-and amphetamine-regulated transcript (CART) [2, 9]. After its synthesis, POMC is cleaved by different enzymes, generating several peptides responsible for the POMC functions [10]. Neurons expressing endogenous melanocortin ligands for POMC and AgRP neuropeptides (antagonists) and neurons containing melanocortin receptors compose the central melanocortin system [8, 11]. This system is strictly involved in the control of food intake, glucose metabolism, and energy homeostasis [12, 13], in conjunction with anorectic hormones, primarily leptin and insulin, composing a complex neuroendocrine system to maintain the correct energy and body weight balance, as recently described by Yang *et al*. [14].

Both POMC and AgRP neurons have the leptin receptor (LepR). When leptin binds to POMC neuronal cell receptors, neuronal depolarization and activation initiate multiple signal

translations related to satiety responses. The signaling mediated by leptin is transduced into the nucleus, producing the anorexic POMC and CART neurotransmitters. [15, 16]. In addition, a cross-inhibitory reaction between AGRP and POMC neurons induces a reduction in orexigenic neurotransmitters in the AGRP neurons.

Insulin is also a crucial hormone for maintaining energy homeostasis by inhibiting pathways associated with NPY/AgRP neurons and their ramifications [16, 17]. Therefore, impairments in the central signaling pathways of insulin [18-20] and leptin [21, 22] are associated with energy imbalance and obesity development. In this context, intermittent fasting (IF) is a protocol popularly used as a strategy to promote weight loss [23] and has become a tremendous scientific topic of interest to elucidate the mechanisms that regulate the hypothalamic molecular responses that will reduce body weight and prevent obesity [24, 25].

Previous investigations in human and animal models analyzed the effects of IF on leptin and insulin sensitivity [26, 27], inflammatory pathways [28, 29], the brain-microbiota axis [30, 31], circadian cycle [32, 33], and autophagic pathway [34]. All these factors seem to be related to adaptations in POMC and AgRP neuropeptides [35] that can improve energy homeostasis through pathways that are not yet fully understood. The present review explored the molecular and physiological adaptations of leptin, insulin, POMC, and AgRP neuropeptides to IF protocols, mostly performed in animal obesity models.

Leptin and insulin in energy homeostasis: molecular pathways linked to POMC and AgRP responses.

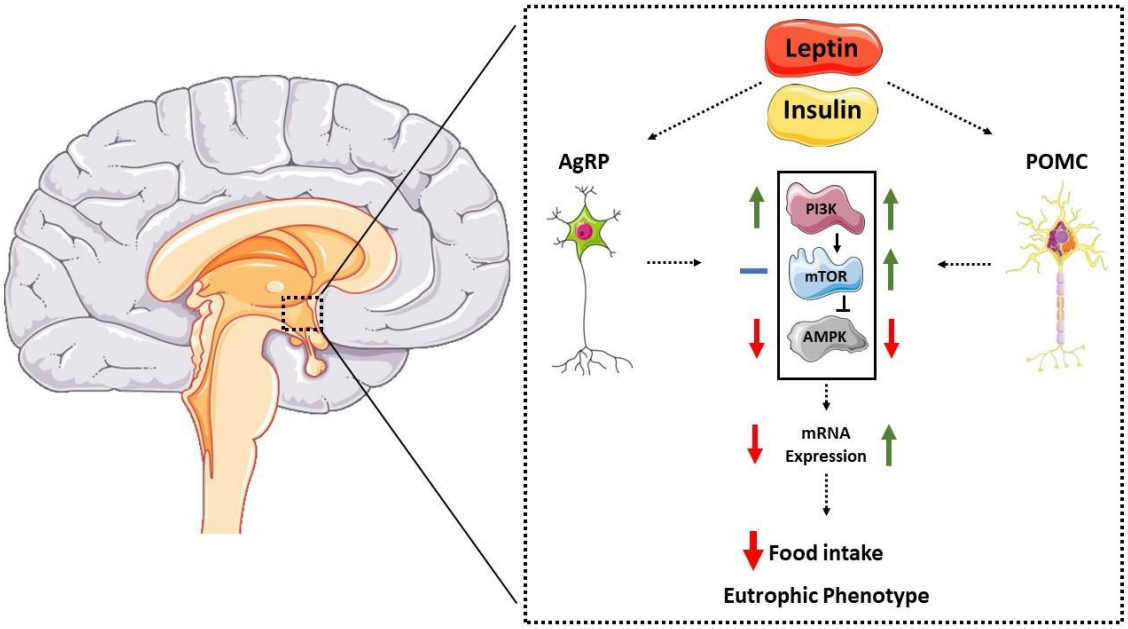

Figure 1. Representative diagram of the interaction between anorectic hormones, molecular pathways, and neuropeptides POMC and AgRP. Anorexigenic hormones (leptin and insulin) act on the hypothalamic arcuate nucleus, reducing AMPK activation in AgRP neurons, thus reducing its expression. In POMC neurons, hormones increase mTOR activity and reduce AMPK, increasing its expression and reducing food intake and eutrophic phenotype.

Insulin and leptin are the main anorectic hormones that act on the arcuate nucleus, activating POMC neurons and inhibiting AgRP neurons [36]. Several studies indicate that the loss of hypothalamic insulin signaling [18] [19] [20] and leptin [21, 22] can induce changes in energy homeostasis, excessive food intake (hyperphagia), and body weight gain, leading to obesity development. The arcuate nucleus is densely rich in leptin receptors [37]. After leptin binds to its receptors in neuronal cells, the intracellular signaling cascade begins. An internal conformational alteration in the LepR attracts the next downstream protein, JAK2 (Janus kinase 2) [38]. JAK is a cytoplasmic cytokine receptor that can autophosphorylate and promote the phosphorylation of its intracellular tyrosine residue Y-938, associated with the recruitment of the phosphatase SHP2 and its extracellular regulator ERK2, and of the residue Y-1077, which recruits the STAT5 transcriptional and signal transduction activator pathway. The primary effects of leptin on energy homeostasis involve the phosphorylation of the Y-1138 tyrosine residue, which creates a STAT3 binding and recruitment site [39]. After its binding and subsequent activation, the STAT3 is transferred to the nucleus of the neuronal cell and promotes the transcription of genes, such as the neuropeptide POMC [40, 41].

Regarding insulin, despite having been discovered in 1921 [42], the complete elucidation of its molecular signaling is still in progress. However, it is known that the insulin receptor (IR) is a tetrameric enzyme that comprises two extracellular alpha subunits and two transmembrane beta subunits. Once the hormone interacts with its receptor, there is activation and consequent phosphorylation of the generated substrates (IR family), leading to activation of its main pathway, the phosphoinositide 3-kinase (Pi3K) pathway, a heterodimeric lipid kinase that binds to tyrosine residues via its SH2 domain, generating PI membrane phosphates with PkB/Akt recruitment [43].

Both insulin and leptin can stimulate the Pi3K pathway in the arcuate nucleus with subsequent phosphorylation of their target proteins, leading to hyperpolarization and activation of POMC neurons [44] and inhibition of AgRP [17]. Mice with genetic Pi3K deletion in POMC cells did not show activation of POMC neurons in response to insulin or intracerebroventricular leptin administration [45]. However, Pi3K deletion in AgRP neurons seems to induce energy expenditure reduction, insulin and leptin resistance, and weight gain [17].

The mechanistic target of rapamycin (mTOR) is one of the Pi3K target molecules through activation of Akt in the hypothalamus (Pi3K/Akt/mTOR pathway) [46]. Both leptin and insulin activate hypothalamic mTOR [47, 48], a serine-threonine kinase with an essential role in brain development [49], which is found in approximately 90% of NPY/AgRP neurons and 45% of POMC/CART in the arcuate nucleus [50]. mTOR is known for acting as a metabolic energy sensor and can integrate the variations in the nutrient serum levels with the endocrine responses [51]. Thus, in food deprivation (fasting) and drastic drops in serum glucose and insulin level, there is a decrease in the phosphorylation of the mTOR active form. On the other hand, increased serum levels of leptin (51) and insulin (53) lead to increased mTOR protein content and expression and reduced food intake in the fed state.

The study of Kocalis *et al*. [52] observed that the deletion of Rictor-mTOR complex (mTORC2) activation, specifically in POMC neurons, can induce hyperphagia and increase adiposity. Interestingly, the specific deletion in AgRP neurons did not affect energy balance, although it led to mild glucose intolerance. It is known that the p70S6k-mTOR kinase further leads to phosphorylation of AMP-dependent protein kinase α2 (AMPK α2) on serine 491, inhibiting its action and thus limiting the effects of leptin on food intake [53]. Thus, in parallel with mTOR activation by food intake, the anorectic hormones leptin and insulin reduce the AMP-dependent protein kinase (AMPK) activity, specifically the AMPKα2 subunit [54, 55]. Like mTOR, AMPK is

also known as a metabolic energy sensor, being considered an essential protein in the complex system of intracellular energy regulation, which is based on the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio [56].

In conditions of depletion of energy reserves such as hypoglycemia and fasting, AMPK is activated in the hypothalamus [57, 58], leading to increased gene expression of NPY/AgRP in neurons and stimulating food intake [54]. AMPK is inhibited in the hypothalamic arcuate nucleus in the fed state in response to increased leptin, insulin, and high levels of serum glucose [54], which consequently inhibits the autophagic pathway in NPY/AgRP neurons, leading to a reduction in food intake by inducing the feeling of satiety and, thus contributing to the eutrophic phenotype [44, 50, 59]. Other molecules and pathways are also stimulated by anorectic hormones and can contribute to energy homeostasis. Further details about the molecules involved in the signaling pathway of insulin and leptin actions in POMC and AgRP neurons in eutrophic conditions were described in the review article by Varela and Horvath [16].

Regarding the PI3K-mTOR-AMPK pathway, anorexigenic hormones increase the activity of the Pi3K and mTOR pathways, leading to activation of POMC neurons [44, 45]. Additionally, leptin and insulin reduce AMPKα2 activity in the hypothalamic region [15, 54]. The p70S6k-mTOR kinase can inhibit the AMPK pathway in the hypothalamus, acting as a counter-regulatory protein [53]. The mechanisms by which these molecules modulate the expression of hypothalamic neuropeptides are not fully understood; however, evidence suggests that the autophagic pathway plays a crucial role in this regulation [59-61]. Furthermore, Claret *et al.* [55] showed that mice with genetic deletion of AMPKα2 in AgRP neurons were grown with the eutrophic phenotype. Interestingly, the specific genetic deletion in POMC neurons led to increased body fat and reduced caloric expenditure despite remaining sensitive to leptin. These results suggest that AMPK also plays a regulatory role in POMC neurons by unknown mechanisms. Therefore, evidence suggests that the hypothalamic autophagic pathway is crucial for activating orexigenic and anorectic neurons [59, 61, 62]. Figure 1 summarizes the data described so far.

Autophagic pathway in POMC and AgRP neurons: a pivotal point in energy homeostasis

It is well known that the neuronal autophagic pathway is crucial for maintaining cellular homeostasis both under basal conditions and in response to stress signals [63, 64]. Several studies indicate that the imbalance between activation and inhibition of the autophagic pathway in the

central nervous system (CNS) is associated with dysregulation of body energy homeostasis and obesity induction and a greater predisposition to the development of various neurodegenerative diseases [61, 65, 66].

Classically, autophagy can be divided into microautophagy, chaperone-mediated autophagy, and macroautophagy, the latter being the most prevalent and commonly referred to as autophagy [67, 68]. The autophagic process begins with capturing cytoplasmic organelles or macromolecules surrounded by a vesicular membrane lining called the autophagosome, which fuses with the lysosome to form autophagolysosome (or autolysosome), and lysosomal enzymes then degrade the sequestered material [69]. The autophagosome formation begins with a pre-phagophore structure, which elongates and expands to form the phagophore, which, in turn, will mature in the membrane vesicle, surrounding the substrate that will be degraded. [69]

The regulation of this entire autophagic process occurs by activating autophagic molecular complexes, starting with activation of the ULK1 (Unc-51 like autophagy activating kinase 1) complex, followed by the activation of phosphatidylinositol 3-kinase (PI3K), which forms a complex with Beclin 1 after dissociating from lymphoma B cell 2 (BCL-2) [70]. Thus, the formed complex activates several proteins of the autophagic family (ATGs) that participate in phagophore elongation and activate the LC3-I protein (light chain 3 of protein 1 associated with microtubules), forming LC3-II. LC3-II is responsible for closing the phagophore and interacting with the p62 protein, which targets the material that the autolysosome will degrade [71, 72]. Figure 2 shows the schematic model of the autophagic pathway.

