POLYPHENOL EFFECTS ON CENTRAL LEPTIN SENSITIVITY IN OBESITY

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Polyphenol effects on central leptin sensitivity in obesity

Doctoral Thesis

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NUTRIGENOMICS RESEARCH GROUP

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FAIG CONSTAR que aquest treball, titulat "Polyphenol effects on central leptin sensitivity in obesity", que presenta Maria Ibars Serra per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a la Menció Internacional de Doctorat .

HAGO CONSTAR que el presente trabajo, titulado "Polyphenol effects on central leptin sensitivity in obesity", que presenta Maria Ibars Serra para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de esta universidad y que cumple los requisitos para poder optar a la Mención Internacional de Doctorado .

I STATE that the present study, entitled "Polyphenol effects on central leptin sensitivity in obesity", presented by Maria Ibars Serra for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university and that this thesis is eligible to apply for the International Doctorate Mention.

Tarragona, 30 de Juny 2017
Tarragona, 30 de Junio 2017
Tarragona, 30th June 2017

El/s director/s de la tesi doctoral
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La vida és experimentar i aprendre a arriscar-se, tot i no saber com sortirà. Crec que per això vaig endinsar-me en el món de la ciència pel qual tenia molta curiositat sense saber massa com aniria i que m'ha portat a fer un doctorat. Si hagués fet cas a aquells que es pensen que tenen una bola de vidre per poder llegir el futur i que tenen molt clar pel que vals i pel que no, ara no estaria fent el que realment m'agrada. Volia entendre més bé la vida, literalment, i estudiar Biologia. Per aquesta etapa de decisions complicades, que ara m'han conduït fins on sóc, vull agrair als meus pares, el seu suport incondicional, i per recordar-me sempre que un ha de fer el que creu que li agrada sempre que pugui, tenint en compte que la dedicació i l'esforç seran la clau per aconseguir-ho i que si no surt bé sempre hi ha alternatives. Per a guanyar coses sempre hi ha part de sacrifici i per això vaig haver de deixar la música que m'havia acompanyat des dels 6 anys, sortir de la zona de confort i anar a totes. Així va ser, en acabar la carrera vaig buscar un grup especialitzat en compostos naturals i salut i vaig marxar un any a Irlanda del Nord, a comprovar si realment el que havia estat estudiant durant quatre anys era com em pensava.

I would like to mention Dr. Chris Gill from University of Ulster (Northern Ireland Centre for Food and Health), to give me that great opportunity to participate in your research, it was the first time I heard the word polyphenols! Thank you for being so helpful during that year. Those were my first steps towards a PhD. Thanks to Noella, Tanya, Kate, Chris, Kevin and Gary; you were my family and friends thank you for your patience, lab hours doing the comet assay, laughs, Guinness and the good "craic"!! Dr. Francina Baldrick and Dr. Adrian McCann, you inspired me with your hard work, thank you for your help, I learned a lot from you.

Després d'un any de massa pluja i vent i investigant una mica, vaig descobrir el Màster de Nutrició i Metabolisme de la Universitat Rovira i Virgili. Va ser

parlar amb la Dra. Cinta Bladé que gràcies al seu entusiasme ho vaig tenir clar, faria el màster i qui sap? potser després el doctorat. Recordo la primera reunió amb el Dr. Lluís Arola, gràcies per deixar-me formar part del grup de Nutrigenòmica i per proposar-me un projecte tant interessant que he pogut continuar més enllà del màster. A partir d'aquí vaig conèixer els que han sigut els meus tutors de tesi al màster i que he tingut la sort de poder conservar durant el doctorat. Dra. Cinta Bladé i Dr. Gerard Aragonès, m'heu guiat, ensenyat, ajudat, aconsellat, i m'heu fet veure el costat positiu de les coses (dels experiments!) i reconduït en moments de dubte. Moltes gràcies, tot el que he pogut aprendre té un valor incalculable. També vull agraïr-vos la llibertat que m'heu donat a l'hora de decidir i opinar sobre els meus projectes. Gràcies a això vaig poder triar un laboratori als Estats Units per a fer l'estada i poder viure el que en diuen "the american dream" durant set mesos. Mesos de somnis fets realitat i també de "hard-work".

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Humphrey Bogart, “sempre ens quedarà París”, amb les crêpes i la felicitat de quan vam anar de congrés. Tota l’energia positiva que transmets segur que l’aprofitaràs fins la recta final!!

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Perquè quasi tot és possible comptant amb la Niurka i la Rosa, sou un exemple d’organització i eficiència i també un referent per a sobreviure al laboratori, espero sortir d’aquí i que se m’hagin encomanat algunes d’aquestes habilitats. No sé si seré capaç de combinar tot això amb la “chispa i sabrosura” que aporteu però ho tindré molt present!

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Gràcies

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Als meus pares, Josep i Emi, al Joan i al Feliu

&

To Jorrit,

So close despite the distance

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"The real discipline is not imposed.

It can only come from within ourselves."

Tenzin Gyatso

The 14th Dalai Lama

Nobel Peace Prize 1989

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CONTENT

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SUMMARY

Obesity is an increasing health problem and a major risk factor for a number of chronic diseases. Up to now, strategies to reduce and prevent obesity were unsuccessful. Therefore, novel approaches to treat obesity need to be developed. In this sense, several animal and human studies demonstrate that polyphenols protect against metabolic disorders including diabetes and cardiovascular disease. Thus, polyphenols emerge as bioactive compounds useful to reduce obesity and its associated metabolic diseases. Energy balance is regulated by leptin in the central nervous system, particularly in the hypothalamus where it activates POMC and inhibits AgRP neurons to produce satiety and promote energy expenditure. However, leptin action appears to be suppressed in obesity which is reflected by increased appetite and reduced energy expenditure. The aim of this thesis was to identify polyphenols that improve leptin sensitivity under obesogenic environments, which could ultimately result in a loss of body weight. We show that a chronic intake of a grape seed proanthocyanidin extract improves leptin signaling by increasing POMC gene expression and reduces food intake without decreasing body weight in obese animals. Furthermore, we investigated other polyphenols that could complement the effects of proanthocyanidins by enhancing body weight loss. Our results show that high doses of resveratrol effectively reduce body weight, fat mass and correct hyperleptinemia in obese animals acting as a leptin sensitizer compound. Additionally, we demonstrate the potential of seasonal fruits rich in polyphenols to modulate hypothalamic leptin signaling and downstream effectors in normal conditions and during obesity. Finally, the role of a novel target to modulate AgRP neurons activity is explained. The outcome of this research provides insights into the design of functional foods that combine bioactive compounds which could potentially be used as anti-obesity therapy.

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I. INTRODUCTION

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1. Obesity and body weight regulation

1.1. Obesity

Obesity and overweight are defined as excessive fat accumulation that increases health risks. Most recent reports indicate that 39% of adults are overweight and 13% obese [1]. Furthermore, childhood obesity rose by approximately 47% in last three decades. There is a high diversity depending on the world region and socioeconomic status. Developed countries had the highest increase rates but this trend is changing and obesity in the developing world is markedly rising [2]. The fact that obesity is associated with high all-cause mortality globally[3] and that up to now approaches to reverse obesity did not succeed [4], underscores the need to take action in preventing this disease.

Many studies focus on the causes of obesity. The genetics of obesity have been broadly investigated. Twin studies weather raised in similar environments or reared apart showed a high correlation for body mass index (BMI) and substantial genetic influence for obesity[5–7] Also,, the effect of genetic influence and family environment on the BMI of adopted children have been examined, reinforcing the ideathat genetics plays an important reole on the BMI [8]. Thes and other studies show that 40-70% of the variance corresponds to genetics and 30% to the environmental factors[7,9].Therefore, an obesogenic environment has also a critical impact on the high prevalence of obesity although it remains subject to genetic predispositions[4,10–13].

Most studies agree that the high prevalence of obesity is due to polygenic disorders rather than a single gene mutation[14]. However, the study of single gene mutations in rodents has been very useful to understand some of the mechanisms of the development of obesity[15].

The main genes involved in monogenic mutations that produce an obese phenotype are the ones encoding for leptin[16], leptin receptor (LEPR)[17], carboxipeptidase E[18], prohormone convertase I[19], proopiomelanocortin (POMC)[20] and the melanocortin receptor 4 (MC4R)[21,22]. The majority of these mutations share a common link. They are relevant to the efficacy of leptin signalling and melanocortin pathway which play a key role on body weight regulation, as demonstrated by many studies [23–26] (explained in next sections of this introduction).

However, the rapid increase in obesity in such a brief period of time suggests that genetic disorders are not the main cause[1427]. The high calorie intake, diet composition changes and decrease in physical activity are undoubtedly promoting the obesity epidemic[122]. Continuous research and development of therapeutic strategies as well as effective public health measures are needed. Thus, the mechanism of body weight regulation is essential to solve the obesity problem.

1.2. Body weight regulation

Energy homeostasis is the balance between energy intake, energy expenditure and energy storage. In most adults, although there is a large variation in daily food intake and energy expenditure, body weight is almost constant, which depends on the regulation of the balance between energy intake, in the form of food and drinks, and energy expenditure, in the forms of basal metabolism, physical activity and adaptive thermogenesis[28,29].

Adipose tissue serves as a crucial integrator of energy homeostasis, because a host of regulating hormones for energy balance can be secreted from the

adipose tissue (namely adipocytokines)[30]. Traditionally, adipocytokines are categorized as pro-storage of energy including resistin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and retinol-binding protein-4 (RBP-4), and anti-storage of energy such as leptin, adiponectin, visfatin and omentin[31] **(Figure 1).** However, the effect of some of these molecules is still controversial depending on the target tissue[32]

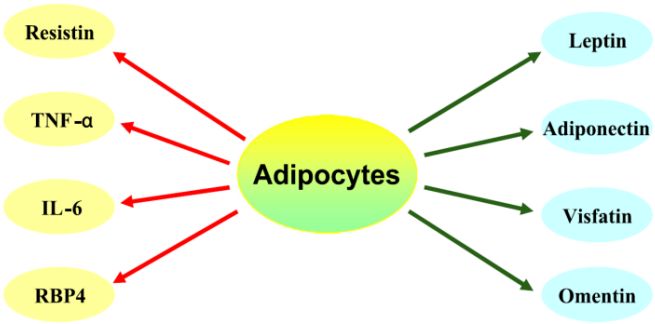


Figure 1. Adipocyte derived secreted proteins. [33]red arrows represent energy storage proteins and green arrows anti-storage proteins. Abbreviations: tumor necrosis factor-α, TNF-α; interleukin-6, IL-6; retinol-binding protein-4, RBP-4.

1.2.1 Brain regulation of food intake and energy expenditure

Most peripheral signals for energy homeostasis, mainly the adipocyte-derived regulating proteins, regulate various types of neuropeptides, which, in turn, modulate energy homeostasis and contribute to the stable state of body weight[34].

The hypothalamus and the brainstem are key areas for the homeostatic control of food intake. Additionally, the reward circuits mediated by neurons in the limbic system, responsible for eating behaviour are important for the acquisition of macronutrients and maintenance of energy homeostasis [35].

The energy regulation occurs through the central melanocortin system, which is a well-described neuronal pathway that receives hormonal and nutritional signals and produces neuronal responses to control food intake and energy expenditure[36,37].

The lesions of specific areas in the brain interfering with the signalling pathways will result in the energy metabolism disorders conducing to the development of obesity and related diseases such as diabetes[38,39].

The brain centres regulating energy homeostasis are the arcuate (ARC), ventromedial (VMH), lateral hypothalamus (LHA), dorsomedial (DMH) and paraventricular (PVN). However, the arcuate nucleus is especially important on the regulation of food intake since it integrates peripheral and brainstem signals[40].

1.2.2 Hypothalamic arcuate nucleus

The ARC is positioned at the base of the 3rd ventricle, above the median eminence (ME) where the blood brain barrier (BBB) is semi-permeable. The ME has fenestrated capillaries that selectively allow the flow of blood-borne compounds. This feature makes the neurons of the ARC highly susceptible to peripheral changes[39,40]. Importantly, the ARC contains neurons co-expressing neuropeptide Y and agouti-related protein (NPY/AgRP) and proopiomelanocortin and cocaine amphetamine-related transcript (POMC/CART) which are part of the melanocortin system[36]. These are called first-order neurons that will respond to the circulating signals and in turn project to second-order neurons located downstream[41].

POMC neurons express and synthetize POMC protein that is further cleaved producing biologically active peptides including adrenocorticotropine (ACTH), and α, β, γ-melanocyte-stimulating hormone (MSH)[37]. Among the melanocortins, the α-melanocyte stimulating hormone (α-MSH) is a peptide with an anorexigenic effect. This peptide acts as an agonist of melanocortin receptor type 3 and 4 (MC3/4R) in various hypothalamic nuclei to reduce food intake and energy expenditure[42].

Studies on the MC3 and MC4 receptors demonstrate different implications on the regulation of energy stores. Only MC4 deficiency produces severe obesity and hyperphagia, in contrast MC3 deficient mice are not obese, but present a higher feed efficiency and almost twice the amount of fat mass with decreased lean mass[43]. Human studies also show that mutations in MC4R produce a rapid development of obesity in early life[44]. Therefore, MC4R has a central role in food intake regulation whereas MC3 is involved in fat stores regulation but its mechanisms of action need further investigation[43,45].

On the other hand, AgRP neurons co-express NPY together with AgRP. Both neuropeptides contribute to increase food intake and promote weight gain, it's been reported that fasting increases mRNA levels of both molecules whereas POMC mRNA levels would be decreased in that condition. Importantly, these neurons are only found in the ARC[46]. AgRP acts as endogenous antagonist of MC4R and inverse agonist of the constitutive activity of MC4R[47]. AgRP shows long-term effects on food intake beyond 24h period to several days, not seen in other orexigenic neuropeptides and agrees with the hypothesis that melanocortins exhibit a tonic inhibition on food intake[48]. Powerful effects on food intake are also produced by NPY although in a different manner. NPY has higher effects on the first 24h compared to AgRP, inducing a short-term feeding response[49]. The signaling of NPY acts through Y (Y1, Y2, Y4, Y5, y6)

receptors and produces inhibitory responses. The Y1 receptors are involved in feeding-induced hyperphagia and together with the Y5 in the PVN they behave synergistically to control energy homeostasis[50,51].

POMC and AgRP neurons have opposite effects on energy balance and both neurons are regulated by leptin, which is the hormone that plays a central role on the regulation of energy homeostasis[52]. Olofsson et al. [53] demonstrated that 70% of AgRP compared to a 10% of POMC neurons are outside the blood brain barrier and are more sensitive to changes in the periphery such as circulating leptin levels.

2. Leptin

The word “leptin” originates from the Greek root “leptos”, which means “thin”, and it was discovered in 1994[54]. Leptin, a 16 kDa polypeptide and the product of the OB gene, is a cytokine-like circulating hormone produced and secreted predominantly by the adipocytes in the white adipose tissue (WAT), acting through their receptors (LEPRs) on specific populations of neurons in the brain, including the hypothalamic, midbrain, and brainstem neurons, and whose main function is to regulate energy stores[55]. Mirroring the body's fat stores, leptin plays a crucial role in the regulation of numerous neuroendocrine functions, from energy homeostasis to a variety of additional processes such as reproduction[56], bone function[57], cardiovascular regulation[58] and immune function [59]. Leptin decreases body weight by both suppressing appetite and promoting energy expenditure by directly targeting the hypothalamic neurons, including the increased expression of the anorexigenic peptide α-MSH, which is derived from POMC cells, and the decreased expression of the orexigenic

peptide neuropeptide Y (NPY) and agouti-related peptide (AgRP) [60–62] **(Figure 2).** Interestingly, besides the central regulation of energy balance exerted by leptin secreted from the adipose tissue, leptin produced in the stomach is gaining interest. Gastric leptin increases in response to a meal and several studies indicate that it might play a role on short-term regulation of food intake[63].

Figure 2. Neuronal mechanisms in energy homeostasis triggered by leptin. Abbreviations: arcuate nucleus, ARC; Agouti-related protein, AgRP; cocaine amphetamine-related transcript, CART; neuropeptide Y, NPY; melanocortin

receptor 4, MC4R; leptin receptor isoform B, OBRB; proopiomelanocortin, POMC; paraventricular nucleus (PVN); neuropeptide Y receptor Y1, Y1; neuropeptide Y receptor Y5, Y5.

2.1 Factors influencing leptin secretion

Various factors influence leptin secretion and expression. The most important factors are the distribution of fat and the status of its energy stores because leptin is mainly expressed in the adipose tissue, and circulating leptin concentrations in the fed state are highly correlated with the degree of adiposity[64,65]. Leptin expression also correlates with feeding and insulin level, as evidenced by the fact that insulin triggers leptin expression directly in isolated adipocytes[66] and enhances leptin levels when injected into rodents[67], as well as the discovery that decreased leptin levels accompanied with low insulin resistance states and circulating leptin concentrations increased after insulin treatment[68]. Glucocorticoids directly induce hyperleptinemia and stimulate leptin synthesis *in vitro* and *in vivo*[69]: For example, leptin expression increases in response to the chronic elevation of cortisol in humans[70]. Glucose and/or its metabolites play permissive roles in the secretion and expression of leptin[71] and leptin signalling[72], and glucose dose-dependently enhances leptin signalling and leptin sensitivity, at least in part, by attenuating the ability of AMP-activated protein kinase (AMPK) to inhibit leptin signalling. Circulating free fatty acids (FFAs) serve as suppressors of leptin secretion that may be associated with the hyposensitivity to food caused by FFAs in circulation[73]. The administration of thyroid hormone decreases leptin levels in rodents, but that is not a major determinant of plasma leptin levels[74]. Meanwhile, there is an interaction effect between leptin, sex hormones and growth hormones, which is likely to result in metabolic disorders involving sex hormones and growth retardation in obese adolescents[75]. Additionally, infections, endotoxins and some cytokines

stimulate leptin synthesis and secretion reported by several studies[76]. In summary, there are many influencing factors contributing to the level of expression and secretion of leptin that provides a wide field for further studies on leptin **(Figure 3).**

Figure 3. Diagram for leptin synthesis, secretion, biological actions, leptin resistance mechanisms and therapies[33]. Notes: +++: high correlation; ++: positive correlation; +: permissive role; − −: inhibition; −: Negative regulation; ●: on study; ▲: require further trials; FFAs: free fatty acids; ER: endoplasmic reticulum; ObRb: leptin receptor b.

2.2 Leptin signalling pathway

Plasma leptin has a central effect on the regulation of feeding behaviour and energy expenditure by activating the hypothalamic leptin receptors (LEPRs or

OBRs). The LEPR, the product of the *Lepr* gene, is a member of the class I cytokine receptor family, and has at least six splice variants, *Obra–Obrf*[77]. Notably, the long receptor OBRB mediates essentially all known physiological effects of leptin in energy homeostasis[78], because genetic deficiency of *Obrb* results in pronounced hyperphagia and morbid obesity in animals[79].

As depicted in **Figure 4**, leptin binding to the domain of extracellular of OBRB triggers the domain of activated box-1 and amino acid at 31–36 chain on OBRB, and the recruitment of the tyrosine kinase Janus kinase-2 (JAK2), resulting in the phosphorylation and activation of JAK2[80]. Activated JAK2 phosphorylates three tyrosine residues in the cytoplasmic domain of OBRB, which includes Tyr985, Tyr1077 and Tyr113[81]. Of these residues, phosphorylated Tyr1138 (pY1138) recruits signal transducer and activator of transcription 3 (a transcription factor, STAT3), which becomes phosphorylated by JAK2[82]. Phosphorylated STAT3 (pSTAT3) then homodimerizes and translocates to the arcuate nucleus in the hypothalamus, where it increases the expression and neuronal excitability of POMC and inhibits that of AGRP and NPY[83]. cause the inhibition of appetite and increasing energy expenditure[84]. This complicated physiological process is regulated by both positive and negative regulators. The negative regulators act as feedback inhibitors of leptin signalling by binding to pY985 and preventing the activation of the JAK2/STAT3 pathway, including suppressor of cytokine signalling protein-3 (*S*OCS3) [85] and protein tyrosine phosphatase 1B (PTP1B)[86].

In addition to the JAK2/STAT3 pathway, leptin mediates its effects through other pathways in the brain and the periphery. Noteworthy the phosphatidylinositol 3-OH kinasa (PI3K) pathway mediates acute leptin effects by targeting forkhead box protein O1 (FoxO1) and the mammalian target of rapamycin/S6 kinase (mTOR/S6K), factors with a relevant role on the

regulation of energy balance[87]. After the activation of the leptin receptor, JAK2 will recruit insulin receptor substrate (IRS) that will activate PI3K and produce anorexigenic effects through the phosphorylation and inactivation of FoxO1, a transcription factor that induces *Agrp/Npy* and inhibits *Pomc* expression. Similarly, PI3K will activate mTOR, a nutrient sensing molecule that contributes to leptin's anorexigenic effect via a reduction of AMPK activity[88,89]. Furthermore, another pathway that is activated by leptin involves the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinaseS (ERK). It is achieved by the SH2-containing protein tyrosine phosphatase 2 (SHP2) that binds pY985 and will recruit growth factor receptor-bound protein 2 (GRB2). All this will contribute to the anorectic effects of leptin[90].

There is limited information about the role of leptin in the periphery. OB-Ra receptors have been detected in several peripheral organs mainly in lungs, kidneys, and lymph nodes and to a lower extent in WAT, liver and skeletal muscle. Similarly OB-Rb can be found in these peripheral tissues but in lower levels compared to OB-Ra. This suggests that leptin has a peripheral action[91]. Additionally, *in vitro* studies with different cell lines and *in vivo* studies in rats demonstrated the existence of leptin signalling in peripheral tissues and, consequently, the actions that mediates at the periphery in different organs. Leptin is able to activate STAT1 and STAT3 and in a lower degree MAPK and PI3K in skeletal muscle, WAT and liver. The role of leptin at the periphery seems to be related with the regulation of adiposity and glucose homeostasis. Referring to glucose metabolism, there are evidences of a cross-talk between leptin and insulin. For instance, in skeletal muscle leptin increases glucose sensitivity and in WAT has opposite effects compared to insulin promoting lipolysis[92].

Figure 4. Leptin signalling pathway and downstream effectors[89](adapted). Abbreviations: AKT, protein kinase B; AMPK, AMP-activated protein kinase; AgRP, agouti-related protein; extracellular signal-regulated kinase, ERK; extracellular signal-regulated kinase, ERK; growth factor receptor-bound protein 2, GRB2; IRS insulin receptor substrate, JAK Janus kinase, Leptin receptor isoform b, LepRb; mammalian target of rapamycin, mTOR; PI3K phos- phatidylinositol 3-OH kinase; POMC, proopiomelanocortin; PTP1B, protein-tyrosine phosphatase 1B; S6K, S6 kinase; SH2-containing protein tyrosine phosphatase 2, SHP-2; SOCS3 suppressor of cytokine signalling 3; STAT signal transducer and activator of transcription

2.3 Leptin and photoperiod

Seasonal changes in light cycles exert an influence on animal's physiology in order to adapt to new conditions, providing an evolutionary benefit to guarantee the survival of the species. Photoperiod effects are mediated by the hormone melatonin which acts in the Hypothalamic-Pituitary-Thyroid axis and affects neuroendocrine systems that control growth, reproduction and energy balance[93].

Leptin secretion and signalling is affected by photoperiod as supported by several studies. In this sense, seasonal rodents such as Siberian hamster (*Phodopus sungorus*) and field vole (*Microtus agrestis*) develop leptin resistance as an adaptive response to overcome the challenges of different seasons. In addition, rat strains sensitive to photoperiod, such as Fisher 344 (F344) rats, increase their body weight in long-day (LD), like summer and spring, whereas reduce it during short-day (SD). Like winter and autumn[93–95]. Thus, .leptin resitance, may occur under physiological conditions such as in seasonal animals or during pregnancy.

Importantly, seasonal leptin resistance in Siberian hamster is characterized by an increase on inhibitors of the leptin signalling cascade, such as SOCS3 and PTP1B. Particularly SOCS3 is thought to be the molecular switch to adapt the physiology to the new season demands[94]. Interestingly, other species like field vole and sheep also increase Socs3 gene expression in LD, when food is abundant and easily available. This is accompanied by an increase in body adiposity that, in turns, increases plasma leptin concentration. This increase of serum leptin levels is not associated with the anorectic effect of this hormone, rather than a mechanism to induce energy storage to achieve future needs during SD season[94,95].