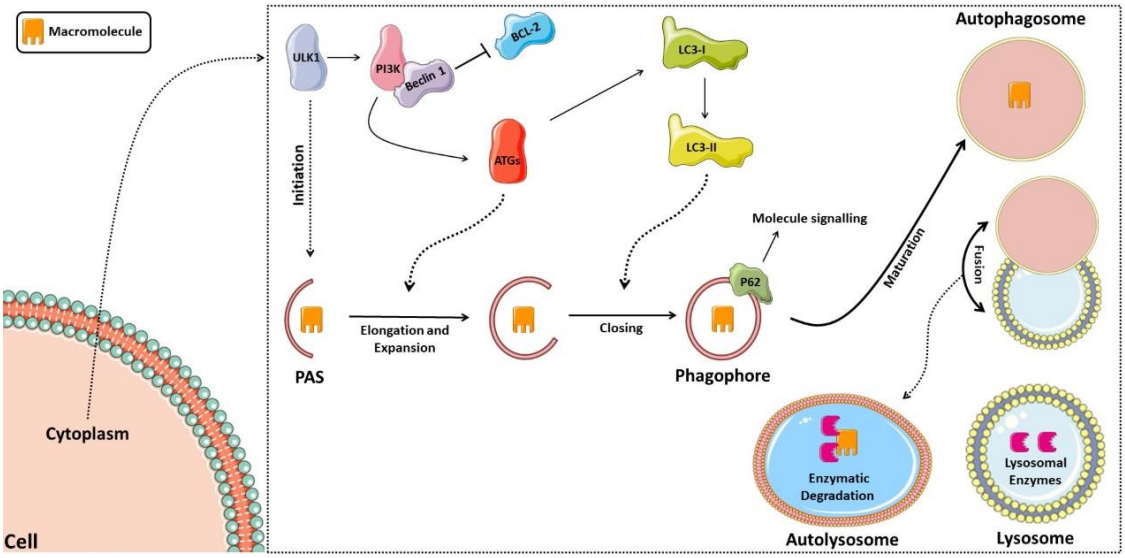

Figure 2. Schematic model of the autophagic pathway. The process starts with activation of the ULK1 complex, then activation of phosphatidylinositol 3-kinase (PI3K), which forms a complex with Beclin 1 after it dissociates from lymphoma B cell 2 (BCL-2). Thus, the complex formed activates several proteins of the autophagic family (ATGs), which participate in the elongation of the phagophore and activation of the LC3-I protein (light chain 3 of protein 1 associated with microtubules), forming LC3-II, responsible for closing the phagophore and interacting with the p62 protein, signaling the material to be degraded. The phagophore matures into the autophagosome, which fuses with the lysosome forming the autolysosome, in which lysosomal enzymes will then degrade the sequestered material.

Evidence suggests that the hypothalamic autophagic pathway is crucial in activating orexigenic and anorectic neurons [59, 61, 62]. AMPK is an activating molecule of the autophagic pathway, while mTOR leads to inhibition [73]. AMPK can activate the autophagic pathway *in vivo* and *in vitro* by phosphorylating raptor-mTOR (mTORC1) [74] and activating the autophagic pathway initiator complex ULK-1 in AgRP neurons [59]. Therefore, AMPK and mTOR directly interact to regulate the autophagic pathway through its complex initiator, ULK-1.[73]

Specifically, in AgRP neurons, the deletion of Rictor (rapamycin-insensitive companion of TOR), a key molecule in the regulation of the MTORC2 complex, did not change the energy balance [52]. However, the deletion of AMPK led to the eutrophic phenotype [55], suggesting that AMPK plays a more expressive role than mTOR in the energy regulation pathways of this neuronal subgroup. An elegant study published by Kaushik and colleagues [61] showed that inhibiting the autophagic pathway, specifically in AgRP neurons, in both cells and mice, through the Agt7 gene deletion, significantly reduces food intake and adiposity. In POMC neurons, studies with knockout mice showed that the activity of mTORC1 and mTORC2 complexes are vital factors for the anorectic effects induced by leptin on the neuron and maintenance of the eutrophic phenotype [52, 75].

While the reduction in AMPK activity in AgRP[59] neurons and the increase in mTOR activity in POMC neurons [52, 75] lead to the eutrophic phenotype and considering that AMPK inactivation and mTOR elevation lead to inhibition of the autophagic pathway [73, 76], hypothetically, inhibition of the autophagic pathway in neurons may be associated with the eutrophic phenotype. However, a study showed that the selective deletion of autophagy-related protein 7 (Atg7) in mouse POMC neurons, interestingly, leads to a reduction in melanocyte-stimulating hormone (MSH) and is also associated with increased adiposity and food intake through mechanisms involving resistance to lipolysis [60]. In addition, Cai *et al.* [77] observed that the suppression of Atg7 in the mediobasal hypothalamus using site-specific lentiviral delivery of

shRNA, without distinction of neuronal subgroups, was accompanied by an increase in hypothalamic inflammation, with activation of IKKB and, consequently, increased food intake and reduced energy expenditure [77].

Supporting these data, the AMPK deletion specifically in POMC neurons [55] and the RICTOR/mTORC2 deletion in the arcuate nucleus [52] are also associated with reduced caloric expenditure and obesity. Together, these data reveal that AMPK and mTOR are correlated with the autophagy pathway, which orchestrates a series of coordinated molecular phosphorylations in neuronal subgroups to provide adequate control of hypothalamic inflammation and energy homeostasis, reinforcing that the hypothalamic molecular pathway of obese individuals needs to be further investigated. Table 1 presents the metabolic phenotypes found according to the deletion or inhibition of molecular pathways in the neuronal subgroups.

Table 1. Molecular pathway deletion or inhibition in the neuronal subgroups and the outcomes.

Neuronal target	Deletion or inhibition of neuronal molecular pathways	Species	Outcome	Reference
AgRP	AMPK	Mice AMPKα2KO	Eutrophic phenotype, light level of glucose intolerance	[55]
	mTOR	Mice lacking Rictor in AgRP	mTORC2 did not change energy homeostasis	[52]
	Autophagic pathway	$Atg7^{F/F}$-AgRP-Cre mice	Better food intake control and eutrophic phenotype	[61]
POMC	AMPK	Mice AMPK α2KO	Hyperphagia, obesity, hyperglycemia	[55]
	mTOR	Mice lacking Rictor in POMC and C57BL/6J POMC-rptor-KO	Rictor/mTORC2: decreased energy expenditure and induced obese phenotype, did not induce leptin resistance mTORC1: limited ROS capacity to inhibit food intake	[52, 75]
	Autophagic pathway	$Atg7^{F/F}$-POMC-Cre mice	Limited lipolysis capacity and obese phenotype	[60]

NPY/AgRP and POMC simultaneously	AMPK	In vitro and in vivo (male C57BL/6)	Dysregulation of autophagic pathway and reduction in body weight	(59)
	mTOR	Mice lacking Rictor in all neurons	Increased adiposity, glucose intolerance, leptin resistance	(52)
	Autophagic pathway	Mediobasal hypothalamus Atg7 K_D mice	Hyperphagia, reduced energy expenditure, and hypothalamic inflammation	(77)

AMP-dependent protein kinase (AMPK), Rapamycin target protein (mTOR), Reactive oxygen species (ROS).

What is intermittent fasting?

Intermittent fasting (IF) and caloric restriction are two distinct forms of dietary restriction associated with improving several metabolic parameters, including body weight control [78]. Previous studies have shown that the obligation to maintain a daily calorie restriction reduces adherence to the caloric restriction protocols [79]. Thus, the presence of *ad libitum* feeding windows in IF protocols emerged as an alternative protocol for dietary restriction interventions. The stress promoted by the low caloric intake is replaced by the metabolic stress induced by intermittent windows of prolonged fasting or alternate days of deficient caloric intake [78, 80]. However, it is essential to highlight that both interventions must be carried out with professional supervision. Overfeeding episodes can occur after the fasting window with the risk of developing eating disorders such as binge eating [81].

Furthermore, caloric restriction programs can also increase the predisposition to the development of psychological disorders [79]. The review of Cerqueira *et al*. [82] pointed out that in animals fed with standardized diets balanced in macro and micronutrients, calorie restriction protocols with daily consumption of 40-60% of energy requirements are associated with micronutrient deficiencies. Deficiency of vitamin B12 and vitamin K, among others, depending on the diet consumed, is observed when the restriction protocols are chronically applied without supplementation with vitamins and minerals [79].

Despite its high popularity, there is no standardization of IF protocols [80]. It is established that protocols do not impose water restrictions. All include periods of food restriction, which may

refer to total deprivation from food consumption during some hours of the day (fasting window) or a full day containing no-energy food. Recently, some papers [80, 83] have been considering IF in three specific categories: a) Complete Alternate Day Fasting (ADF) - consists of days with ad libitum feeding intercalated by whole days of food restriction, b) Modified alternate-day fasting (MADF) or alternate-day modified fasting (ADMF) - with two non-consecutive days of total food restriction within the week, or two days of food intake of about 20% of the total caloric necessity with meals distributed throughout the day c) Time-restricted feeding (TRF) - consisting of a protocol with a fasting window (usually 16 hours) followed by a food intake window of approximately 8 hours, with the meals distributed within this period, according to individual needs. The main point of the protocols is not to change the average weekly caloric intake but to change the frequency of food consumption [78, 80].

In this sense, the application of IF protocols generally does not change the average calorie intake due to post-fasting compensatory overfeeding. Thus, only a slight reduction in the average percentage of daily intake (84, 85) contributes to the protocol being considered an alternative strategy to improve weight loss and induce positive metabolic adaptations generated by energy stress [84, 85]. However, it is important to mention the warning that IF protocols are not recommended in cases of malnutrition, pregnancy, gastric ulcers, elite athletes, and patients at risk of hypoglycemia, among others [86-88].

Unlike the globally disseminated IF protocols for weight loss and improvement in health-related aspects [23, 24, 89], Ramadan fasting is a protocol with spiritual purpose practiced by Muslim followers of Islam [90]. Once a year, according to the Islamic calendar, Muslims abstain from any food or drink, including water, during the period of daylight, having all their meals in the evening or just before sunrise [91]. This practice extends for about 30 consecutive days once a year during the Islamic lunar month, which can occur in different seasons depending on the year [92] and on the latitude of the geographic region. The fasting window can vary from 11:00 am to 6:00 pm [93]. During this practice, most Muslims eat about two bulky meals within 24 hours, one just after sunset and the other just before sunrise [93], resulting in a slight but significant reduction in the total calorie intake [94, 95] regarding loss or maintenance of body weight [96]. Table 2 illustrates the main differences between intermittent fasting, caloric restriction, and Ramadan fasting.

Table 2. Main differences between intermittent fasting, caloric restriction, and Ramadan fasting.

	Intermittent Fasting protocols	Caloric restriction	Ramadan fasting
Caloric Intake	Considering the food consumption throughout the week, there is a slight caloric intake restriction [97]	TRF: intake of 40% to 60% of the total energy expenditure, or daily restriction of 500 kcal to 1000 kcal [82] ADF: includes days containing absolute fasting of food [83]. MADF: includes two days a week with no-energy intake or days with severe restriction of food intake (less than 25% of daily necessity)[80]	Slight caloric intake restriction (300 kcal) [95]
Meals daily distribution	2 to 7 food restriction windows weekly. Usually composed by 16h-fasting or two days of the week with a caloric intake lower than 20% of the TCI [78]	Daily caloric restriction with a variable number of meals (TRF, MADF) [82] or days without any meals (ADF, ADF)	Fasting during daylight period (from 11 AM to 6 PM). Generally two meals a day, one after sunset and one before sunrise [93]
Related risks	Binge eating and hypoglycemia [81]	Vitamin and mineral deficiency [82]	Risk of dehydration and accidents at work [98]
Liquid intake	No restriction [84]	No restriction [82]	Restricted, including water restriction. Liquid intake is allowed only at night [90]

Intermittent fasting as a possible adjuvant in the treatment of obesity: modulations in neuroinflammatory, and leptin and insulin pathways

Obesity is a multifactorial disease usually associated with hyperphagia, hyperinsulinemia, and hyperleptinemia. The high levels of leptin and insulin in the cerebrospinal fluid of obese individuals indicate a chronic state of resistance to the actions of these hormones in the CNS [19, 99]. It is essential to highlight the diet quality profile as a significant possible factor in the pathophysiology of obesity. Increased exposure to a high-fat diet (HFD) is associated with a reduction in hypothalamic mTORC1 and leptin resistance [100]. There is evidence that an acute lipid infusion for 24h or exposure to a HFD over 8 – 20 weeks induces markers of inflammation in the hypothalamic NPY/AgRP neurons, which may contribute to a significant alteration in NPY/AgRP expression or content [101] and also, six days of exposure to a high-fat diet can induce leptin resistance in mice with a predisposition to obesity [102].

Several studies indicate that the practice of IF for periods longer than one month can improve insulin resistance and reduce its serum levels, contributing to regulation of glucose metabolism [26, 103-105]. A recent meta-analysis evaluated 545 participants, most overweight or obese, and observed that IF protocols are associated with a reduction in the body mass index (BMI) and leptin serum levels, lowering fasting blood glucose, and improving insulin resistance. These results suggest that IF may contribute to prevention/improvement in the resistance of the anorectic hormone observed in obese individuals [106].

Although not fully elucidated, the mechanisms by which IF acts in the insulin signaling pathway are probably different from those observed in caloric restriction protocols, since benefits associated with IF can be observed even when there is no reduction in calorie intake and weight loss [84, 107-109]. In addition, there is some evidence that IF protocols may produce more significant beneficial effects on glucose regulation and fasting insulin [103, 109].

It is known that obesity is associated with the chronic low-grade inflammatory process, not only peripheral but also central, highlighted by increased expression of several inflammatory proteins related to impairments in the hypothalamic signaling of leptin and insulin, such as the suppressor of insulin signaling cytokine 3 (SOCS3) [108, 110, 111]. Despite SOCS3 being part of a negative feedback system related to this signaling cascade, when it reaches a high concentration induced in an inflammatory scenario, SOCS3 significantly impairs the anorexic leptin cascade. This cytokine can bind to an intracellular region of LepR, attenuating the ability of JAK2 to

autophosphorylate and recruit the STAT3 pathway [112]. In addition, the C-terminal portion of SOCS can recruit the ubiquitin transferase system, promoting the degradation of JAK receptor complexes [113]. Thus, SOCS3 impairs the reduction in the activity of the AMPK protein threonine 172 by leptin [114], stimulating autophagic activity in AgRP neurons and appetite [59]. It is also known that SOCS3 can impair the insulin signaling pathway by binding directly to the insulin receptor [115] and/or degrading both substrates of insulin receptors 1 and 2 (IRS1/2) [116]. The study of Mori *et al.* [110] observed that hypothalamic suppression of SOCS3 could prevent central insulin resistance generated by the chronic high-fat diet.

In this sense, although several studies show that IF protocols can reduce plasma levels of pro-inflammatory proteins in obese or overweight individuals [27, 117], few studies have assessed the adaptation of inflammatory proteins in the hypothalamic region. Spezani *et al*. [27] evaluated the effects of a 24-hour fasting protocol interspersed with days of *ad libitum* high-fructose diet in mice with induced obesity (eight-week protocol with a high-fructose diet). After four weeks of intervention, a reduction in the expression of hypothalamic SOCS3 was observed. However, animals fed a standard diet and submitted to an IF protocol showed an increase in SOCS3 compared to control animals with a standard diet without application of the IF protocol [27]. Controversially, the study of Zangh *et al*. [118] did not observe changes in the expression of hypothalamic SOCS3 or alteration in plasma insulin in female mice fed with a standard diet and submitted to chronic IF protocols for 24h performed only one to two times a week, during a period of 13 or 42 days.

It is also known that the increase in tumor necrosis factor-alpha (TNFα) attenuates the anorectic effect of leptin and increases the expression of SOCS3 in the hypothalamus [119]. Despite studies showing that caloric restriction [117] and Ramadan fasting [120] can reduce plasma TNFα levels, particularly in obese or overweight individuals, a recently published meta-analysis [28] evaluated serum levels of inflammatory markers in response to different IF or caloric restriction protocols. After applying the exclusion criteria, the meta-analysis included only one study with obese individuals and IF (alternating every 24-h between consuming 25% or 125% of energy needs), which did not reduce TNFα levels [121]. However, it is crucial to consider the lack of papers published in this area.

Regarding animal studies, Spezani *et al*. [27] evaluated the effects of IF for 24h. The authors observed that obese mice submitted to fasting curiously showed a greater expression of hypothalamic TNFα when compared to the control group. Therefore, the data are still

contradictory, and further studies are needed to assess the content and expression of TNFα, specifically in the hypothalamus of obese animals submitted to different IF protocols.

Another relevant inflammatory pathway involved in the etiology of obesity is the IKKb/NF-kb pathway [111]. Zhang *et al*. [111] showed that mice submitted to a high-fat diet developed obesity accompanied by increased concentrations of IKKB, which can activate the nuclear factor kb (Nf-kb), leading to endoplasmic reticulum stress in the hypothalamus and consequent resistance to leptin and insulin [111]. IKKB phosphorylation in the mid-basal portion of the hypothalamus can impair the action of insulin by inducing tyrosine phosphorylation and the consequent inactivation of the insulin receptor (IR). In addition, it can limit the activity of its target proteins: phosphatidylinositol-3-kinase (Pi3K) and protein kinase B (Akt) [111, 122], which are involved in the control of the hypothalamic autophagic pathway [70]. In a complementary way, the increase in IKKB contributes to elevation of the expression of hypothalamic SOCS3 [111], impairing the central signaling of leptin and insulin [41, 123]. However, although IKKB is a protein widely studied in obesity models, no investigations have evaluated IKKB in the hypothalamic region in response to IF protocols.

The IKKb/NF-kb inflammatory cascade can also be activated by lipopolysaccharides (LPS) when bound to their Toll 4 membrane receptor (TLR-4) [124, 125]. Previous studies have shown that obese individuals present increased levels of LPS in the bloodstream, causing a condition called metabolic endotoxemia, which is associated with systemic inflammation and an increased risk of developing chronic diseases [126], favoring the development/worsening of obesity [126, 127]. Additionally, prolonged treatment with LPS seems to increase JNK and limit the hypophagic effects in response to central insulin administration, regardless of the increase in body weight [128]. Although not fully understood, the increase in LPS plasmatic levels is probably due to intestinal dysbiosis and changes in the permeability of the intestinal wall [129, 130].

Dietary factors seem to modulate endotoxemia, and the use of prebiotics could contribute to attenuating its progression (131), while chronic exposure to a high-fat diet intake could worsen progression [126]. In this sense, a recent review article proposed that IF protocols can also be used as a nutritional strategy, with effects on the brain-microbiota axis of obese individuals [131]. An elegant study demonstrated that the removal of the intestinal microbiota with the use of antibiotics reduced the protective effects of IF on the cognitive function of the evaluated mice, with the subsequent administration of microbiota metabolites, such as short-chain fatty acids and 3-acid

propionic indole, which were able to improve cognitive function and insulin sensitivity [30]. Together, we hypothesized that IF might play a supporting role in attenuating inflammation in the CNS through actions on the microbiota-brain axis. However, this hypothesis needs to be evaluated.

Additionally, it is known that during acute fasting periods, there is an increase in β-hydroxybutyrate (βHB) production [132], leading to increased phosphorylation of IRS1 and Akt in their active forms, a reduction in serum insulin levels, and a better response to the intraperitoneal insulin tolerance test [133], as well as being able to modify hypothalamic leptin and insulin signaling pathways in type 2 diabetic rats [134]. It is essential to highlight that βHB is also involved in inflammatory control [135]. The oral administration in Crohn's disease patients exerts an anti-inflammatory response through downregulation of NF-kb [136]. Cerniuc *et al.* [137], evaluating an IF protocol (2 non-consecutive days of total fasting per week) in healthy women, also identified a significant increase in blood βHB levels. To date, despite not directly evaluating insulin response associated with βHB production and hypothalamic responses in IF protocols, data suggest that the increase in butyrate levels may also contribute to improving insulin sensitivity in response to IF.

Although the relationship of neuroinflammation with insulin and leptin signaling in response to IF protocols needs to be further explored, data suggest that: 1) the mechanisms of action of IF seem to be different from those observed in calorie restriction protocols [108, 109]; 2) IF seems to be able to improve insulin and leptin sensitivity [105, 106]; 3) IF seems to be able to modulate inflammatory pathways in the brain [27, 29] and attenuate the levels of LPSs in the plasma [29], which we hypothesize could be associated with an improvement in hormonal and neuronal sensitivity; and 4) the increase in βHB production in IF [137] may also contribute to better insulin sensitivity considering the relationship of βHB and the insulin pathway [133, 134].

The influence of the circadian cycle on the modulation of leptin and insulin pathways in different IF protocols

Although several studies show that IF protocols are capable of improving insulin and leptin sensitivity, it is essential to emphasize that the time when the fasting window and the eating window are performed significantly interferes with metabolic responses and autophagic stimulation due to their influence on the hormonal rhythm guided by the circadian cycle [33, 138]. However, the habit of skipping breakfast is associated with greater consumption of food at night (breakfast skipping and late-night eating pattern), increasing the risk of developing insulin resistance and cardiometabolic risk [33, 139, 140].

Overweight or obese individuals submitted to IF protocols evaluated the differences between the protocol carried out with the food window from 8 AM to 2 PM (Early Time-Restricted Food – eTRE) with a second protocol containing the last meal at 8 PM, both on a controlled diet. After four consecutive days of intervention, the authors observed increased BMAL1 expression in the morning, activation of Akt2, reduced fasting plasma insulin, and glucose concentrations in the eTRE group compared to the group that had the last meal at 8 PM [33]. These results corroborate another study carried out with humans by the same research group that observed that the eTRE group improved insulin sensitivity, assessed by the glucose tolerance test, compared to the group fed at night [107].

The IF protocol performed without respecting the circadian cycle can induce a dysregulation in the expression of circadian cycle leading to a significant increase in the natural peak of mRNA expression of genes involved in glucose regulation (*Gck, Slc2a2, Pdk4*), and also lead to a higher plasma leptin levels when compared to an IF protocol applied respecting the circadian cycle (with a distributed feeding window in the active period of mice) [32]. On the order hand, an IF protocol respecting the circadian cycle seems to reverse the obese and hyperphagic phenotype of heterozygous knockout mice of brain-derived neurotrophic factor (BDNF) and re-established insulin sensitivity and brain BDNF levels after three weeks of intervention [141]. Thus, due to its influence on the circadian cycle, the effects of IF on endocrine responses and body weight may vary according to the time of day in which each food and fasting window is held. It seems better for healthy improvements not to skip breakfast and to start the fasting window at a time close to sunset to improve sensitivity to anorectic hormones and help prevent obesity [139, 142, 143].

Therefore, it is essential to point out that these data warn us regarding popular IF models disseminated in social media that encourage avoiding breakfast and starting the eating window at lunch. It is essential to reinforce the importance of a scientific basis to achieve better dietary prescriptions at the individual and population levels. This topic was deeply explored in the recent review published by Moon *et al*. [144].

Intermittent fasting and the autophagic pathway

Autophagy is an essential mediator of physiological responses associated with the generation of ROS and cellular protein damage [145], being directly involved in maintaining energy homeostasis through the increased expression of neuropeptides [146] and in the control of the neuronal inflammatory response [77]. Energy stress and IF-induced oxidative stress can activate the

autophagic pathway [147] through the increase in sirtuins (SIRTs) [148, 149], associated with increased phosphorylation of AMPK in threonine [150, 151], correlated with phosphorylation of the ULK1 protein and autophagic pathway [152]. Therefore, the application of IF protocols is a non-pharmacological alternative capable of activating the autophagic pathway [33, 34].

During prolonged fasting, lower glycemic values and changes in the adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio induce SIRTs activation in tissues such as the kidney, skeletal muscle, and blood samples from overweight individuals [147, 149, 153]. Sirtuins are known to induce the autophagic pathway through phosphorylation of AMPK, FOXO1, or deacetylation of autophagic family proteins [147, 151]. A study evaluating the IF and autophagic pathway in adults observed an increase in serum levels of SIRT1 and the autophagosomal membrane component LC3A, thus suggesting autophagic stimulation, accompanied by improved insulin sensitivity [33].

AMPK is an essential protein also associated with the neuronal autophagic pathway and energy homeostasis [59]. An interesting study by Kaushik *et al*. [61] observed that acute fasting could increase the content of free fatty acids in the hypothalamus, with consequent phosphorylation and activation of AMPK and ULK1, increasing autophagic flow in AgRP neurons with hunger induction. Furthermore, the authors observed that impairment of the autophagic pathway in cultures of hypothalamic cells through the deletion of the protein related to autophagy 7 led to a reduction in AgRP levels, food intake, and adiposity [61]. The increased availability of fatty acids can induce the hypothalamic autophagic pathway and increase NPY expression [146].

Additionally, the acute fasting protocol can also lead to phosphorylation of mTOR and its target protein, ribosomal protein kinase S6 (S6K) at serines 240 and 244 in the hypothalamus, thus inactivating the mTOR/S6k pathway [50]. During fasting, the increase in AMPK associated with the reduction in mTOR contributes to the regulation of food intake [50, 53] and activation of ULK1 and the autophagic pathway [152]. These data corroborate the work of Chaix *et al.* [154], who evaluated IF protocols in obese mice, and observed an increase in the levels of the homolog of ATG8, Gabarap1, a key regulator of autophagic flow during fasting. The animals showed significant weight loss compared to the control group, although food intake did not show any significant difference.

Although several studies have shown the effects of caloric restriction programs or fasting periods on the autophagic pathway, it is essential to emphasize that, to our knowledge, few studies

have assessed the effects of IF protocols on the autophagic pathway. To date, we understand that fasting periods in general lead to increased availability of free fatty acids in the hypothalamic region, reduced levels of glucose and serum amino acids, leading to activation of sirtuins [33] and the AMPK pathway [150], and inactivation of the mTOR pathway, thus stimulating the autophagic complex [50, 73]. Currently there are no studies evaluating the autophagic pathway in the hypothalamic nucleus in response to chronic IF protocols.

Intermittent fasting, POMC, and AgRP neuropeptides

The mechanisms by which IF alters the expression of hypothalamic neuropeptides are not fully understood; however, it is known that IF can improve sensitivity to leptin and insulin [106] and stimulate the autophagic pathway [33, 147], which is related to the activation of hypothalamic neuropeptides and energy homeostasis [27, 55, 59, 60]. Another relevant factor is the increase in reactive oxygen species induced by the IF protocol [148]. ROS in the hypothalamus is also a factor that leads to the electrical activation of neuropeptides POMC and inactivation of Npy/AgRP [155].

The suppression of reactive oxygen species decreases the activation of POMC cells and increases the activity of NPY/AgRP neuropeptides [155]. During fasting, the mechanisms of oxidative protection performed in the mitochondria protect the exacerbated increase in ROS in AgRP neurons. Uncoupling protein 2 (UCP2) is a protein abundantly expressed in arcuate nucleus neurons associated with energy homeostasis and involved in controlling oxidative stress in mitochondria [156, 157]. A study shows that the function of UCP2 via AMPK seems to be a key point for the electrical activation of NPY/AgRP neurons during a fasting period [158]. In contrast, ROS levels are low during fasting in POMC neurons, and the transient increase in ROS favors satiety and the action of leptin via mTOR [75, 159]. However, these data were evaluated using acute fasting protocols. There is still no evidence about the content of ROS in neuronal groups and its impact on energy homeostasis in response to IF protocols.