Photoperiod also affects neuropeptides that are regulated by leptin. Mercer *et al.* reported that Siberian hamster exposed to SD displayed a repression of *Pomc* and an overexpression of *Agrp* in the ARC, whereas *Npy* was not affected[96]. Besides, leptin gene expression in adipose tissue and OBRB in the ARC were downregulated. The possible biological meaning is the convenience of this modification to counteract the negative energy balance state that Siberian hamster is undergoing in SD periods[97]. In contrast, studies from Ross *et al*. using F344/N rats a sub-strain more sensitive to photoperiod, described an

overexpression of *Npy* and repression of *Agrp* without *Pomc* modification in the ARC in response to SD[98]. Remarkably, NPY has been shown to act as a negative regulator of growth axis. Thus, the overexprresion of NPYagrees with the growth reduction and the low mRNA levels of growth hormone releasing hormone (GHRH) displayed in this sub.stari of rats during SD. Furthermore, F344/N rats reduce the food intake in SD,which is consistent the repression of AgRP [93].

Other studies have focused on the effect of photoperiods on F344 rats fed a high-fat diet. In this situation, the gene expression of neuropeptides in the ARC nucleus and blood leptin levels are unaltered by photoperiod and. Therefore, it is remarkable that the HFD that developed obesity do not allow any changes in orexigenic and anorexigenic neuropeptides in the ARC under each of two photoperiods[93].

In conclusion the function of hypothalamic neuropeptides in different photoperiods cannot always be anticipated by its known role in energy balance. This introduces new questions of how leptin works in a physiological context involving different photoperiods. The study of these models could give valuable insights on how to reverse leptin resistance in obesity.

2.4 Leptin sensitivity

Noticeably, the reduced abilities of peripheral leptin and central OBRB to collaborate in the suppression of appetite and the promotion of energy expenditure can be taken as crucial risk factor for the development of obesity. Therefore, elevated peripheral leptin level is likely to control obesity. However, the hope that leptin might be a “miracle cure” in the treatment of obesity was

remote. Rather than being leptin deficient, most obese humans and animals have high levels of circulating leptin[99,100],and increased leptin fails to prevent the development of obesity[101–103]. Meanwhile, treatment with leptin alone in obese individuals fails to counteract common obesity[104]. This apparent leptin ineffectiveness and hyposensitivity has been identified as leptin resistance. A large number of studies demonstrated that most individuals with diet-induced obesity manifest leptin resistance characterized by increased leptin levels in the blood and decreased leptin sensitivity[105–110]**.**

In view of the great influence of leptin resistance on the development of obesity, it is an important subject for treating obesity to elucidate the mechanisms of leptin resistance. Currently, several non-genetic mechanisms have been proposed to explain leptin resistance, including defective leptin transport across the blood–brain barrier (BBB) and attenuation of leptin signalling, ER stress, inflammation, loss of sirtuin 1 activity and others[89,111]**(Figure 5).**

Figure 5. Cellular mechanisms of leptin resistance[89](adapted). Abbreviations: IL-6R, interleukin 6 receptor; IRS insulin receptor substrate, JAK Janus kinase, Leptin receptor isoform b, LepRb; myeloid differentiation primary response gene 88, MyD88; mammalian target of rapamycin, mTOR; NF-kB, nuclear factor kappa-light-chain enhancer of activated B cells; IKK-b IKK-e, inhibitors of nuclear factor kB; PI3K phos- phatidylinositol 3-OH kinase; PTEN, phosphatase and tensin homolog; PTP1B, protein-tyrosine phosphatase 1B; SFA, saturated fatty acid; SOCS3 suppressor of cytokine signalling 3; STAT signal transducer and activator of transcription; TCPTP, tyrosine-protein phosphatase non-receptor type 2; Toll-like receptor 4, TLR4; TNF-R, tumor necrosis factor receptor 1.

2.4.1 Defective transport of leptin across the BBB

The results of a large number of studies displayed that obese individuals exhibit high peripheral leptin levels but relatively lower cerebrospinal fluid (CSF) concentrations, suggesting that defective leptin transport into the central nervous system (CNS) across the BBB is part of the mechanism of leptin

resistance[112–117]. Indeed, leptin mediates the inhibition of appetite and increased energy expenditure only when circulating leptin is transported across the BBB and ultimately binds to its receptors initiating the signals for energy homeostasis in some hypothalamus neuron populations. Triglycerides act as the regulators for the process because decreasing triglycerides can strengthen the anorectic effect of leptin by enhancing leptin transport across the BBB[118]. Acute phase C-reactive protein (CRP) is also likely to contribute to leptin resistance by preventing leptin from crossing the BBB[119].

The process of leptin transport balance is regulated by two LEPRs, OBRA and OBRE. OBRA mediates leptin transport across the BBB [117,120] OBRE inhibits leptin transport by counteracting the function of OBRA[121]. Under normal conditions, the actions and number of these two receptors are in balance. Thus, subsequent studies are needed to clarify the equilibrium correlation between OBRA and OBRE under normal and obese states.

2.4.2 Attenuation of OBRB signalling

The attenuation of OBRB signalling is mainly due to two parallel molecular mechanisms including the up-regulation of suppressor of cytokine signalling (SOCS3) in the cytoplasm and that of protein tyrosine phosphatase-1b (PTP1B) in the endoplasmic reticulum. Both of them are all involved in the regulation of OBRB signalling pathway, particularly JAK2/STAT3.

SOCS3 is a critical protein that inhibits the signal transduction process of various cytokines in the body, including leptin. The most crucial event for the inhibition of appetite and increased energy expenditure is the leptin-mediated STAT3 phosphorylation. This factor increases POMC expression as well as

inhibits the activities of NPY and AGRP. By binding to Tyr985 of OBRB and JAK2, SOCS3 inhibits the leptin-induced phosphorylation of STAT3 signalling through a feedback negative mechanism. Mice with deletions of *Socs3* in the whole brain or in POMC neurons are resistant to diet-induced obesity[122,123]. In addition, the incidence of diet-induced obesity and leptin resistance is decreased in rats with hypothalamic *Socs3* silencing by RNAi[124].

When obesity is accompanied by hyperleptinemia, or decreased response to leptin administration, we use the term "leptin resistance" [125]. However, studies from Ottaway *et al.* [126]reveal that responsiveness to endogenous leptin is preserved in hyperleptinemic DIO mice therefore, maintaining leptin-mediated suppression of food intake. Their findings suggest that the reduced OBRB signalling is due to the increase in SOCS3 levels instead of decreased leptin action or a defect on OBRB functionality[126]. Referring to this, Myers et al. stated that leptin resistance could be defined as the failure of pharmacologic (exogenous) leptin to increase OBRB signalling and physiologic responses in obesity[127]. Agreeing with this hypothesis, others propose that pSTAT3 activated neurons in DIO mice would produce a constant response to endogenous leptin.. Nevertheless, the role of the STAT3 phosporylation in leptin resistance is contradictory. Whereas some groups show that DIO mice present high pSTAT3 levels, compared to lean controls, others found decreased pSTAT3 levels in obesity[87]. Why obesity develops leptin resistance in animals that fully respond to leptin is a question that remains to be answered. Therefore, new approaches are needed to study the mechanisms to increase leptin sensitivity.

PTP1B is a class 1 non-receptor protein tyrosine phosphatase , which is attached to the cytoplasmic face of the endoplasmic reticulum [128]. PTP1B binds to and dephosphorylates JAK2, thereby inhibiting leptin signalling[129]. A high-fat diet is accompanied by elevated *Ptp1b* expression, suggesting that PTP1B

may play a crucial role in the aetiology of leptin resistance[130]. Both systemic, neuron-specific and POMC neuron-specific deletions of *Ptp1b* improve leptin sensitivity and protect individuals from diet-induced obesity[131,132]. The exact correlation between the expression and activity of PTP1B and leptin resistance remains unclear; however, this particular correlation is the focus of gene therapy for leptin resistance in the future.

3. Polyphenols

Polyphenols are plant secondary metabolites that despite not being essential for growth and development they play important roles protecting against UV radiation, herbivore aggressions, providing defence against pathogens, or acting as pollinators attractants among other functions[133].

Polyphenol consumption has been linked to beneficial effects on health[134,135]. Interestingly, a 5 year follow up of PREDIMED study shows that a polyphenol rich-diet is negatively associated to body weight and obesity in elderly subjects at high-cardiovascular risk[136]. Furthermore, there are evidences that polyphenols are able to cross the BBB and may have neuroregulatory actions on energy expenditure and food intake which are negatively associated to diet-induce obesity in animal and human studies[137]. There is limited information about the mechanisms of action of these bioactive compounds on leptin signalling pathway. Therefore, it is a promising research field, since it could bring interesting insights on how to prevent and reduce obesity.

In this section the structure and classification of the main classes of polyphenols are presented. Besides, polyphenols used on the studies contained in this thesis

will be discussed in more detail from a health perspective, focusing on their effects in energy balance and leptin signalling pathway.

3.1 Structure and classification

Phenolic compounds and polyphenols have one or more aromatic ring with a hydroxyl group respectively. Flavonoids and non-flavonoids are usually referred to as polyphenols. Nonetheless, a few compounds mentioned, including phenolic acids, have only one phenolic ring. In this section the term polyphenols will be used indistinctively. They range from low to large molecular weight and are usually conjugated with sugars and organic acids. They are classified in two main groups: **flavonoids** and **non-flavonoids**. According to the number of phenol rings and the structural elements binding the rings different subgroups can be designated **(Fig. 6).** Polyphenols occur in plant-derived foods and beverages[138].

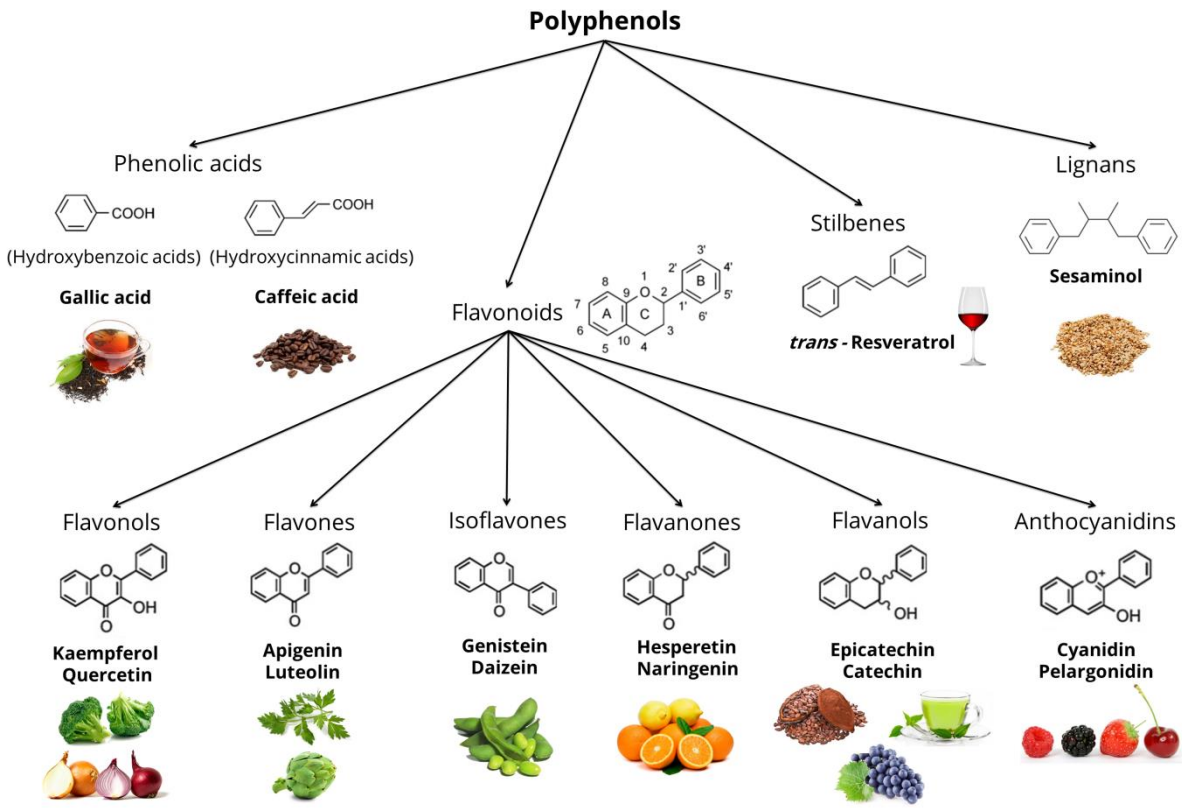


Figure 6. Basic structural skeletons of phenolic compounds. Representative molecules of each subgroup and dietary sources.

3.1.1 Phenolic acids

Phenolic acids are the main non-flavonoids that are considerably abundant in diet. They can be divided into hydroxybenzoic acids (protocatechuic and gallic acid) and hydroxycinnamic acids (coumaric, caffeic and ferulic acid). Among them, gallic acid is the most common form and is mainly found in black tea, green tea, grapes and wine, as non-sugar galloyl ester. Ellagic acid and ellagitannins are also present in a variety of fruits including raspberries, strawberries and pomegranate and are usually glycosylated. Hydroxycinnamic

acids are also named chlorogenic acids when esterified with tartaric or quinic acid. Coffee and grapes represent important sources[133,139].

3.1.2 Stilbenes

Stilbenes are substances produced by plants as a response to injury, pathogens or stress. They are found in very low amounts in human diet. Resveratrol (3,5,4'-trihdroxystilbene) is the main compound and appears as *cis* and *trans* isomers as well as conjugated with glycosides. *Trans*-resveratrol **(Fig. 7)** is the predominant form and it is mainly found in red wine and peanuts. Minor quantities have been identified in spinach, red cabbage, berries and pistachio. Although its presence in diet is scarce resveratrol is important since numerous health effects have been reported in animal studies using high doses compared to those achieved by a dietary intake[133,138].

Figure 7. Molecular structure of *trans*-resveratrol.

Resveratrol has gained interest in the obesity research field since the evidences suggesting that this molecule could have anti-obesity properties. Resveratrol is a potent activator of sirtuin 1 (Sirt1), a deacetylase involved in energy homeostasis and that is induced by caloric restriction and exercise. Therefore, resveratrol is able to mimic this conditions, that are linked to improved health and negative energy balance[140].

Several mechanisms of action have been described to explain the beneficial effects of resveratrol, involving the regulation of adipogenesis, *de novo* lipogenesis, lypolisis, thermogenesis and fatty acid oxidation in peripheral tissues[141]. However, little is known about the modulation of central energy homeostasis and leptin signalling pathway by resveratrol. Nonetheless,it has been reported that the treatment with resveratrol (30 mg/kg per day) rescue hyperleptinemia and pSTAT3 levels in the ARC of the offspring of high-fat diet, without affecting food intake [142].,Furthermore, in vitro(142) and other vivo(143) studies show reduced leptin levels with resveratrol treatment. In contrast, a mice study using intraperitoneal doses of 100 mg/kg of resveratrol reported supressed food intake and downregulation of *Npy* and *AgRP* genes[143]. Intriguingly, a meta-analysis of randomized controlled trials do not find changes on plasma leptin of obese and non-obese subjects supplemented with resveratrol [144]. Furthermore, leptin concentration in plasma of these subjects was independent of the length of treatment and the dose.[144].

The mechanisms by which resveratrol affects energy homeostasis still need to be clarified; especially, in what refers to leptin signalling pathway in the hypothalamus. For this reason, in this thesis different doses of resveratrol were used to investigate the anti-obesity effects and the modulation of leptin signalling in the hypothalamus in dose-response experiments. This topic will be addressed in **Chapter 2.**

3.1.3 Lignans

Lignans are phytoestrogens, together with isoflavones. Due to structural similarities with estrogens, they can act as agonists or antagonists of estrogen receptors. The parent forms of lignans, such as secoisolariciresinol and

matairesinol, are all converted to enterodiol and enterolactone by human gut microbiota. *In vitro* and *in vivo* studies support a beneficial role of these metabolites. Flaxseed and sesame seeds are rich sources of lignans whereas only traces can be found in grains, cereals, some fruits and vegetables[133,139].

3.1.4 Flavonoids

Flavonoids are the broadest group of polyphenols and are found all through the plant kingdom. They contain a total of 15 carbons with two aromatic rings linked through a three-carbon bridge **(Fig. 6).** The most abundant and principal subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. They are usually conjugated with sugars as glycosides[138].

Flavonols are the most extensive group of flavonoids. The main compounds are kaempferol and quercetin and are usually found forming glycosides with glucose or rhamnose. They are found in different amounts in foods depending on variety or seasonal changes and accumulate in leaves or the skin since their production is enhanced by light. Yellow and red onion are an important source of flavonols[133,138,139].

Flavones include apigenin and luteolin as principal compounds. Structurally flavones are similar to flavonols, but they lack the oxygen at C-3 **(Fig. 6).** They can undergo several modifications such as hydroxylation, methylation, gylcosylation and alkylation. Their distribution is limited to celery, parsley, some herbs and the skin of citrus fruits. Small quantities are found in rooibos tea.

Isoflavones are found in leguminous plants, mainly in soybeans, which contain considerable amounts of daidzein and genistein. Due to their structural

similarity to estrogens, they are considered phytoestrogens as the non-flavonoid lignans. Isoflavones may have pseudohormonal effects since they have the ability to bind estrogen receptors. Glycosidated forms are more common, except in fermented soy products which can be rich in aglycones[133,139].

Flavanones are present in high amount only in citrus fruit. Small amounts are found in tomatoes and some aromatic herbs. The main aglycones are hesperetin in oranges, naringenin in grapefruit, and eriodictyol in lemons. However, they are usually glycosylated, hydroxylated or methylated. The most common form is hesperetin-7-O-rutinoside (hesperidin) that is tasteless. In contrast, neohesperidoside conjugates confer the characteristic taste of bitter oranges. Interestingly, the majority of these compounds are found in the solid parts of citrus fruits, mainly the peel[133,139].

Flavanols, are structurally complex. They occur as monomers such as catechin and epicatechin that can undergo hydroxylation and form gallocatechins and be esterified with gallic acid **(Fig. 8).** Green tea and chocolate are the main sources of flavan-3-ol monomers although they are also present in red wine. Contrarily to other flavonoids they are not glycosylated in foods.

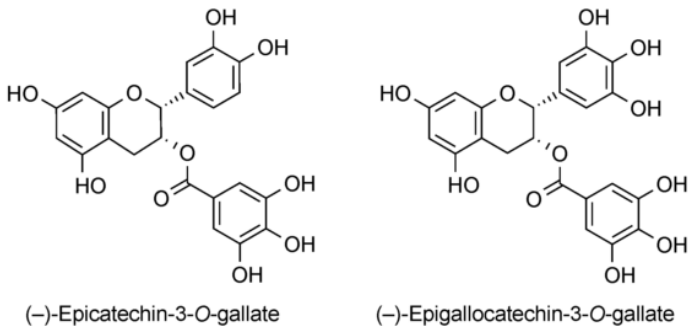


Figure 8. Basic structural skeletons of flavanol monomers[138].

Additionally, they can form oligomers and polymers known as condensed tannins or proanthocyanidins (PACs) **(Fig. 9).** The ones that consist only in (epi)catechin units are named procyanidins and are the most abundant class in plants. Remarkably, proanthocyanidins are the main source of polyphenols in human diet, being present in grapes, apples, pears, kakis, peaches and beverages such as red wine, cider, tea and beer[138,139]. Since proanthocyanidins are abundant in foods, our research group have focused years on the study of these compounds for their potential to improve metabolism and health.

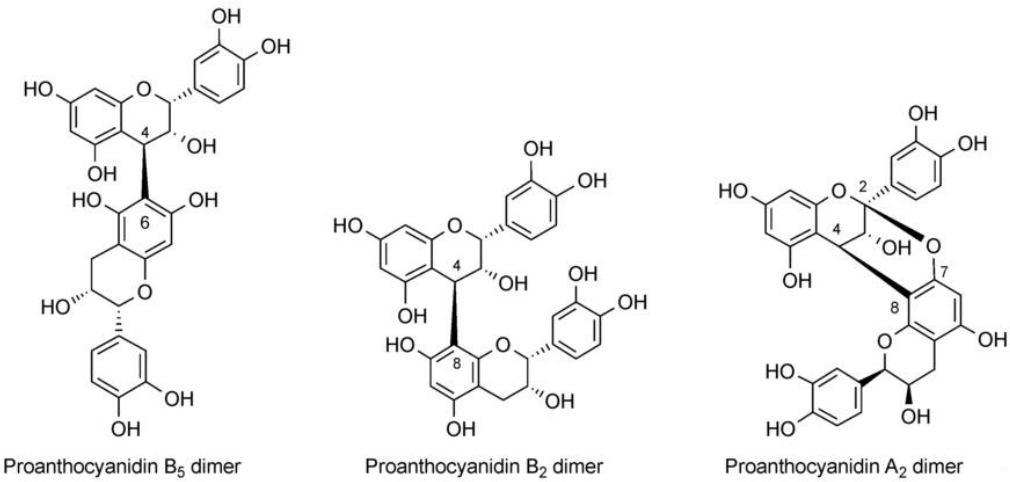


Figure 9. Basic structural skeletons of proanthocyanidins[138].

The Nutrigenomics Research Group has extensively studied the effects of PACs in metabolism, mainly in peripheral tissues. PACs have been studied for its antioxidant, anti-inflammatory and hypolipidemic effects [145]. Specially, it has been reported that a grape seed proanthocyanidin extrat (GSPE) rescues dyslipidemia in HFD fed rats by supressing lipogenesis and increasing cholesterol efflux in liver, reducing fatty acid accumulation[146]. Many of these effects are mediated by the modulation of gene expression through the regulation of the nuclear receptors farnesoid-X receptor (FXR) and Small Heterodimer Partner (SHP), the transcription factor SREBP1 and some microRNAs (mirRNA), such as mirR-33a and miR122 in liver [147,148]. Moreover, GSPE is able to induce lipolysis in 3T3-L1 adipocytes, modulate insulin-signalling pathway in diabetic rats, exerting an anti-hyperglycemic effect, improve muscle oxidative capacity in obese rats and decrease blood pressure in animals with metabolic syndrome [149–153].

According to this data, GSPE is able to improve many of the features of metabolic syndrome such diabetes, dyslipidemia and hypertension. Nevertheless, none of the previous studies have shown that GSPE could a reduce body weight at physiological doses. However, some studies have reported that a high and acute dose of GSPE is able to reduce food intake[154]. . Accordingly, GSPE could exert some of these effects partly via the modulation of leptin signalling in the hypothalamus. Thus, the study of specific mechanisms involving the main factors controlling food intake was required. This topic will be addressed in **Chapter 1.**

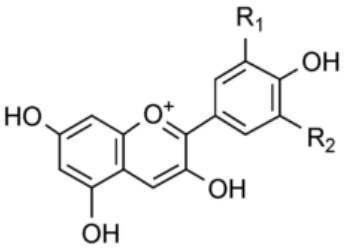
Anthocyanidins are widely distributed in plants and give the red, blue and purple colors characteristic of some fruits and flowers. Cyanidin is the most present in foods, followed by pelargonidin, delphydin, peonidin, petunidin and malvidin. To prevent their degradation, they are forming sugar conjugates in

plants, which are known as anthocyanins, and they can also be esterified with phenolic and organic acids **(Fig. 10).** Dietary intake of anthocyanins comes mainly from red wine and berries, although they are also present in leafy and rood vegetables like aubergines, onion, and cabbage and radish. Usually anthocyanins predominate in skin with the exception of cherries and strawberries where they also occur in the flesh.

Anthocyanins are particularly interesting since some studies show their obesity reducing properties. Prior et al. showed that the supplementation with an anthocyanins-rich extract from blueberry decrease body weight in mice fed a high-fat diet and fat mass mainly due to a decrease in retroperitoneal and epididymal fat pads[155]. Furthermore, serum leptin levels were also reduced. Interestingly, mice that consumed anthocyanins showed a reduction on the ratio of leptin to adipose tissue which suggests a direct effect of this extract on the production of leptin in the adipose tissue[155]. In contrast, another study from the same group, using black raspberry anthocyanins, do not reveal any of the previous effects[156].