Low serum leptin values during fasting contribute to an increase in the expression of AgRP and NPY [158, 160, 161], stimulating the autophagic pathway and consequently inhibiting activation of the hypothalamic-pituitary-thyroid axis to reduce caloric expenditure and save energy [162]. However, contrary to the results observed after a single fasting period window, the study of Chausse *et al*. [163] submitted eutrophic rats to a 24-hour IF protocol and observed that rats that underwent IF for three weeks, even with an excellent response to leptin, curiously showed increased expression of the orexigenic neuropeptide AgRP both during fasting periods and on

feeding days. Increased energy expenditure, reduced energy efficiency factor, and lower weight gain were also observed when compared to the control group. These findings suggest that AgRP neuron responses to the IF protocol may differ from those observed after a single fasting window, and further studies are needed.

It seems that during the IF protocol, the response of neuropeptides can change. A study with eutrophic mice evaluating the response to IF protocols after 13 days and again after 42 days found that the first intervention had an increase in food intake in the feeding windows accompanied by an increase in NPY mRNA expression. However, interestingly, after 42 days, there was a reduction in NPY mRNA expression that returned to baseline values. There was no difference compared to the control group (without IF intervention), thus suggesting a new late hypothalamic adaptation of NPY in response to the chronic application of the IF protocol [(118)], which may be related to the popularly described hunger adaptation. Regarding POMC neuropeptides, there were no significant changes.

Additionally, the time that the fasting window is performed can also influence the expression of neuropeptides. Animals exposed to a high-fat diet for two weeks and then submitted to the IF protocol of 16h for one week, with the feeding period performed during the rest period, disregarding the circadian cycle, showed increased hypothalamic expression of the orexigenic genes NPY and AgRP. Higher food intake and higher serum levels of leptin were also observed, thus suggesting possible resistance to leptin compared to animals that followed the IF protocol with the feeding window performed in the active period (referring to the night period for mice). Therefore, it can be concluded that when the IF is carried out with the food period during the rest period, disregarding the physiological circadian cycle, there is a possibility that the protocol can trigger dysregulation of the neuroendocrine mechanisms of hunger control, which may harm health [(32)].

Few studies have assessed the effects of IF and neuropeptide expression in obese individuals. Gotthardt *et al*. [(35)] studied obese mice submitted to an IF protocol where the mice were food deprived every other 24-hour period beginning at 9:00 AM (fasting day), 2 hours into the light cycle, for four weeks. The results showed increased expression of mRNA of hypothalamic NPY and increased energy expenditure compared to the control group that consumed a high-fat diet *ad libitum*. Regarding the expression of POMC neurons, the group that performed the IF showed a significant reduction in the expression of the POMC neuropeptide when compared to the control

group with an *ad libitum* high-fat diet, which was also accompanied by a reduction in serum levels of leptin, improvement in insulin sensitivity, and weight loss.

Interestingly, a study evaluating the effects of IF in animals fed with a standard diet observed that after four weeks of application of the IF protocol (24 h fed, 24h fasting), the group with standard diet and fasting presented reduced expression of POMC when compared to its respective control (standard diet without fasting) [27]. However, the authors also looked at the effects of IF on two other types of diet: obese animals fed a high-fat diet, and obese animals fed a high fructose diet. After four weeks of applying the IF protocol (24 h fed, 24h fasting), the fasted obese animals showed increased expression of POMC in both protocols [27].

Therefore, we suggest that the content of neuropeptides in IF seem to occur differently from that observed in caloric restriction protocols, and the adaptations of the CNS seem to differ according to 1) the duration of time that the IF protocol is being applied, with the orexigenic neuropeptide NPY being able to return to baseline values as a late adaptation [118]; 2) it is essential that the distribution of feeding and fasting periods respects the circadian cycle to avoid possible health risks [32]; 3) concerning POMC neuropeptides, the IF protocol interestingly seems to reduce the expression of POMC neurons in some models [27, 35], but the results are still contradictory. Therefore, further studies are necessary to elucidate the effects of IF on hypothalamic responses and energy homeostasis. In addition, it is necessary to investigate long-term changes. Figure 3 summarizes the possible effects of IF as an adjuvant treatment to partially rescue hypothalamic responses in obesity.

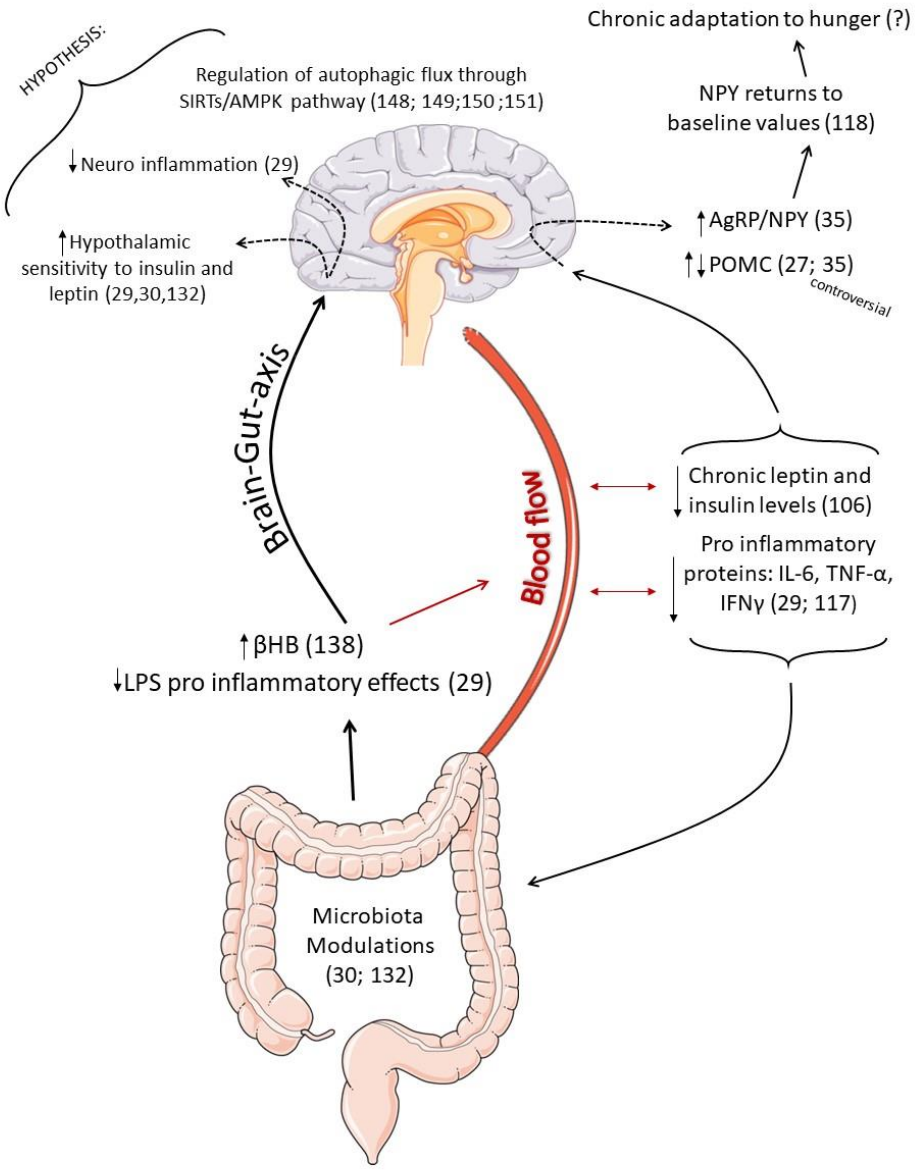

Figure 3. Schematic representation of the possible effects of intermittent fasting (IF) as an adjuvant treatment to partially rescue hypothalamic responses in obesity. Once IF prevents the pro-inflammatory effects in the hippocampus caused by LPS [(29)], and it is also able to increase the production of β-Hydroxybutyrate (βHB) [(138)], correlated to inflammatory control [(136)] and insulin sensitivity, it is possible to hypothesize that modulations in the microbiota may be helpful to reduce hypothalamic inflammation and increase hormonal sensitivity. Low serum leptin and insulin values during fasting contribute to an increase in the expression of AgRP and NPY. However, interestingly, the NPY mRNA expression returned to baseline values as a chronic response to IF, which may be is an adaptation to hunger. Furthermore, some studies found an increase in the expression of POMC neuropeptide, which is controversial. Such neuronal sensitivity to the IF protocol is perhaps also associated with better regulation of hypothalamic autophagic

response since IF can activate the autophagic pathway in other tissues [148], and autophagic flux is an essential mediator of neuropeptide responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

Evidence indicates that IF protocols can be used as a strategy to promote weight loss, as they induce an increase in energy expenditure [35, 163] and improve the peripheral response to anorectic hormones [33, 164], which can significantly interfere in the hypothalamic autophagic pathway [33] and also in the expression of neuropeptides [27, 35]. Thus, the literature reviewed allows us to hypothesize that IF could help to reestablish, at least in part, the control of hypothalamic molecular responses in obese individuals, alleviating neuroinflammation and improving hypothalamic sensitivity to anorectic hormones, thus helping to improve reestablishment of energy homeostasis. However, when the IF protocol is performed without considering the circadian cycle, it can impair energy metabolism regulation [32]. These associations require further research in the area, especially when the responses of the autophagic pathway and hypothalamic neuropeptides are considered when obese individuals undergo long periods of IF. In conclusion, considering the favorable results of IF in obesity, the protocol may be an adjuvant treatment to partially rescue hypothalamic responses in obesity.

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Competing interests

The authors declare that they have no competing interests.

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3. MANUSCRIPT II

Original Article: Intermittent fasting associated with exercise training reduces body mass and alleviates hypothalamic disorders induced by high-fat diet intake.

Intermittent fasting associated with exercise training reduces body mass and alleviates hypothalamic disorders induced by high-fat diet intake

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ABSTRACT

Consumption of a high-fat diet causes hypothalamic inflammation leading to dysregulation of the leptin pathway, which in turn compromises the modulation of hypothalamic neuronal activities and predisposes obesity development. Intermittent fasting (IF) and exercise training (ET) have been pointed out as efficient strategies to modulate hypothalamic inflammation and neuronal activity. However, no data evaluated whether the combination of these strategies could induce better results on the re-establishment of hypothalamic homeostasis disrupted by high-fat diet intake. Firstly, male C57BL/6 mice were separated into two groups: sedentary mice fed a standard diet (CT) and sedentary mice fed a high-fat diet (HF). At week 8, part of the HF group was submitted to different interventions for 6 weeks: HF-IF = HF diet mice submitted to IF; HF-T = HF diet mice submitted to ET; HF-IFT = HF diet mice submitted to IF and ET. We observed that all interventions decreased body weight gain induced by high-fat diet intake associated with a reduction in calorie consumption in week 14. Only the HF-IFT group improved serum insulin, leptin, resistin, and Tnf-alpha levels concomitantly with a decrease in hypothalamic inflammation. The HF-IFT group also increased Pomc mRNA expression associated with improved pSTAT3 expression in ARC and VMH. Our data indicate that the beneficial effects of the combination of IF and ET on energy homeostasis are associated with an increase in leptin sensitivity in the ARC and VMH, which is likely due to an improvement in hypothalamic inflammatory pathways in those nuclei.

Keywords: obesity, intermittent fasting, hypothalamus, leptin, physical exercise.

1. INTRODUCTION

In healthy individuals, plasma leptin levels can bind to leptin receptors (LepR) expressed in hypothalamic arcuate nucleus (ARC) neurons initiating the intracellular cascade of the Janus kinase pathway2/Signal transduction and transcription activators 3 (JAK2/STAT3) [1]. Activation of this cascade in proopiomelanocortin (POMC) neurons induces POMC cleavage and releases the anorexigenic alpha-melanocyte-stimulating hormone while inhibiting synthesis and secretion of the orexigenic agouti-related protein (AgRP)/ neuropeptide Y (NPY), leading to the enhancement of satiety and increased energy expenditure [2]. Furthermore, leptin signaling in the ventromedial nucleus (VMH) has important effects on energy and glucose homeostasis control [3].

Several investigations indicate that the increase of inflammatory proteins [4-6] and/or dysregulation of autophagic activation [7, 8] compromise leptin signaling, leading to hypothalamic resistance to leptin and insulin, which predisposes to obesity. Besides activation of the JAK2/STAT3 pathway following leptin binding to LepR, an increase in the suppressor of cytokine signaling-3 (SOCS3) expression is observed, which in turn inhibits tyrosine phosphorylation of the LepR, resulting in negative modulation of leptin signaling [9, 10]. However, the chronic increase of hypothalamic SOCS3 content, as observed in obesity, predisposes to central resistance to leptin [11, 12]. Increased expression of SOCS3 can also be observed during the inflammatory process, including that involved with the recruiting of Toll-like receptor 4 (TLR4) signaling [9, 10]. Thus, TLR4 and SOCS3 are two inflammatory proteins related to the regulation of the JAK2/STAT3 pathway [11, 13]. The autophagic processes also play an important role in the hypothalamic action of leptin [14], regulation of AgRP [8], and POMC [15] neuron activity. Indeed, autophagy inhibition in the mediobasal hypothalamus, using site-specific delivery of lentiviral shRNA against Atg7, increased hyperphagic behavior and reduced energy expenditure [16].

Non-pharmacological strategies such as intermittent fasting/IF [14, 17] and physical exercise [14, 15] can improve peripheral sensitivity to insulin and leptin. Recently, Cho and coworkers [17] showed the efficiency of IF protocols in reducing serum insulin and leptin levels, as well as adiposity in adults. Although the data related to the effects of IF on the central inflammatory response are still contradictory, it was reported that IF protocol can modulate the expression of SOCS3 in the hypothalamus [15, 16] and also reduce TLR4 mRNA in the hippocampus of rats treated with lipopolysaccharide [18], suggesting that IF can be used to alleviate hypothalamic inflammation in obese animals.

Regarding physical exercise, a recent review demonstrates that a 2-week protocol can decrease body fat mass associated with lower levels of circulating leptin [19]. In addition, the endurance exercise is also able to reduce hypothalamic TLR4 total content and increase phosphorylation of the JAK/STAT3 pathway, inducing higher POMC mRNA expression compared to sedentary obese mice [20]. However, to the best of our knowledge, there are no data examining the effects of the combination of IF and physical exercise on the impairment of the hypothalamic molecular pathways caused by the chronic ingestion of a high-fat diet. Therefore, we investigated whether the combination of IF and physical exercise would be more effective in alleviating hypothalamic dysfunctions in high-fat diet mice in comparison with these interventions applied alone.

2. MATERIAL AND METHODS

2.1 Animals

The experimental procedures were performed according to the Brazilian Society of Science in Laboratory Animals and approved by the Ethics Committee in the Use of Animals of the School of Physical Education and Sport of Ribeirao Preto under protocol number 2017.5.55.90.0. Eight-week-old male C57BL/6J mice from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were housed in groups of 3 animals per cage with *ad libitum* access to filtered water and controlled temperature (22 ± 2 °C) on a 12:12-h light-dark normal cycle. The mice were assigned into five groups based on the diet provided and the type of intervention used: standard diet (CT), high-fat diet (HF), HF submitted to intermittent fasting (HF-IF), HF submitted to the physical exercise (HF-T), and HF submitted to both intermittent fasting and physical exercise (HF-IFT). The standard diet (3.39cal/g, 25.9% proteins, 63.4% carbohydrates, and 10.6% lipids – nuvilag-cr1, Curitiba, Paraná, Brazil) or HFD (4.73Kcal/g, 45% fat, 20% protein, and 35% carbohydrates acquired from PragSoluções Biociências company, Jaú, São Paulo, Brazil) were offered throughout the experimental period.

2.2 Experimental procedures

2.2.1 Intermittent fasting protocol

After 8 weeks of high-fat diet consumption, the animals in the HF-IF and HF-IFT groups were food-deprived for 24h starting at 12:00 am. On the next day, these groups had 24h of food available (24h fed/24h fasting protocol). This procedure was carried out 3 times per week [21]. During the 24-hour fasting period, the animals received water *ad libitum*, and on days without

fasting, they received access to a high-fat diet and water *ad libitum*. Table 1 illustrates the distribution of fasting windows throughout the week.

Table 1. Arrangement of fasting/fed windows throughout the week.

	Sunday	**Monday**	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**
Before midday	Food *ad libitum* period	Fasting period	Food *ad libitum* period	Fasting period	Food *ad libitum* period	Fasting period	No intervention
After midday	Fasting period	Food *ad libitum* period	Fasting period	Food *ad libitum* period	Fasting period	Food *ad libitum* period	

2.2.2 Training protocols and physical tests

The animals of the HF-T and HF-IFT groups were submitted to resistance training on alternate days with endurance training (combined training), totalizing three aerobic training sessions and two resistance training sessions per week, over 6 weeks, starting at 10:00 AM. Before starting the training protocol, the animals were adapted to the respective exercise models. After the adaptation period, mice performed the physical tests to determine the respective training loads as described in the following topics.

2.2.2.1 Incremental load test on the treadmill

The animals were adapted to physical exercise on a treadmill (Insight Ltda, Ribeirão Preto, São Paulo, Brazil), for 4 days, 10min/day at the speed of 3m/min and subsequently performed the incremental load test as standardized by Ferreira *et al*. [22]. The maximum velocity (Vmax), defined as the speed (m/min) of exhaustion of the animal, was used to prescribe aerobic training intensity, with progressive adjustment of the training volume (time in min). The animals of the HF-IFT group performed the incremental load test in fasting.

2.2.2.2 Endurance training protocol

The aerobic training protocol was based on the study by Ferreira *et al*. [22]. The mice run at 60% of Vmax with a progressive increase in training volume (time), starting with 20min with an increment of 5min per week, reaching 45 minutes in the last week.

2.2.2.3 Resistance training protocol

The mice performed 4 days of adaptation to climbing the ladder (Insight Ltda, Ribeirão Preto, São Paulo, Brazil), without the use of an external load [23]. The resistance training protocol was adapted from Khamoui *et al*. [24]. After warming up the exercise (a repetition without load), the load was applied at the base of the tail of each animal referring to 50% of the body mass in the first week, followed by increments of 7.5% of body weight per week, reaching 87.5% of body weight in the last week. The animals performed 4 sets of 3 repetitions per session. The passive rest between repetitions was 60 seconds, and between sets was 2 minutes.

2.2.3 Intermittent fasting and exercise protocol

The first fasting window in the week started on Sunday at 12:00 am, aiming to intercalate fasting periods with food access *ad libitum* in the morning periods. Thus, fasted mice performed aerobic training on Mondays, Wednesdays, and Fridays. Strength training was performed without previous fasting periods on Tuesdays and Thursdays. Table 2 illustrates the disposition of the days and types of training interspersed with the fasting period throughout the week of the HF-IFT group.

Table 2. Arrangement of training and fasting windows throughout the week.

	Sunday	**Monday**	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**
Before midday	#	Endurance training	Resistance training	Endurance training	Resistance training	Endurance training	No intervention
After midday	*						

The areas colored in #blue represent the feed *ad libitum*. The areas colored in *****pink represent the fasting period.

2.2.4 Caloric intake and body mass determination

The food intake and body mass were evaluated weekly. Because animals were housed in 3 per cage, we divided the total amount of food consumed in a week by 3 to estimate the food consumption per animal. The caloric intake was determined based on the caloric value (kcal/g) of each diet [kcal/day = average food intake per week [g]× 3.39cal (control) or 4.73cal (HFD)]. [25]

2.2.5 Intraperitoneal insulin tolerance test (ITT)

The ITT was performed at the end of the experiment, 24h after the last intervention (training or fasting). At 11:00 am, the animals were housed in a clean cage without food. After 6h, a small incision was made in the tail of the animal, the glycemia was measured with a glucometer (*Accu-chek; Roche Diagnostic Corp*., Indianapolis, IN, USA), and the animals received an intraperitoneal injection of insulin (1.5 IU/$^{kg-1}$, *Eli Lilly*, Indianapolis, IN, USA). Later, the glycemia was measured every 5 minutes in a 30-minute time interval. The area under the curve was determined using the trapezoidal principle [26].

2.2.6 Serum and hypothalamus extraction

The animals were anesthetized by intraperitoneal administration of a mixture of xylazine (10 mg/kg body weight), and ketamine (100 mg/kg body weight). Next, the animals were decapitated, and the blood and the brain were collected. The serum was separated for Elisa assays and the hypothalamus was isolated and immediately frozen in liquid nitrogen for posterior immunoblotting and real-time polymerase chain reverse studies (RT-PCR), as described below.

2.2.7 Determination of serum leptin, insulin, resistin, and Tnf-alpha levels.

Serum levels of insulin, leptin, resistin, and Tnf-alpha were determined by enzyme immunoadsorption assay (ELISA) using Multiplex Biomarker Immunoassays (cat. MADCYMAG- 72k-04, MerckMilipore, Darmstadt, Germany) according to the manufacturer's instructions. The mean fluorescence intensity (MFI) was detected by the Multiplex plate reader for all measurements (Luminex System, Bio-Rad Laboratories, Hercules, California, USA) using a Luminex system, Bio-Rad Laboratories software.

2.2.8 Immunoblotting

The immunoblotting technique was performed as previously described [27]. Specific antibodies used include Jak2 (catalog-3230S), Phospo-Jak2 (Tyr 1007/1008, catalog -3771S), FOXO1, (catalog -9454S) and Atg7 (catalog-2631) from Cell Signaling Technology (Cell Signaling Technology, Danvers, Massachusetts, USA); TLR4 (catalog -sc-293072), GAPDH (catalog -sc-365062), and SOCS3 (catalog-sc-518020) from Santa Cruz Biotechnology (Santa Cruz, Dallas, Texas, EUA); Phospo-FOXO1 (ser256, catalog-OAAJ02704) from Aviva (Aviva Systems Biology Corporation, San Diego, California, USA) and Beclin (catalog-66665) from Proteintech, Rosemont, USA. Routine chemical reagents were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). After the band intensities were measured, all proteins (phosphorylated or not) were normalized by a control protein (GAPDH). Then the

phosphorylated/non-phosphorylated ratio was calculated. A Ponceau S-stain was used as an experiment loading control. All Western blot experiments are available in Supplementary File 1. Figure and supplementary figure panels were checked for data integrity using the Proofig pipeline, https://www.proofig.com.

2.2.9 Quantitative Real time-PCR (RT-PCR)

Total RNA extraction from the hypothalamus was performed with TRIZOL (Trizol Reagent, 15596026, Invitrogen, Carlsbad, CA) as previously described [28]. The reverse transcription reaction (1μg of total RNA) was performed using Superscript III Reverse Transcriptase. The *real-time* PCR was performed using oligonucleotides acquired from Integrated DNA Technologies (IDT) for *Pomc* (Mm.PT.58.5917321), NPY (Mm.PT.58.29444574), *Agrp* (Mm.PT.58.45969609.g), *Lepr* (Mm.PT.58.33275723) and beta-*Actin* (Mm.PT.39a.22214843.g). The threshold cycle (Ct) was used to calculate the relative expression of the target gene and results were evaluated as stated by the ΔΔCt method.

2.2.10 Transcardiac perfusion, brain collection, and immunostaining

The animals were fasted for 12h and right after treated with an intraperitoneal injection of leptin (1μg.g-1), as previously described in the literature [29]. After 45 minutes, the animals were anesthetized by intraperitoneal administration of xylazine mixture (10 mg/kg body weight) and ketamine (100 mg/kg body weight). After that, mice were transcardially perfused with 0.9% saline phosphate buffer followed by 10% formalin solution (Sigma–Aldrich, Taufkirchen, Germany). The brain was removed and post-fixed in 10% formalin and balanced in sucrose solution 30% in PBS. After dehydration, the brains were cryo-sectioned in 30um slices. Next, the sections containing ARC and VMH were processed by immunohistochemistry for pSTAT3 and p62. The samples were balanced in PBS 1X buffer, treated for 10 min in 0.3% oxygen peroxide solution in PBS, and then incubated for 60 min in a nonspecific binding blocking solution containing 5% normal donkey serum. Then the sections were incubated for 48h (4ºC) with rabbit anti-pSTAT3 (Tyr705 – cell signaling, 30835S) (1:1000) or rabbit anti-SQSTM1/p62 (1:1000, cell signaling, 23214S) antibodies (Cell Signalling Technology, MA, USA). Subsequently, brain sections were incubated for 1h in a solution containing biotinylated donkey anti-rabbit antibody (1:400, Vector Laboratories, Newark, California) and after washing, incubated for 1h in ABC solution (PK-6100, Vector laboratories). The reaction was revealed using a commercial DAB kit (SK-4100, Vector

laboratories). Finally, the sections were placed over the slides, DPX (44581, Sigma) was applied, and the slides were coverslipped for further examination using a Nikon Eclipse C2si Confocal microscope. The images were captured in 10x for better identification of the region, and later 25x for better analysis of the right and left sides. The number of pSTAT3 or p62 positive cells was obtained by counting the black nucleus or brow cytoplasmatic staining, respectively. The timeline referring to all protocols performed is summarized in figure 1.

2.2.11 Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to verify normality and the Levene test to verify homogeneity. Outlier tests (Grubb´s test) were performed and, when found, these points were omitted as significant outliers for the analysis in the process. When data from the same animal was consistently identified as an outlier by the software in different parameters, data concerning this animal was removed from all the analysis and not considered as part of the study. For comparison between the CT and HF groups, the Student's test was performed for independent samples. The One-Way ANOVA test was used to examine the effects of the interventions in HF diet-fed animals on the other parameters studied. When the analysis indicates significance, the Bonferroni *post hoc* test was used. The level of significance adopted was $p \leq 0.05$. Statistical analyses were performed in GraphPad Prism 8 software.