Recently, Wu et al reported that high doses of blueberry anthocyanins decrease the body weight by a 19.4% and serum leptin levels of obese mice after 8 week treatment[157] . Another study from the same group, using a high dose of anthocyanins extracted from sweet cherry also showed a 11.2% reduction on body weight decreased serum leptin levels of high-fat diet fed mice [158]. Thus, the source of anthocyanins could be relevant on the capacity of these polyphenols to reduce body weight. Despite to that, the study of anthocyanidins could bring promising insights to target obesity. To date, the anthocyanin effects on hypothalamic regulation of leptin has yet to be investigated since the majority of studies have focused on the effects of anthocyanins on leptin secretion from adipose tissue[159]. Therefore, in this thesis an anthocyanin rich

extract, or fruits rich in these compounds, have been used to investigate their effects to modulate the hypothalamic leptin system. This topic will be addressed in **Chapter 2 and 3.**



Pelargonidin (**35**) $R_1 = H, R_2 = H$
Cyanidin (**36**) $R_1 = OH, R_2 = H$
Delphinidin (**37**) $R_1 = OH, R_2 = OH$
Peonidin (**38**) $R_1 = OCH_3, R_2 = H$
Petunidin (**39**) $R_1 = OCH_3, R_2 = OH$
Malvidin (**40**) $R_1 = OCH_3, R_2 = OCH_3$

Figure 10. Basic structural skeletons of main anthocyanidins and Cyanidin conjugated with rutin[133].

3.2 Intake, bioavailability and metabolism

Bioavailability refers to the proportion of the nutrient that is digested, absorbed and metabolized through physiological pathways. Depending on the structure of the polyphenols the bioavailability will be different. These compounds have to be hydrolyzed by intestinal enzymes or colonic microflora before being absorbed[160].

The absorption can vary among phenolic compounds. F**igure 11** displays the basic mechanism of absorption of flavonoid glycosides in the small intestine. Firstly, the sugar is cleaved by the action of lactase phloridzin hydrolase (LPH) and the aglycone may enter by passive diffusion inside the enterocyte. Alternatively, flavonoids can be hydrolyzed by the cytosolic b-glucosidase

(CBG) once they have been transported inside the cell via sodium-dependent glucose trans- porter 1 (SGLT1)[133]. The body recognizes flavonoids as xenobiotic, so they are subject to phase-II in the small intestine or later in the liver. In the small intestine, specialized enzymes such us sulfotransferases (SULTs), uridinedi- phosphate glucuronosyltransferases (UGT), and catechol-O- methyltransferases (COMTs) will produce sulfate, glucuronide and/or methylated forms[133,161]. Efflux of polyphenols back into the lumen can also occur and involves multidrug resistance proteins (MRP) and the glucose transporter GLUT2[133]. Therefore, polyphenol metabolites reaching systemic blood and tissues are different from dose present in food[160]. The majority of polyphenols reach the maximum concentration in plasma after 1-2h of their ingestion[162,163].

Figure 11. Proposed mechanisms for the absorption and metabolism of polyphenols in the small intestine. CBG, cytosolic b-glucosidase; COMT,

catechol-O-methyl transferase; GLUT2, glucose transporter; LPH, lactase phloridzin hydrolase; MRP1-2–3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenol sulfate/glucuronide/methyl metabolites; SGLT1, sodium-dependent glucose transporter; SULT, sulfotransferase; UGT, uridinediphosphate glucuronosyltransferase[133].

Polyphenols that are not metabolized by small intestine are metabolized by colonic microflora into aglycones and phenolic acids. The rate and site of absorption depends on the chemical structure, degree of glycosylation/acylation, conjugation with other phenolics, size and degree of polymerization[163]. Polyphenol determination in the ileal fluid of ileostomists after ingestion of food rich in phenolic compounds shows that important amounts arrive to the colon were they might play a physiological role[164]. These studies are critical to understand the absorption and metabolism of polyphenols and to elucidate which are the bioactive metabolites that exert beneficial effects.

After their absorption, circulating flavonoids bind to albumin and are transported to the liver where will be metabolized [165]. There are evidences that flavonols and their metabolites may also accumulate in several organs such as heart, lungs, liver adipose tissue and muscle[166]. Finally, the excretion is through xenobiotics detoxification pathway. This restricts the potential toxic effects and helping their biliary and urinary elimination[165].

4. Effects of Polyphenols on leptin signalling

Several studies investigated the effect of polyphenols on leptin signalling pathway in the hypothalamus and the peripheral tissues. Interestingly, some of them demonstrated that certain polyphenols are able to modulate leptin signalling and could potentially increase leptin sensitivity in obesity.

Next, we present a review written by members of the Nutrigenomics Research Group that focuses on bioactive food compounds that interact with leptin system and may improve leptin resistance. Importantly, among the cited compounds are polyphenols and phenolic compounds.

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Modulation of leptin resistance by food compounds

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UNIVERSITAT ROVIRA I VIRGILI
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Abstract

Leptin is mainly secreted by white adipose tissue and regulates energy homeostasis by inhibiting food intake and stimulating energy expenditure through its action in neuronal circuits in the brain, particularly in the hypothalamus. However, hyperleptinemia coexists with the loss of responsiveness to leptin in common obese conditions. This phenomenon has been defined as leptin resistance and the restoration of leptin sensitivity is considered to be a useful strategy to treat obesity. This review summarizes the existing literature on potentially valuable nutrients and food components to reverse leptin resistance. Notably, several food compounds, such as teasaponins, resveratrol, celastrol, caffeine and taurine among others, are able to restore the leptin signaling in neurons by overexpressing anorexigenic peptides (POMC) and/or repressing orexigenic peptides (NPY/AgRP), thus decreasing food intake. Additionally, some nutrients, such as vitamins A and D, can improve leptin transport through the blood brain barrier. Therefore, food components can improve leptin resistance by acting at different levels of the leptin pathway; moreover, some compounds are able to target more than one feature of leptin resistance. However, systematic studies are necessary to define the actual effectiveness of each compound.

1. Introduction

Overweight and obesity, the epidemic of the 21st century according to World Health Organization (WHO) information, are defined by chronic disease with an elevated accumulation of adipose tissue due to an imbalance between energy intake and energy expenditure, which affects both physical and psychosocial health. Currently, obesity is considered a health problem in both developed and developing countries, significantly increasing in prevalence in many nations worldwide with the expectation that more than 300 million people will be obese in 2035. However, presently, there is no successful long-term treatment for obesity, with the exception of bariatric surgery, which is expensive and risky. Thus, society needs innovative anti-obesity strategies that cause a significant reduction in food intake and/or an increase in energy expenditure with higher efficacy, safety, and selectivity. In this sense, the discovery of leptin in 1994 [1] opened a new field within the therapeutic strategies driven to combat obesity, and fortunately, leptin therapy has been found to be relevant for patients with very low leptin or leptin deficiency [2]. However, the administration of leptin is absolutely inefficient in decreasing the body weight of obese humans who are not leptin-deficient but instead have high levels of circulating leptin associated with loss of responsiveness to leptin [3]. This hyposensitivity to leptin is, currently, identified as leptin resistance, and its prevention and treatment could represent a major challenge in obesity research for the next decade.

Understanding the biological function of leptin and its receptors is an important step to identify potential dietary food compounds that could provide new strategies for restoring leptin sensitivity. Accordingly, dietary food compounds, such as amino acids, terpenoids, and flavonoids, act through a variety of mechanisms to improve leptin sensitivity. Herein, we review the roles of dietary

food compounds that have demonstrated a clear improvement in leptin signaling, as well as briefly summarize the latest advances in the molecular mechanisms involved in leptin resistance, to supply new ideas for the management of obesity.

2. Biology of leptin

Leptin, which is a 16-KDa circulating protein with 167 amino acids synthesized from the LEP gene, is mainly secreted by white adipose tissue (WAT) and acts in the brain to regulate energy homeostasis [4]. The quantity of leptin released into circulation is directly proportional to the amount of body fat in the organism, reflecting the status of long-term energy stores [5]. Apart from WAT, there are other tissues with the capacity to secrete leptin, such as the placenta, mammary glands, ovaries, skeletal muscle, stomach, pituitary gland, lymphoid tissue and brown adipose tissue. Notably, leptin is secreted in a pulsatile fashion and displays a circadian rhythm. Its levels fluctuate according to changes in calorie intake, decreasing during starvation and increasing in overfed and obese states [6]. Additionally, women have higher levels of circulating leptin than men because of their higher estrogens levels, which increase the leptin serum concentration; meanwhile, male androgens suppress the leptin serum levels [7]. In addition to sex steroids, circulating leptin levels are also modulated by other hormones, including catecholamine, insulin, glucocorticoids and cytokines[8–10].

To date, six isoforms of the leptin receptor are identified, including five short isoforms (namely LEPRa, LEPRc, LEPRd, LPERe and LEPRf) and one long isoform (LEPRb) [11]. All of the isoforms have an extracellular domain to link leptin, but only LEPRb has the complete intracellular domain required to

activate the cellular signaling cascade of leptin. LEPRb belongs to the gp130 class I cytokine receptor family and is the main receptor implicated in leptin signaling in neurons. The highest expression of LEPRb in the brain is located in the hypothalamus, particularly in the arcuate nucleus and ventromedial hypothalamus. However, LEPRb is also expressed in many extra-hypothalamic brain regions, such as the ventral tegmental area, hippocampus and brainstem [12], as well as in peripheral tissues[13].

After crossing the blood-brain barrier (BBB) through a receptor-mediated process, leptin directly targets two different neuronal populations in the arcuate nucleus, one co-expressing the proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) and the other co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) [14]. Leptin stimulates POMC/CART expression and inhibits AgRP/NPY expression, reducing food intake and increasing energy expenditure, which consequently decrease body weight. In addition, leptin also inhibits feeding by reducing the expression of the melanin-concentrating hormone (MCH) and orexins in the lateral hypothalamic area, as well as by enhancing the expression of brain-derived neurotrophic factor and steroidogenic factor-1 (SF-1) in the ventromedial hypothalamus [15].

Leptin is also implicated in the regulation of other physiological functions, such as glucose and lipid metabolism, reproduction and sexual maturation, thermogenesis, heart rate and blood pressure, the hypothalamic-pituitary-adrenal system, neuroendocrine and neuroprotection functions, thyroid and growth hormones, angiogenesis and platelet-aggregation, hematopoiesis, immune and pro-inflammatory responses and bone remodeling.

3. Molecular leptin signaling

When leptin interacts with LEPRb, the conformational change and dimerization induced in the receptor promote the activation of JAK2 and its auto-phosphorylation. Moreover, JAK2 phosphorylates three tyrosine (Tyr) residues, which include Tyr985, Tyr1077 and Tyr1138, in the cytoplasmic domain of the receptor, activating it and initiating different intracellular signaling pathways.

Phosphorylated Tyr1138 (pY1138) recruits the signal transducer and activator of transcription 3 (a transcription factor, STAT3), which also becomes phosphorylated [16].Subsequently, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to POMC and AgRP promoters, stimulating POMC expression and inhibiting AgRP [17].This is what is known as the JAK2/STAT3 signaling pathway, which is regulated by both positive and negative regulators. The suppressor of cytokine signaling protein-3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) act as feedback inhibitors of leptin signaling by binding to pY985 and preventing the activation of the JAK2/STAT3 pathway [18,19]. However, the Src-homology 2 domain 1 (SH2B1) markedly enhances JAK2 activity, which is conducive to the activation of this signaling pathway [20].

In addition to the JAK2/STAT3 signaling pathway, the activation of LEPRb also activates the extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K) pathways (Figure 1). The ERK pathway is activated through the recruitment of the protein tyrosine phosphatase non-receptor type 11 (PTP11 or also called SHP2) to pTyr985 of LEPRb [21], whereas the activation of the PI3K pathway is mediated by the phosphorylation of insulin receptor substrate 2 (IRS2) [22]. The PI3K pathway also affects the neuronal activity and neuropeptide release of AgRP and POMC neurons [22].