3. RESULTS

3.1 The combination of IF and physical exercise was more efficient in the control of hormonal parameters in comparison with these interventions alone

The HF group had higher body weight (Figure 2A), AUC for ITT (Figure 2D), insulin (Figure 2E), leptin (Figure 2F) and resistin (Figure 2G) serum levels compared to the CT group. When the HF group was compared to the HF-T, HF-IF, and HF-IFT groups, the mice submitted to the different interventions had lower body mass (Figure 2A), calorie intake (Figure 2B), and AUC for ITT (Figure 2D). The HF-IF and HF-IFT groups had lower serum insulin levels compared to the HF group. Also, the HF-IF group had lower levels compared to the HF-T group (Figure 2E). The HF-IFT group had lower serum leptin levels compared to the HF group. In addition, the HF-T group had lower levels compared to HF, HF-IF, and HF-IFT groups (Figure 2F). The HF-T, HF-IF, and HF-IFT groups had lower serum resistin levels compared to the HF group (Figure 2G). The HF-IFT group had lower serum levels of Tnf-alpha compared to the HF and HF-IF groups (Figure

2H). In summary, only the HF-IFT group led to a reduction in the four evaluated serum parameters compared to the HF group.

3.2 The combination of IF and physical exercise reduces SOCS3 and increases pFOXO1 and pJAK2 proteins in the hypothalamus of HF diet-fed mice

The HF group had lower hypothalamic content of Atg7, pFOXO1, and pJAK2 (Figures 3A, 3B, and 3F, respectively), but higher hypothalamic content of SOCS3 (Figure 3D) compared to the CT group. The HF-IF group had lower content of Atg7 compared to the HF-T and HF-IFT groups (Figure 3A), and lower content of Beclin compared to the HF-T group (Figure 3B). In addition, the HF-IF group had a lower level of TLR4 compared to HF and HF-IFT groups (Figure 3E), and a lower level of SOCS3 compared to the HF-T group (Figure 3D). The HF-IFT group had a higher content of pJAK2 compared to the HF, HF-T, and HF-IFT groups (Figure 3F).

3.3 Restoring pSTAT3 expression in the ARC and increasing in VMH after leptin stimulation in HF diet-fed mice submitted to the combination of IF and physical exercise

After intraperitoneal leptin stimulation, we observed increased pSTAT3 expression in ARC of the CT group compared to the HF group. Interestingly, after leptin administration, the HF-IFT group presented a higher expression of pSTAT3 in ARC compared to HF, HF-T, and HF-IF groups (Figure 4A). In addition, we also observed increased pSTAT3 expression in the VMH of the HF-IFT group compared to the HF, HF-T, and HF-IF groups (Figure 4B) after leptin treatment.

3.4 The expression of p62 in the arcuate hypothalamic nucleus was increased in response to IF, physical exercise, and the combination of both interventions

Regarding p62, after intraperitoneal leptin stimulation, we observed increased expression of this protein in the ARC of the HF-T, HF-IF, and HF-IFT groups compared to the HF group (Figure 5A). In the VMH, leptin stimulation increased p62 expression in the HF group compared to the CT group. In addition, we observed higher p62 expression in the VMH of the HF-IFT group (Figure 5B) compared to the HF group after leptin treatment.

3.5 The different interventions modify hypothalamic mRNA expression of peptides involved in energy homeostasis control

The HF-T group showed higher hypothalamic mRNA expression of *Agrp* compared to the HF group (Figure 6A). Interestingly, we observed that the HF-IF and HF-IFT groups showed higher mRNA expression of the *Pomc* in the hypothalamus compared to the HF group (Figure 6C). In

addition, the HF-T and HF-IF groups showed higher mRNA expression of *Lepr* compared to the HF group (Figure 6D). There was no difference in the expression of *Npy* mRNA between the experimental groups. (Figure 6B).

DISCUSSION

In the current manuscript, we demonstrated the combination of IF and physical exercise reduced body mass and calorie intake, as well as increased peripheral insulin sensitivity, which can be linked to decreased hypothalamic inflammation, and leptin sensitivity improvement. Although both interventions used separately also counteract the negative effects of HF diet consumption on energy and glucose homeostasis, neither one was effective in normalizing hypothalamic leptin sensitivity. Despite initially seeming a dispensable effect, since the IF and physical exercise ameliorates body weight and ITT independent of the re-establishment of hypothalamic leptin signaling, it is important to emphasize that the modulation of hypothalamic leptin actions is crucial to the appropriate control of the pathways involved in the regulation of energy and glucose homeostasis, as well as motivated behavior [30]. Therefore, the defect in modulating this neurocircuitry could negatively affect the maintenance of body weight loss after reducing or interrupting the interventions.

The beneficial effects of intermittent fasting on body weight and plasma glucose levels in obese individuals or obese rodent models were previously reported in other investigations [31-33]. In addition, we observed that those changes in high-fat diet fed mice submitted to IF were associated with reduced plasma insulin and resistin levels. Consistent with the increased insulin sensitivity in the HF-IF group, we verified reduced plasma insulin levels. It is known that resistin is linked to insulin resistance development via TLR4 pathway activation [5, 34]. Thus, the reduction in the serum levels of this hormone can participate in the improvement of insulin sensitivity and weight loss.

It is important to point out that the partial decrease of hypothalamic inflammation in the IF mice, which is represented by the reduced amount of TLR4, also seems to be associated with the increased *Pomc* [34] and *Lepr* mRNA expression, which could account for the effects on the reduction of food intake and glycemia. Saturated fatty acids are abundant in high-fat diets, and it is well known that these lipids can bind to TLR4 and cause hypothalamic inflammation [35, 36], slowing metabolism and favoring energy storage. The disturbance in TLR4 signaling is associated with protection from weight gain and obesity-associated insulin resistance [36-38]. Similar to our

findings, it was described that IF can reduce the content of TLR4 in the hippocampus of animals treated with LPS [18]. Therefore, the partial reduction in hypothalamic inflammation associated with the increased expression of *Lepr* mRNA can facilitate the leptin actions on ARC neurons, which in turn can contribute to the increased *Pomc* mRNA expression.

POMC neurons have traditionally been related to anorexigenic properties [39]. Despite evidence that arcuate POMC neurons are a heterogeneous neuronal population (i.e., part of POMC neurons coexpress NPY and AgRP) [40, 41], studies using DREADDs to modulate these neurons reported reduced food intake and body weight after their selective activation [42, 43]. However, in mice fed a high-fat diet and submitted to four weeks of IF (24h fed/24h fasting), divergent results of changes in *Pomc* mRNA expression were described (15, 21). Furthermore, it has been reported that selective activation of POMC neurons in the ARC reduces pyruvate-induced hyperglycemia [44]. However, other data are showing no effect [45] or increase [46] in glycemia after activation of POMC neurons. Our findings corroborate the positive effects of IF on energy and glucose balance.

Despite acute fasting protocol being associated with increased activation/mRNA expression of NPY/AgRP neurons [46], the chronic IF effects appear to be distinct from those observed after an acute fasting session. Zhang *et al*. [16] compared the differences in NPY expression after a short (13 days) or prolonged IF protocol (42 days). The authors reported that mice submitted to IF for 13 days had an increase in NPY expression and food intake, while the NPY expression content returned to baseline values in the group that fasted for 42 days, suggesting an adaptation of these neurons [15]. Our data corroborate the hypothesis that a chronic application of IF leads to a later adaptive response in the expression of NPY, as well as AgRP.

Similar to IF, physical exercise has been used as a nonpharmacological strategy for the treatment of obesity and diabetes [47]. The effects of physical exercise on energy and glucose homeostasis can be attributed, at least in part, to the increase in energy expenditure and metabolic rate [48]. In our investigation, we verified an improvement in leptin and resistin levels with a slight effect on the hypothalamic function. Leptin levels are proportional to the energy stores and, therefore, the reduction in this parameter can be probably associated with decreased body mass [49]. Hyperleptinemia is required for the development of leptin resistance [50]. Despite we have observed no difference in activation of hypothalamic pSTAT3 in the HF-T group, its reduced

plasma levels may be involved in the changes in body mass and glycemia. We consider the reduced resistin levels also contributed to the beneficial effects mentioned above.

Recently, Ropelle and coworkers [51] reviewed the chronic effects of physical exercise on hypothalamic homeostasis restoration, contributing to the reduction in body mass and increase in insulin sensitivity in high-fat diet fed mice. In the current investigation, we observed increased mRNA expression of *Agrp* and *Lepr* in the hypothalamus of mice submitted to physical exercise. Indeed, Marinho *et al*. [20] reported that chronic endurance exercise prevented hypothalamic inflammation by reducing SOCS3 and TNF-alfa contents, as well as increasing IL-10 in obese mice.

Evaluating the combination of intermittent fasting with physical exercise is the main novelty of this investigation. Therefore, we verified this combined intervention restored energy and glucose homeostasis, which were accompanied by the improvement of all hormones/cytokine analyzed parameters. Knowing peripheral low-grade inflammation is associated with obesity, the reduction in the Tnf-alpha systemic levels can be linked to inflammation improvement, favoring the peripheral actions of insulin and leptin [52]. As previously described, the hypothalamic resistance to leptin signaling interferes with the appropriate control of energy and glucose balance. After binding and activating its receptor, leptin recruits/phosphorylates JAK2 that in turn phosphorylates and activates different pathways [53]. STAT3 activation is essential to the effects of leptin on energy homeostasis. Indeed, STAT3 deletion specifically in Lepr-positive neurons causes obesity similar to that observed in mice deficient in leptin or Lepr [54]. Herein, we demonstrated that only the mice performing both IF and physical exercise increased hypothalamic pJAK2 protein levels and had higher phosphorylation of STAT3 in the ARC and VMH after leptin stimulation.

It is well known that in the ARC, leptin can inhibit *Agrp* and *Npy* mRNA expression [55], as well as their activities [56]. Interestingly, in HF-fed animals, there is persistent activation of NPY neurons and a failure of leptin to reduce AgRP/NPY neuronal activity [57, 58]. Prolonged or chronic feeding with a lipid-rich diet also decreases the immunoreactivity for AgRP in the paraventricular nucleus [59], an essential brain area involved in the control of endocrine and autonomic responses. Regarding POMC neurons, it was reported that about 30% of them can be activated by leptin [60, 61]. In mice fed for 20 weeks with an HF diet, there was no release of alpha-MSH and AgRP after leptin stimulation [57]. However, these animals have a reduced ratio

of alpha-MSH/AgRP secretion, indicating a predominating effect of AgRP on MC4r signaling [57]. Finally, it was recently demonstrated that the STAT3 pathway in VMH neurons plays a relevant role in protection against high-fat diet-induced obesity [62], improvement in insulin sensitivity, and glucose uptake in skeletal muscle [63]. Given the consistent effect of the combination between IF and physical exercise on the recovery of leptin sensitivity in the ARC and VMH, additional studies must be performed to determine the length and extension of its effects on energy and glucose homeostasis.

The phosphorylation of FoxO1, which results in nuclear to cytoplasmic translocation of this transcriptional factor, is also important to the control of metabolism [64]. In the ARC, nuclear FoxO1 acts to increase *Agrp* reporter activity, while inhibiting *Pomc* reporter activity [65]. Interestingly, in our study, we observed increased pFoxO1 in mice that were submitted to the combination of intermittent fasting and physical exercise. It was described that delivery of adenovirus encoding a constitutively nuclear mutant FoxO1 (mutation of three Akt-mediated phosphorylation sites) into the ARC of mice increased food intake, body weight, plasma leptin, and insulin levels [66]. The increased FoxO1 activity also inhibits the ability of leptin to suppress food intake and *Agrp* mRNA expression, with no effect on *Pomc* transcription [65]. However, it was described that nuclear FoxO1 ablation in POMC neurons decreases food intake, while mice selectively expressing a constitutively nuclear FoxO1 in POMC neurons are obese and hyperphagic [67, 68]. FoxO1 is also expressed in VMH neurons [65, 69], where specific deletion of FoxO1 in Sf1 neurons induces a lean phenotype associated with increased energy expenditure [69]. In addition, these animals have improved glucose tolerance due to increased insulin sensitivity in skeletal muscle and heart [69]. The current increase of hypothalamic pFOXO1 can also be involved in the improved leptin sensitivity and mRNA expression in the ARC of HF-IFT mice.

The imbalance between activation and suppression of the autophagic pathway in the central nervous system is associated with dysregulation of body energy homeostasis and the development of obesity and other metabolic disorders [70]. The low-grade inflammation characteristic of obesity is associated with the modulation of the inflammatory and autophagic pathways [71, 72]. An essential autophagic protein, also related to leptin sensitivity, is the p62 [73], which is highly expressed in arcuate POMC neurons. It was demonstrated that p62-/- mice develop hyperphagic behavior and alteration in the distribution of brain STAT3, limiting the action of leptin [73]. The current increase of p62 in ARC may be involved in the evidenced POMC mRNA elevation in the

HF-IF and HF-IFT groups. On the other hand, the p62 accumulation associated with a blockage or even a reduction of autophagosome formation, installing a pathological p62 accumulation [74], was reported in POMC neurons in Atg7 knockout mice (7,34).

In the present study, the three interventions led to a higher accumulation of p62 in the ARC compared to the HF group, which we understand to be a positive effect due to leptin action, considering that all the interventions were efficient in reducing caloric ingestion and body weight gain induced by the high-fat diet. This data combined with the lower levels of serum leptin indicates a positive effect of the training protocol applied alone or combined with IF in reestablishing the hypothalamic autophagic responsiveness to leptin action. Regarding the increase in p62 in the VMH in the HF group, we understand it as a negative adaptation since there was STAT3 reduction in ARC after intraperitoneal application of leptin compared to the CT group, and also lower values of Atg7 in the physiological state (i.e., without leptin stimulation) which is related to autophagosome malformation and increased p62.[75] These data corroborate the investigation of Portovedo *et al*.[76], who observed hypothalamic autophagic pathway impairment after 16 weeks of high-fat diet intake[76].