Notably, there is evidence that this pathway inhibits PTP1B and forkhead box protein O1 (FoxO1). FoxO1 stimulates the expression of NPY and AgRP, inhibits the expression of POMC, and blocks STAT3 action in these neurons; therefore, the inactivation of FoxO1 via PI3K allows STAT3 to bind to POMC and AgRP promoters [23].

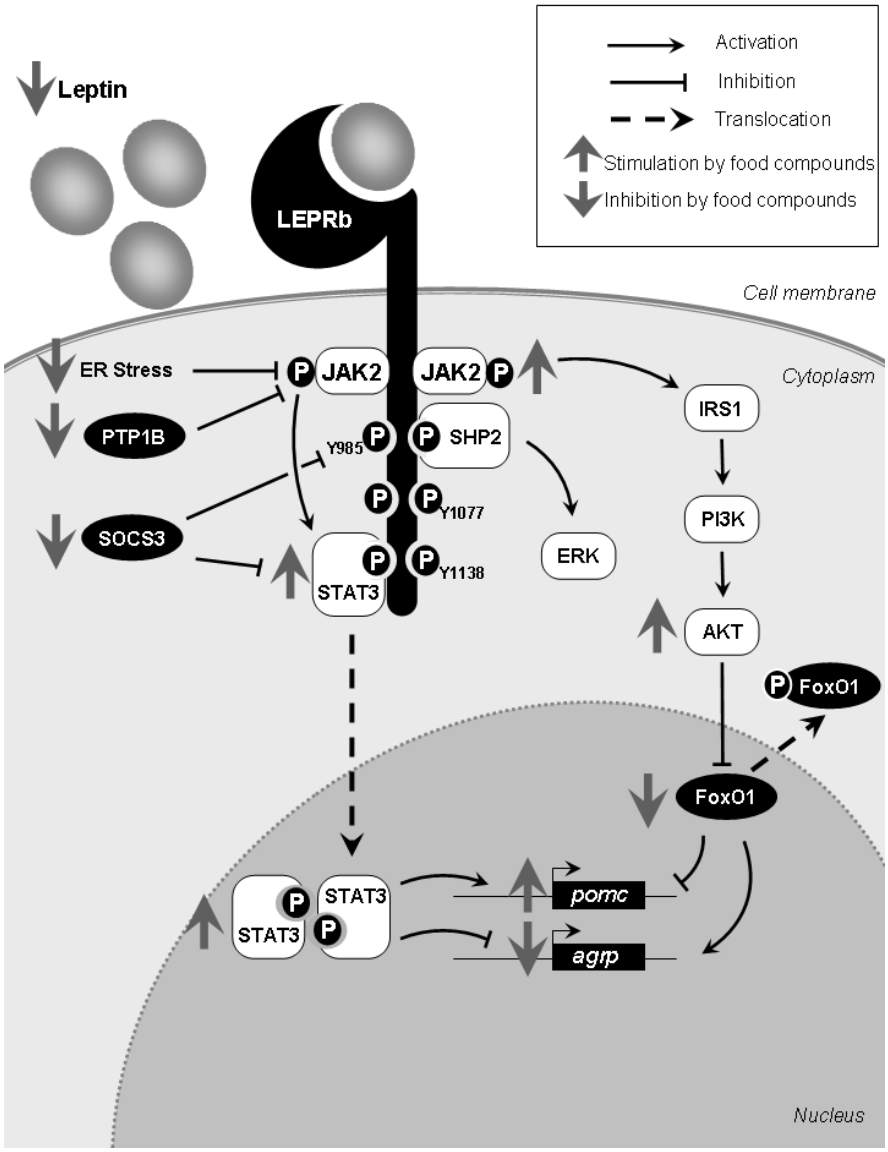

Figure 1: Schematic representation of the main components and regulators of leptin pathway targeted by food compounds. Leptin signalling pathway is

modulated by the effect of several food compounds on targets from different upstream and downstream levels.

Finally, the AMPK and mTOR pathways are also involved in the regulation of leptin signals. Specifically, leptin inhibits AMPK in several hypothalamic regions stimulating hypothalamic acetyl-CoA carboxylase (ACC) action, consequently decreasing food intake and body weight [24].

4. Mechanisms of leptin resistance in obesity

As previously explained, the term leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Although the exact mechanisms that lead to leptin resistance are still unclear, some have been proposed, including impaired leptin transport across the BBB and the disruption of the leptin signaling cascade within neurons from specific brain areas. Besides, hypothalamic inflammation, endoplasmic reticulum (ER) stress and loss of Sirtuin1 activity also promote leptin resistance.

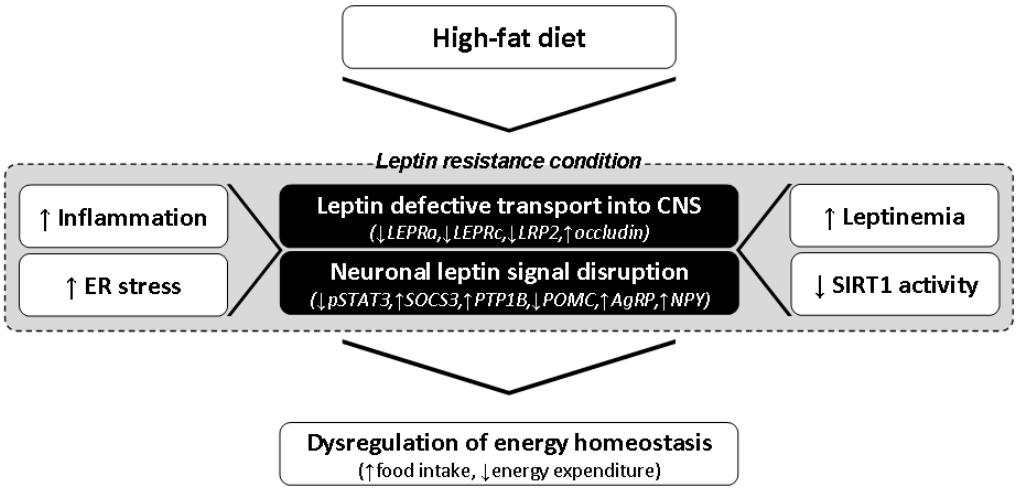

Figure 2: Schematic diagram for molecular mechanisms of leptin resistance. The molecular basis for leptin resistance is not yet completely understood but it have been mainly attributed to several mechanisms. These include reduced transport across the blood-brain barrier and the enhancement of intracellular processes that attenuate cellular signalling such as inflammation, endoplasmic reticulum (ER) stress, hyperlepinemia and sirtuin 1 dysfunctionality. CNS: central nervous system; ER: endoplasmic reticulum; SIRT1: sirtuin 1.

4.1 Defective leptin transport across the blood-brain barrier (BBB)

Most known functions of leptin within the central nervous system (CNS) are presumably mediated by leptin produced in the periphery. Therefore, leptin must be transported across the BBB. Several studies have shown that this is most likely conducted using a specific and saturable transport system that is located at both the endothelium of the cerebral microvessels and the epithelium of the choroid plexus [25]. LEPRa and LEPRc short form leptin receptor isoforms have both been shown to be highly expressed in the microvessels of the brain and are suggested to facilitate the BBB transport of leptin [26]. Furthermore, low density lipoprotein-related protein 2 (LRP2), also known as megalin, has been identified as potential novel leptin transporter protein at the choroid plexus epithelium. It functions by binding circulating leptin and transporting the hormone into the CNS [27].

Several reports in obese humans and rodents, in which the leptin levels in the cerebrospinal fluid are significantly decreased compared with control group, have suggested that leptin resistance is associated with a defect in the transport of leptin through the BBB [28].From these studies some hypotheses have been proposed as causes of this defective transport of leptin. For example, polyunsaturated fatty acids have been reported to induce peripheral leptin

resistance via an increase in the expression of hypothalamic occludin, one of the main proteins of the tight junctions, reducing paracellular transport of leptin into the brain [29]. Alternatively, an impaired expression of the transporters LEPRa and LEPRc at the BBB has also been suggested, but this aspect is quite controversial because some studies have suggested that a decreased capacity of the transporter to bind and transport leptin into the brain is the major cause of the defective leptin transport [30,31]. Additionally, some studies indicate that high levels of both triglycerides and protein C reactive (PCR) also reduce leptin transport across the BBB [29]; therefore, in states of hypertriglyceridemia and/or inflammation, such as obesity or starvation, decreased leptin transport into the CNS is expected.

4.2 Attenuation of the leptin signaling cascade in the hypothalamus

Another mechanism involved in the development of leptin resistance is the disruption of LEPRb signaling in the hypothalamus. In fact, this mechanism has been considered to be one of the leading factors and the primary defect that induces central leptin resistance. This loss of leptin signaling is mainly due to two parallel molecular mechanisms, including the up-regulation of SOCS3 and PTP1B.

As explained before, SOCS3 is a key protein that inhibits the signal transduction process of various cytokines in the body, including leptin. By binding to Tyr985 of LEPRb and JAK2, SOCS3 inhibits the leptin-induced phosphorylation of STAT3 through a negative feedback mechanism [18]. In this sense, the incidence of diet-induced obesity and leptin resistance is significantly

decreased in rats with brain-specific deletion of SOCS3 [32]. Moreover, PTP1B is a non-receptor protein tyrosine phosphatase located in the cytoplasmic face of the endoplasmic reticulum (ER) in the hypothalamic regions enriched with leptin-responsive neurons [19]. PTP1B dephosphorylates JAK2, thereby inhibiting leptin signaling. The expression of PTP1B is increased by high-fat feeding and inflammation, suggesting that PTP1B may play a crucial role in the etiology of leptin resistance[33]. Additionally, brain-specific deletions of PTP1B improve leptin sensitivity and offer protection from obesity [34]. Thus, as important targets to increase leptin sensitivity and improve obesity, SOCS3 and PTP1B have emerged as their inhibition may facilitate this process.

In addition to the JAK2/STAT3 signaling cascade, other signaling pathways such as the P13K/Akt and AMPK pathways, as mentioned above, jointly participate in the LEPRb signal transduction. Consequently, the study of regulators in these secondary pathways, including the phosphatase tensin homolog deleted on chromosome 10 (PTEN) and ACC, among others, can also potentially provide new targets for the management of leptin resistance.

4.3 Hypothalamic inflammation

Most evidence indicates that inflammation, both in peripheral tissues and the hypothalamus, is a cause of the development of leptin resistance in obesity. Specifically, the inflammatory pathway IκB kinase-β/nuclear factor-κB (IKKβ/NF-κB) is activated in the hypothalamus of rodents fed a high-fat diet (HFD) [35]. Notably, genetic inhibition of this pathway in the neurons of the arcuate nucleus [36] or AgRP neuron-specific deletion of IKKβ [37]protects from HFD-induced obesity, enhances leptin signaling and reduces SOCS-3

expression. Remarkably, the promoter of SOCS-3 has two putative motifs for binding NF-κB [37], thus connecting hypothalamic inflammation with leptin resistance.

Toll-like receptor 4 (TLR4), a membrane receptor that functions in the innate immune system, is activated by saturated fatty acids in the hypothalamus, triggering the IKKβ/NF-κB pathway [38]. Moreover, genetic and pharmacological inhibition of TLR4 restored leptin signaling in rodents fed a HFD [38]. Therefore, TLR4 is proposed to act upstream from IKKβ/NF-κB in the inflammatory process induced by a HFD in the hypothalamus.

Besides IKKβ/NF-κB pathway, c-Jun N-terminal kinase (JNK) is another pro-inflammatory signalling component up-regulated in the arcuate nucleus of murine fed a HFD [35]. However, the actual implication of JNK on inducing leptin resistance and obesity is controversial.

Recently, 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), which regulates key aspects of immunity, has been also involved in the development of leptin resistance[39]. Specifically, it has been described that 15d-PGJ2 inhibits the leptin-induced phosphorylation of STAT3 *in vitro* and the leptin-induced anorexia *in vivo* [39].

In addition to leptin signaling dysfunction, inflammatory processes are also responsible for structural changes in the hypothalamus, which alter the hypothalamic circuits. Interestingly, neuronal injury has been observed in specific brain areas that regulate food intake in obese humans and rodents [40].