CONCLUSION

Our study supports the use of IF, physical exercise, and their combination as important interventions for the treatment of obesity/diabetes, either in combination with drugs or as the primary therapy. Despite being equally effective in terms of improving body weight and insulin sensitivity, only the combination of both interventions was able to reduce hypothalamic inflammation and improve leptin sensitivity in both ARC and VMH. The recovery of molecular hypothalamic homeostasis suggests the combination of both interventions as a more effective therapy to face metabolic disorders. Figure 7 summarizes the main results of the present investigation.

Authors' contributions

LCO, ASRS, and RR designed the paper. LCO, ASRS, and RR wrote the paper. LCO, GPM, FPO, MMM, ASCV, and ALR performed the experiments and/or data collection. LCO and GPM designed the figures of the manuscript. LCO, GPM, FPO, MMM, ALSCV, ALR, LLKE, GRT, CM, DEC, ERR, LPM, JRP, ECF, RR, and ASRS contributed to data analysis, discussion, and/or supported financial costs. All authors have read and approved this manuscript.

FIGURE CAPTIONS

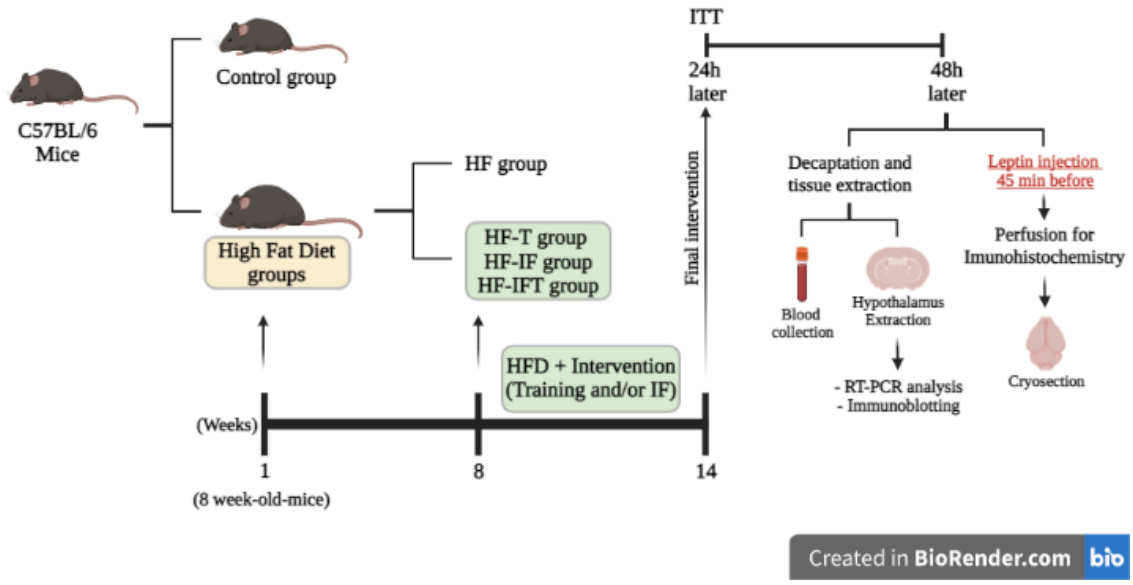

Figure 1. Experimental design. The 8-weeks-old mice were fed a control or high-fat diet for 8 weeks. In week 8, part of the HF group was subjected to three days of adaptation tests to the exercise models, followed by incremental load tests. The animals were subjected to IF and/or physical exercise from week 9 to the end of week 14. The ITT was performed one day after the interventions ended, and the animals were decapitated or perfused from sample collections the next day. Created with Biorender.com.

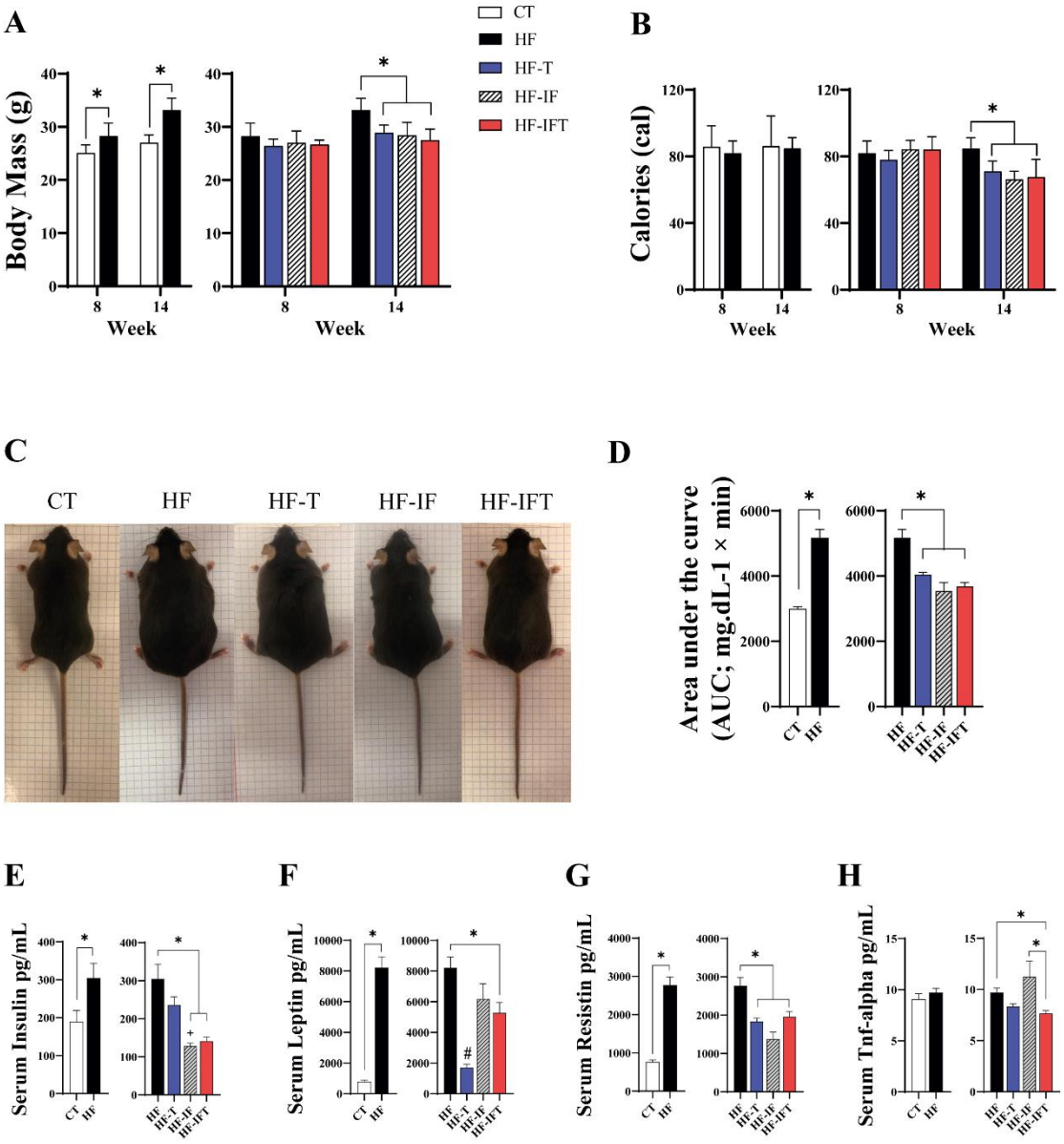

Figure 2. Metabolic and hormonal parameters in control diet (CT) and high-fat diet (HF) fed animals submitted to intermittent fasting (HF-IF), training (HF-T), and intermittent fasting combined with training (HF-IFT). (A) Body mass and (B) calorie intake in week 8 and week 14, (C) representative images of the mice of the different groups, (D) area under the curve of ITT, (E) serum insulin, (F) serum leptin, (G) serum resistin and (H) serum Tnf-alpha. The data are expressed as the mean ± SEM; n= 4-6 mice per group for serum levels and ITT; n= 10-12 mice per group for body mass and caloric ingestion. $^{*}p\leq0.05$, $^{+}p\leq0.05$ vs. HF-T $^{\#}p\leq0.05$ vs. vs HF, HF-IF and HF-IFT.

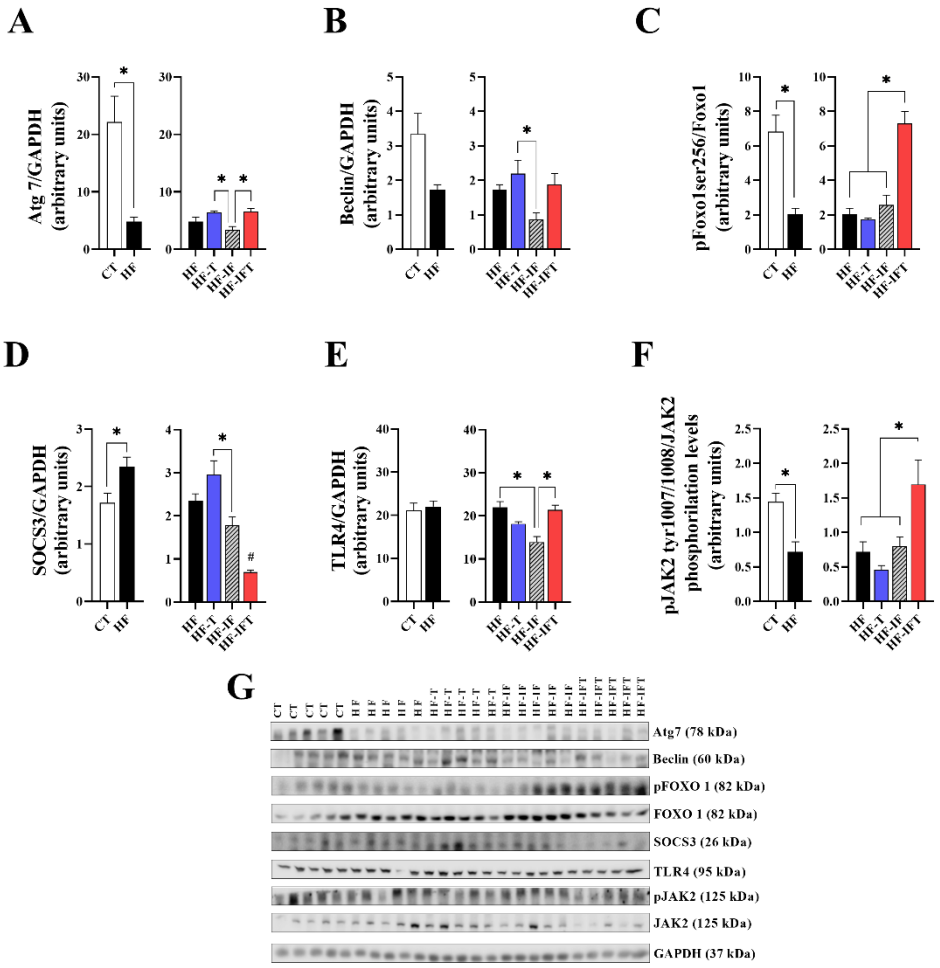

Figure 3. Analyses of total content and protein phosphorylation in the hypothalamus of animals fed with control diet (CT) and high-fat diet (HF), as well as animals fed with HF diet and submitted to intermittent fasting (HF-IF), training (HF-T), and intermittent fasting combined with training (HF-IFT). Hypothalamic protein levels of (A) Atg7/GAPDH, (B) Beclin/GAPDH, (C) phospho-FOXO1 (pFOXO1)/FOXO1, (D) SOCS3/GAPDH, (E) TLR4/GAPDH, and (F) phospo-JAK2 (pJak2)/JAK2. The bands represent immunoblotting experiments to evaluate the hypothalamic response (arbitrary units). Loading was evaluated by re-probing the membranes with anti-GAPDH. Representative membranes of the experiments (G). The data are expressed as the mean ± SEM; n = 4 - 5 mice per group. $^{*}p \leq 0.05$ and $^{\#}p \leq 0.05$ vs. HF, HF-T and HF-IF.