4.4 Endoplasmic reticulum stress

The transmembrane proteins are synthesized and folded by ER in order to form active proteins in the ER lumen. Miss-folded and un-folded proteins are eliminated via proteasome complex. An imbalance in this process causes an accumulation of defective proteins in RE lumen, forming ER-stress and, consequently, the induction of unfolded protein response (UPR) [41]. If there is a short-term ER-stress, the UPR restores the ER homeostasis reducing the protein synthesis, increasing their folding capacity and degrading the miss- and un-folded proteins [41].

Notably, ER stress has also been suggested to be an inducer of leptin resistance. In this sense, long-term ER stress inhibits the leptin signaling pathway, leading to hyperleptinemia and obesity [37], and several studies have shown that obese animals display significant ER stress in multiple tissues (i.e., liver, adipose, and brain tissues) [42,43]. Therefore, the inhibition of ER stress increases leptin sensitivity and reduces food intake and body weight [44]. At molecular level, hypothalamic ER stress results in decreased post-translational conversion of POMC to α-MSH in HFD fed rats [45], thus connecting ER stress and leptin resistance.

The pro-inflammatory IKKβ/NF-κB pathway may act both upstream and downstream from ER stress and experimental evidences show that hypothalamic IKKβ/NF-κB pathway and ER stress positively feedback each other under conditions of overnutrition [35] further worsening leptin resistance.

4.5 Sirtuin1 activity

Recently, a reduced activity of sirtuin 1 (SIRT1), which is a NAD+dependent protein deacetylase, has been implicated in the appearance of leptin resistance [46,47]. On the contrary, the activation of hypothalamic SIRT1 increases energy expenditure and reduces food intake [46]. SIRT1 can improve leptin sensitivity by decreasing the levels of PTP1B [46], SOCS3 [46] and FOXO1 [48]. Moreover, SIRT1 activation reduces inflammation [49] and ER stress [50], two dysfunctions that also induce leptin resistance. Therefore, SIRT1 appears as a new promising target to improve leptin resistance.

5. Food compounds useful for counteracting leptin resistance

Several nutrients and food components with the ability to reverse leptin resistance have been described. This review compiles food compounds that are able to reduce hyperleptinemia, promote leptin transport across the BBB or modulate leptin cascade in the hypothalamus. However, food compounds able to reduce inflammation and ER stress or to increase SIRT1 activity but without experimental evidence of promoting hypothalamic leptin sensitivity, are not included in this review.

5.1 Food compounds controlling circulating leptin levels

Hyperleptinemia is one of the characteristics of leptin resistance, and numerous studies have been conducted to identify compounds with anti-hyperleptinemic activity. It should be noted that most of these studies are performed in animal

models, using basically rats and mice. Table 1 summarizes a list of food compounds and extracts that have shown the ability to decrease the levels of circulating leptin in *in vivo* studies.

Among the molecules listed in Table 1 numerous phenolic compounds can be found. This group of compounds, which is widely distributed in fruits and vegetables, has been shown to reduce the level of circulating plasmatic leptin in a large range of *in vivo* studies, using different types of models and treatments. This capacity has been observed by both using pure phenolic compounds, such as resveratrol [51], oleuropein [52] and myricetin [53], as well as some precursors or derivatives of phenols, such as polydatin [54] and KMU-3 [55] (a synthetic derivative obtained from gallic acid). In addition, some polyphenolic rich-extracts, obtained from natural sources such pecans [56], brown algae [57] and peach and plum juices [58], have shown the same behavior. Among all of these studies carried out with phenolic compounds, it is worthwhile to highlight that some of them have been conducted in humans. For example, fraxin, a glucoside of an *o*-methylated coumarin, and curcumin have been confirmed to reduce hyperleptinemia in overweight and obese humans [59], thus emphasizing their suitability for use in the formulation of functional foods directed towards weight reduction. Taking into consideration all of these studies, it seems clear that phenolic compounds are a very interesting family of molecules for finding new molecules from natural sources that could be used to improve hyperleptinemia and leptin resistance.

Table 1. Food compounds that reduce hyperleptinemia

Class	Compound/s	Dietary source	Experimental model	Reference
Phenolic compounds	Resveratrol	Grapes, red wine	Wistar rats	51
	O-coumaric acid	Vinegar	Male Wistar rats	107
	7-O-galloyl-D-sedoheptulose	*Cornifructus*	*db/db* mice	108
	Neohesperidin	Citrus	Male KKAyand C57BL/6 mice	109
	Polydatin	*Polygonum cuspidatum*	Male Sprague Dawley rats	54
	Myricetin	Vegetables, fruits, nuts, berries, tea, red wine	Male C57BL/6J mice	53
	Fraxin	*Fraxinus sp*	Elderly overweight/obese human	59
	Gingerol	Ginger	Male Wistar rats	110
	Oleuropein	Olives	C57BL/6JOlaHsd mice fed HFD	52
			C57BL/6 mice fed HFD	111
	Polyphenol-rich extracts	Pecan nut	Male Wistar rats	56
		Brown algae	High-fat diet induced obese mice	57
		Peach and plum juice	Zucker rats	58
		Zygophyllum album	Female Wistar rats	112

Terpenes	Thymol	Thyme	High-fat diet-induced obese mice	61
	Lycopene	Tomatoes, watermelon, papaya, orange	Male Wistar rats	63
	Teasaponin	Tea	Male C57Bl/6J mice	62
	Ginsenoside Rb1	Ginseng	Male C57Bl/6 mice	88
PUFAs	DHA, EPA	Fish oils, golden algae oil	Male C57BL/6J mice	55
	CLA	Beef, lamb, dairy foods	Human and mice	67
Soluble fiber	Pectin	Fruits	Male Wistar rats	96
Other	Protein lysates	Rice bran	A high carbohydrate diet-induced obese rats	65
	Isothiocyanates	*Moringa oleifera*	Male C57BL/6J mice	60
	Mate aqueous solution	*Ilex paraguariensis*	Male Wistar rats weaned prematurely	102

CLA, conjugated linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFAs:polyunsaturated fatty acids

In addition to phenolic compounds, other plant secondary metabolites, such isothiocyanates [60] and some terpenoids including thymol [61], saponins [62] and lycopenes [63] among others, have also been proven to be effective in reducing circulating leptin levels when administered to rodents. Furthermore, other compounds different from plant secondary metabolites have shown effects reversing leptin resistance. For example, some peptides [64] and protein hydrolysates [65], as well as polyunsaturated fatty acids (PUFAs), such as docosahexanoic acid (DHA) [66], eicosapentaenoic acid (EPA) [66] and conjugated linoleic acid (CLA) [67], also exhibit anti-hyperleptinemic action. Interestingly, some of them have been proven in humans. For example, this is the case for the CLA isomer t10c12-CLA, thus highlighting its suitability and potential to be used in treatments directed towards the reversal of leptin resistance [67].

Although there are several studies describing a reduction in the leptin level due to the effect of natural compounds, only some of them describe the mechanisms by which this reduction is produced. One of these mechanisms is through the repression of the leptin gene. Notably, cranberries [68] and KMU-3 (a synthetic gallic acid derivative) [55] have the ability to repress leptin gene expression in the adipocyte cell line 3T3 L1 (Table 1). However, to confirm the contribution of leptin gene repression on the anti-hyperleptinemic effect of a food component, it would be necessary to determine whether the expression of the leptin gene in WAT correlates with the reduction in the leptin level induced by a specific food compound in *in vivo* models. In this sense, one promising phenolic compound that can be highlighted is oleuropein, found in olives. In the study performed by van der Stelt *et al.* [52] a reduction in the levels of serum leptin is correlated with a reduction of the expression of the leptin gene in epididymal WAT of mice fed HFD supplemented with oleuropein compared to HFD mice.

Another target to take into account to reverse leptin resistance involves the CB1 cannabinoid receptor (CB1R). CB1R is the main cannabinoid receptor found in the brain, and it is present in endocrine cells and other peripheral tissues [69], such as pancreas, fat, liver and skeletal muscle tissues [70]. It is known that the use of brain-penetrating CB1R antagonists can cause neuropsychiatric side effects, but a selective targeting of peripheral CB1R results in an improved hormonal-metabolic profile without the observation of the brain secondary consequences [70]. The antagonistic action of CB1R causes an increase in appetite, insulin resistance and an increase in the hepatic lipogenesis, suggesting its implication in obesity [70]. Therefore, there are synthetic CB1R antagonists that increase the leptin sensitivity [70] or CB1R inverse agonists that reverse the leptin resistance, decreasing leptin expression and secretion by adipocytes and increasing leptin clearance via the kidney [71]. Although, to date, no natural compounds have been described as CB1R antagonists, the search and use of molecules from natural sources that antagonize this target could be one mechanism to reverse leptin resistance.

As indicated above, many food compounds have been described as anti-hyperleptinemic. However, food compounds could improve leptin sensitivity by a direct action in the brain, targeting hypothalamic leptin signalling and leptin transport across the BBB, or by a secondary effect resulting from body weight reduction, as a result of targeting peripheral tissues such as liver and adipose tissue. In this sense, serum leptin levels strongly correlate with the reduction of body weight induced by several polyphenols in rats fed a HFD [72]. Therefore this review focuses on food compounds that target hypothalamic leptin signalling and leptin transport across the BBB.

5.2 Food compounds that modulate leptin transport across the brain blood barrier

As stated in previous sections, leptin resistance can be the consequence of the impairment of its transport across the BBB, reducing leptin accessible to the CNS. Thus, increasing leptin transport across the BBB could be a good strategy to increase central leptin sensitivity. Despite that several transporters have been described; most studies have focused on the capacity of food compounds to increase the expression of the transporter megalin. Some of these compounds are listed in Table 2. Notably, increased bile acid production due to a lithogenic diet [73] produce an overexpression of megalin in mice, whereas vitamin A and vitamin D [74] are effective in producing the overexpression of megalin in several cell lines. Interestingly, synthetic PPARα and PPARγ agonists induce the expression of megalin [75]. Therefore, it can be hypothesized that food components that could act as PPAR agonists, such as PUFAs [76], coumarins [77], flavonoids [78] or even polyphenol rich extracts from fruit juices [58], could be good candidates to improve leptin transport across the BBB.

Table 2. Food compounds that modulate leptin transport across the brain blood barrier

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Fraxin	*Fraxinus sp*	Upregulates clusterin gene expression	HUVEC cells	[80]
	Quercetin	Onion, broccoli	Increases LEPRa protein levels	HUVEC cells	[82]
Vitamins	Retinoic acid	Sweet potatoes, carrot	Upregulates LPR2 gene expression	Male C57BL/6J mice	[74]
	Cholecalciferol	Fish liver oils, fatty fish species, beef liver			
Bile acids	Cholic acid	Lithogenic diets	Upregulates LPR2 gene expression	RPT, JEG-3 and EC-F9 cells	[73]
	Chenocholic acid				

LEPRa, leptin receptor isoform a; LPR2, low density lipoprotein-related protein 2 also known as megalin

In addition to megalin, other proteins can modulate the transport of leptin across the BBB. In this sense, clusterin (also called ApoJ) is a plasma leptin-binding protein for which megalin has been identified as an endocytic receptor [79]. Additionally, clusterin modulates leptin signaling in cell lines expressing LepRb [79]. Therefore, increasing the expression and/or the activity of clusterin could be a good way to increase leptin interactions with LepRb and megalin. Notably, the coumarin fraxin upregulates clusterin gene expression in cell lines [80]. As an overexpression of clusterin induces the overexpression of megalin [81], fraxin is considered a potential stimulator of leptin transport across the BBB.

It is also important to highlight the findings obtained by Indra *et al*. [82], in which the administration of quercetin, a flavonol found in many fruits, produced an overexpression of LepRa in HUVEC cells. This finding is very important due to the involvement of this receptor in the transport of leptin through the BBB. However, as quercetin is actively sulphated and glucoronidated upon intestinal uptake, further experiments should be carried out using these quercetin metabolites in this cell line to obtain stronger conclusions.

5.3 Food compounds enhancing leptin signalling in the hypothalamus

Several food compounds, most of them from vegetal sources, have the capacity of reaching the hypothalamus, where they regulate leptin signaling pathways. These food compounds can either cross the BBB or pass through fenestrated capillaries of circumventricular organs (CVO) and medial eminence (ME) and target arcuate nucleus (ARH)neurons, which express LEPR[83], i.e., POMC/CART and AgRP/NPY neurons, where they can act at different levels.