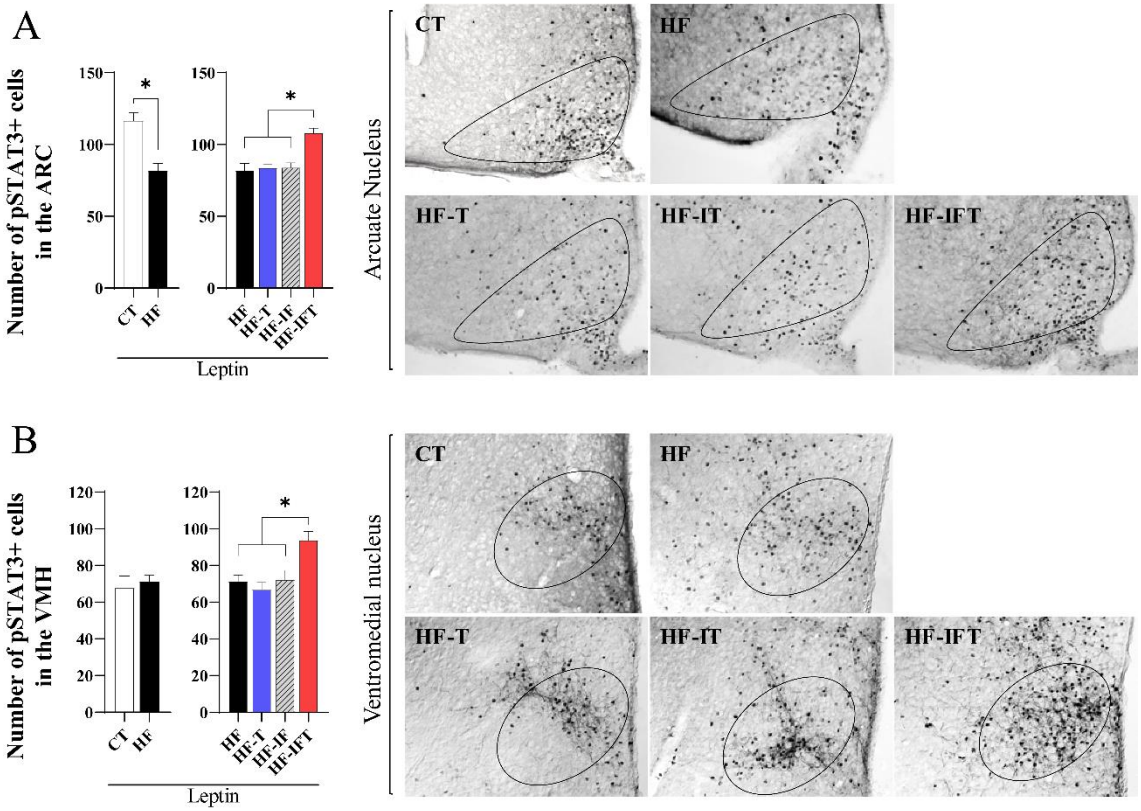

Figure 4. STAT3 phosphorylation in the arcuate (ARC) and ventromedial (VMH) nuclei of control diet (CT) and high-fat diet (HF) fed animals submitted to intermittent fasting (HF-IF), training (HF-T), and intermittent fasting combined with training (HF-IFT) that received intraperitoneal injection of leptin (1µg.g-1, i.p.). (A) The number of pSTAT3+ cells in ARC and (B) VMH nuclei. The data are expressed as the mean ± SEM; n = 6-8 mice per group. $^{*}p \leq 0.05$. Representative photomicrographs of pSTAT3 immunostaining in the ARC (upper right panel) and VMH (bottom right panel) of all the groups.

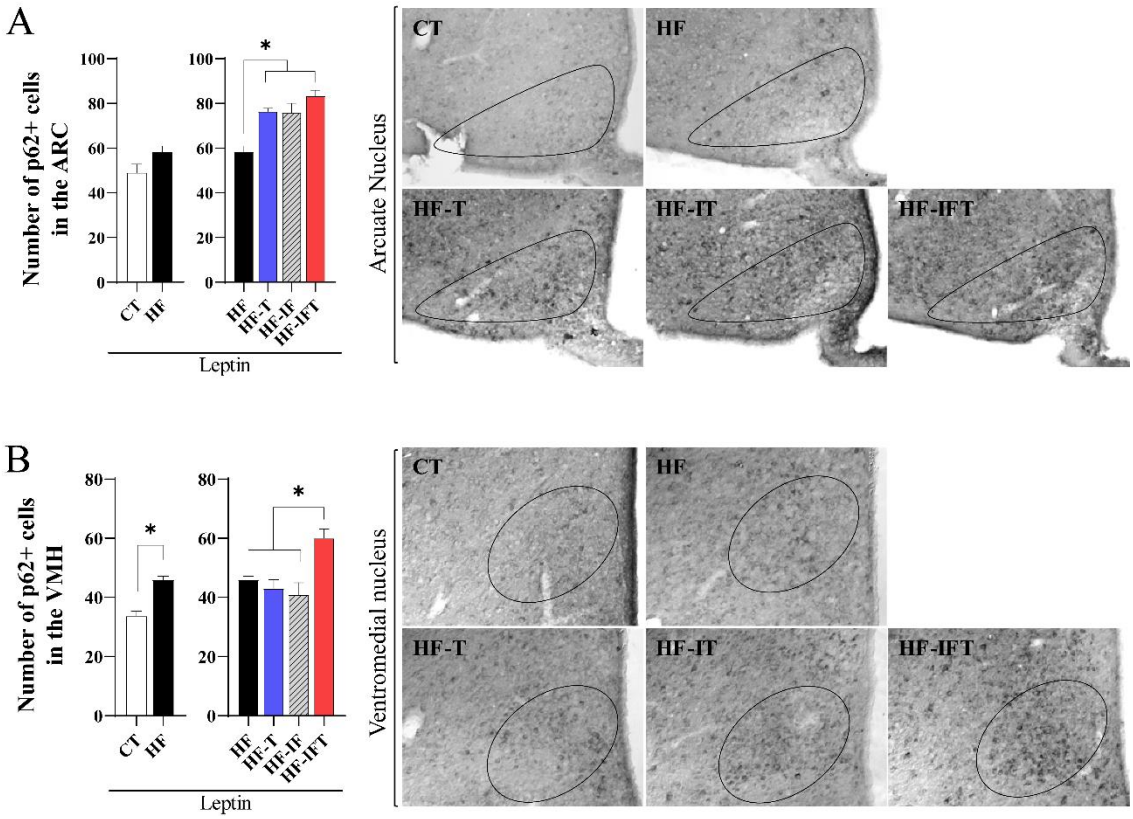

Figure 5. P62 immunoreactivity in the arcuate (ARC) and ventromedial (VMH) nuclei of control diet (CT) and high-fat diet (HF) fed animals submitted to intermittent fasting (HF-IF), training (HF-T), and intermittent fasting combined with training (HF-IFT) that received intraperitoneal injection of leptin (1µg.g-1, i.p.). (A) The number of p62+ cells in ARC and (B) VMH nuclei. The data are expressed as the mean ± SEM; n = 5–8 mice per group. $^{*}p \leq 0.05$. Representative photomicrographs of p62 immunostaining in the ARC (upper right panel) and VMH (bottom right panel) of all the groups.

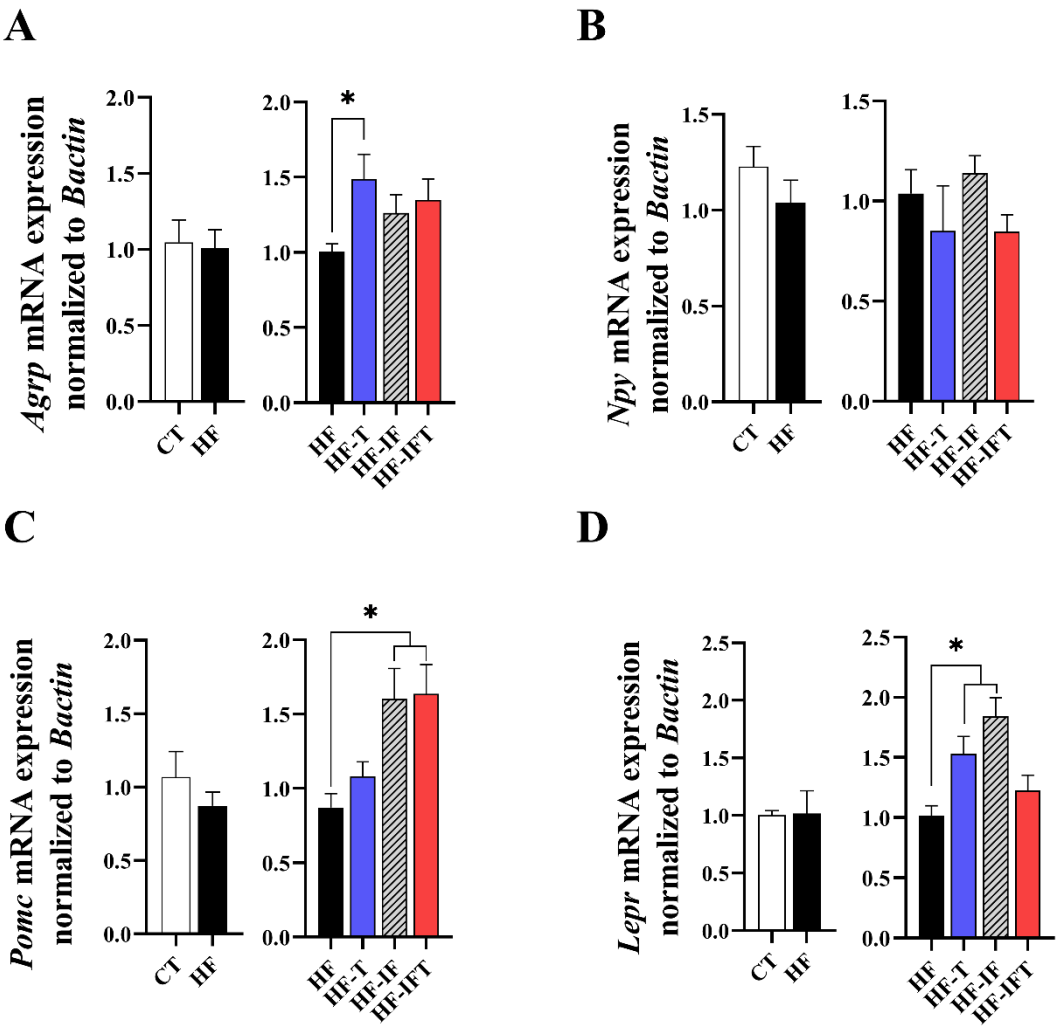

Figure 6. Relative mRNA expression in the hypothalamus of control diet (CT) and high-fat diet (HF) fed animals submitted to intermittent fasting (HF-IF), training (HF-T), and intermittent fasting combined with training (HF-IFT). (A) *Agrp*, (B) *Npy*, (C) *Pomc*, and (D) *Lepr* mRNA expression in the hypothalamus. The data are expressed as the mean ± SEM; n = 8 mice per group. $^{*}p \leq 0.05$.

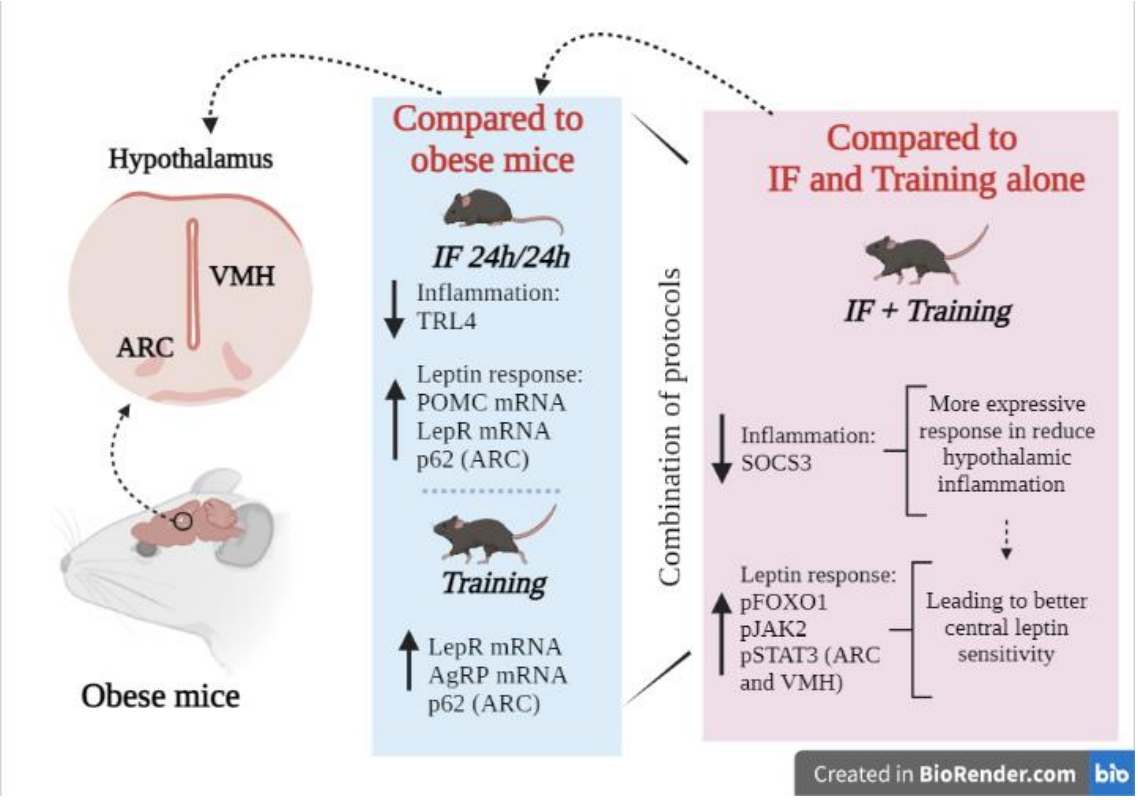

Figure 7. Schematic representation summarizing the main findings of the present study. Compared to the HF group, the groups that performed isolated IF or physical exercise presented distinct hypothalamic effects that may have contributed to appetite control and resistance to weight gain induced by the HF diet. The combination of IF and exercise leads to lower hypothalamic SOCS3 content that may be associated with the higher hypothalamic pJAK2 content and expression of pSTAT3 in the ARC and VMH compared to the HF-IF and HF-T groups, which brings up the hypothesis that the protocol may have more expressive molecular effects on central resistance to leptin. Created with Biorender.com.

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