Some compounds improve central leptin sensitivity by increasing the expression of LEPRb, the phosphorylation of STAT3 and the expression of downstream components, mainly neuropeptides [84]. Additionally, some food compounds target the negative regulators of the leptin cascade, such as SOCS3 and PTP1B, reduce ER stress and/or modulate other leptin cascades, such as PI3K/Akt [85]. Food components that modulate leptin signaling activity are summarized in Table 3.

Table 3. Food compounds involved in the modulation of the leptin signaling cascade

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Tissue	Reference
Phenolic compounds	Curcumin	Turmeric	Reduces LEPRb phosphorylation Reduces STAT3 phosphorylation	Male Sprague Dawley rats	Hepatic stellate cells	[113]
	Resveratrol	Grapes, red wine	Increases STAT3 phosphorylation	Male Wistar rats	Hypothalamus	[84]
			Increases AMPK phosphorylation	Male Wistar rats	Liver, muscle	[95]
	Fucoxanthin	Brown algae	Increases AMPK phosphorylation	Male C57BL/6J mice	eWAT	[97]
Terpenes	Teasaponin	Tea	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male C57BL/6J mice	Hypothalamus	[62]
	Ginsenoside Rb1	Ginseng	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Males C57Bl/6 mice	Hypothalamus	[88]
			Increases AKT phosphorylation	Male Long-Evans rats	Hypothalamus	[94]
	Celastrol	*Tripterygium wilfordii* *Celastrus regelii*	Increases STAT3 phosphorylation Upregulates SOCS3 mRNA levels	Male C57BL/6J mice	Hypothalamus	[89]

			Reduces ER stress			
Soluble fibre	Pectin	Fruits	Increases STAT3 phosphorylation Increases AMPK phosphorylation	Male Wistar rats	Liver, eWAT	[96]
Plant extracts	Triterpenoids	*Schisandra chinensis*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[114]
	Phenolic compounds	*Cyclocarya paliurus*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[115]
	Xanthones and fllavonoids	*Cudrania tricuspidata*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[116]
Others	Caffeine	Coffee beans, tea bush, kola nuts	Increases STAT3 phosphorylation Reduces ER stress	SH-SY5Y-Ob-Rb cells	Liver, eWAT	[90]
	Leucine	Soybeans, beef	Increases LEPRb phosphorylation Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male Sprague-Dawley rats	Hypothalamus prWAT	[117]
	Taurine	Shellfish, turkey dark meat	Increases STAT3 phosphorylation Reduces ER stress	Male C57Bl/6 mice	Hypothalamus	[91]
	Safranal	Saffron	Inhibits PTPB1 activity	Male C57BL/6J mice	C2C12 myoblast	[118]

Abbreviations: AKT, protein kinase B; AMPK, 5' adenosine monophosphate-activated protein kinase; ER, endoplasmic reticulum; LEPRb, leptin receptor; PTP1B, protein-tyrosine phosphatase 1B, SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; prWAT, perirenal white adipose tissue; eWAT, epididymal white adipose tissue.

To the best of our knowledge, only leucine has been described as a food component that increases the expression of LEPRb [86]. Currently, the best marker of leptin signaling activity is the level of pSTAT3 which is the transcription factor that mediates leptin anorexigenic actions [87]. Notably, several food compounds increase the level of pSTAT3 in the hypothalamus of rodents fed a high-fat diet, such as teasaponin [62] and ginsenoside Rb1 (the main bioactive compound of ginseng) [88], which also inhibit SOCS3, thus increasing leptin sensitivity. Interestingly, resveratrol increases pSTAT3 levels in the hypothalamus concomitantly with a decreased adiposity [51]. Other compounds, such as celastrol (a pentacyclic triterpene extracted from the roots of thunder god vine) [89], caffeine [90] and taurine [91] are also STAT3 activators, and their actions seem to be mediated through the decline of ER stress in the hypothalamus.

Other studies have focused instead on negative factors that attenuate leptin receptor signaling, such as PTP1B. However, only *in vitro* studies had found potential natural compounds that would inhibit this target [92]. Therefore, the capacities of these compounds to increase leptin sensitivity in the hypothalamus are speculative and more research is needed to confirm these findings.

Current studies have shown that the activation of PIK3/Akt and AMPK pathways is essential to maintain energy homeostasis, as they are involved in the anorexigenic effect of leptin [93]. Notably, ginsenoside Rb1 activates AKT in both the hypothalamus of obese rats and in hypothalamic cell lines [94]. Moreover, it has been hypothesized that SIRT1 activation would inhibit proteins involved in leptin resistance, thus reducing ER stress [47]. Therefore, compounds that either directly or indirectly activate SIRT1 , such as resveratrol, could increase central leptin sensitivity [95].

Other studies have focused on the capacity of several food compounds, which lower body weight and visceral fat mass, to modulate leptin signaling in peripheral tissue. For example, it has been confirmed that pectin [96] and fuxocanthin [97] increase pSTAT3 levels and AMPK activity in adipocytes, thus relating it with the reversal of leptin resistance.

First order neurons POMC and AgRP, will project to second order neurons in the paraventricular nucleus (PVH), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and lateral hypothalamic area (LHA), where they produce the anorexigenic and orexigenic effects, respectively [98]. Leptin anorectic effects are mediated by CART and POMC neurons. The later produces α-MSH peptide, which binds to the MC3/4 receptor in second order neurons and will inhibit food intake [99,100]. Many natural compounds are able to increase either POMC or CART levels. In this sense, apigenin [101], ginsenoside Rb1 [88], teasaponin [62], taurine [91], leucine [86] and yerba mate extracts [102] have been shown to increase the expression of POMC in the hypothalamus and to reduce food intake and body weight (Table 4). Moreover, some of these compounds, including resveratrol [103], ginsenoside Rb1 [88] and taurine [91], are also able to inhibit the orexigenic neuropeptides AgRP and NPY, suggesting an increased effectiveness to modulate food intake and energy expenditure. However, celastrol, which reduces body weight in DIO mice, increases AgRP mRNA levels [89]. Therefore, more studies are necessary to thoroughly understand this mechanism.

Table 4. Food compounds targeting neuropeptides that regulate energy homeostasis

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Apigenin	Fruits, vegetables	Upregulates POMC mRNA levels Upregulates CART mRNA levels	N29-2 and SH-SY-5Y neuronal cells	101
	Resveratrol	Grapes, red wine	Downregulates AgRP mRNA levels Increases NPY protein expression	N29-4hypothalamic cells	51
Terpenes	Teasaponin	Tea	Upregulates POMC mRNA levels	Male C57Bl/6 mice	62
	Ginsenoside Rb1	Ginseng	Downregulates AgRP mRNA levels Upregulates POMC mRNA levels	Male C57Bl/6 mice	88
			Increases NPY protein expression Upregulates POMC mRNA levels	Male Long-Evans male rats	94
	Celastrol	*Tripterygium wilfordii* *Celastrus regelii*	Upregulates AgRP mRNA levels	Male C57Bl/6 mice	89
Others	Retinoic acid	Sweet potatoes, carrot	Increases methylation levels in the POMC gene	Leukocytes of obese men	119
	Taurine	Shellfish, turkey dark meat	Increases NPY protein expression Upregulates POMC mRNA levels Upregulates CART mRNA levels	Male C57Bl/6 mice	91

Abbreviations: AgRP, Agouti-related protein; CART, cocaine-and amphetamine-regulate transcript; NPY, neuropeptide Y, POMC, proopiomelanocortin;

5.4 Food compounds against leptin resistance: a holistic overview

In the previous sections, food compounds have been listed according to the level where they improve leptin resistance. However, it is important to take into account that some of these compounds act at several levels of the leptin pathway. Thus, some food compounds stand out from the others. For example, resveratrol, a phenolic compound obtained from the skin of grapes, is able to reduce the circulating level of leptin not only by reducing its secretion but also by promoting the activation of STAT3 in the hypothalamus, thereby increasing leptin sensitivity and to down-regulating AgRP and NYP. Others compounds that can be highlighted are teasaponin and ginsensoside, which reduce hyperleptinemia, activate hypothalamic STAT3, increase POMC mRNA levels, and inhibit SOCS3. In addition, ginsensoside increases p-FOXO1 and inhibits PTP1B.

It is also worthwile to take into account that some food compounds also modulate proinflammatory cytokines. These cytokines contribute to the development of leptin resistance, and some food compounds reduce proinflammatory cytokines, such as tesaponin and ginsensoside.

Therefore, the selection of a compound taking into consideration its influence within the entire set of mechanisms involved in the onset of leptin resistance is the best way to assure its functionality. Figure 1 shows the leptin pathway components and regulators targeted by food compounds.

6. Methods for the identification of new natural compounds with anti-leptin resistance activities.

To deeply study the mechanisms involved in the development of leptin resistance and its reversal using natural compounds, several strategies have been followed by researchers in the last two decades. These strategies involve a broad variety of techniques, ranging from the more theoretical ones, which are based in the use of bioinformatics-aided tools, to biological studies that evaluate the *in vivo* response in different models.

Traditionally, both *in vitro* cellular models and *in vivo* studies have been used to study leptin resistance. Focusing on cell models, different points of view can be followed. For example, it is very important to evaluate if the compounds being studied are able to cross the BBB and reach the selected targets. To do this, a specific cellular model using endothelial cells should be used. Until now, several endothelial cell lines are described in the bibliography for the simulation of the BBB, such as RBEC1, GP8/3.9, GPNT and RBE4 [104]. Other studies are focused on the evaluation of the signaling cascade pathway. These studies are carried out using astrocyte cultures and neuronal cell lines.

Regarding the *in vivo* studies aimed to evaluate leptin resistance, several models of metabolic syndrome using animals with a non-functional leptin pathway have been used. In general, these animals can be classified into two groups: genetically altered or diet-induced altered animals. In the first group, all of the animals (in general mice and rats) that present a modification in any of the genes involved in the onset of leptin resistance can be included. These modifications can be either from natural origin or generated in the laboratory, including knock-out animals that had a deficiency in the leptin receptor or

animals that do not produce leptin (ob/ob mice, db/db mice, etc.). In the other group, animals in which leptin resistance originates by means of the nutritional composition of the diet are included. For example, a high fat diet produces the onset of leptin resistance in animals. This last group of animal models is very interesting to simulate the most typical situation that originates leptin resistance in humans.

The most novel approach to study leptin resistance is the so-called *in silico* strategy, which is based in the use of bioinformatics. This set of techniques is focused on the search through the virtual screening of new potent molecules from natural products with the capacity to directly act in one of the mechanisms that is implicated in leptin resistance, including molecules that have the capacity to directly bind to therapeutic targets and consequently either inhibit or activate them. To carry out these *in silico* studies and successfully do the virtual screening, some prerequisites have to be accomplished. For example, the existence of crystallized structures within databases is necessary to know their 3-D conformations, which is essential in this process. The existence of ligands described for the selected targets is also required to complete the design of the pharmacophore, which contains the information of the biological conformation and electrostatic features that the ligands should have to interact with the binding site of the target proteins. To date, some bioinformatics results focusing on the reversal of leptin resistance have been carried out. For example some papers have reported molecules with the ability to inhibit PTP1B [105,106], whereas no results against SOCS3 and SH2B1 have been found. Following this virtual screening, the selected set of molecules that fulfil the prerequisites should be used in an in vitro assay to confirm the theoretical activity described.

Taking into consideration this wide spectrum of strategies, the most logical sequence of action in the search for compounds that could revert leptin

resistance is as follows: start with *in silico* studies to select potentially active food compounds, test the molecules selected in the virtual screening *in vitro* by using several cellular models, and finally test the most actives ones in *in vivo* conditions, first in animals models and then in humans, to determine their efficacies.

Concluding remarks

Leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Notably, numerous food components, mainly polyphenols, are able to reduce hyperleptinemia, suggesting that these compounds could improve leptin resistance. However, only a few studies have focused on the mechanism by which these food components could primarily restore leptin sensitivity. The results of these studies indicate that food components can reverse leptin resistance by increasing leptin access to the brain and/or activating the intracellular signaling cascade of leptin in the hypothalamus. Nevertheless, the conclusions that could be extracted from these studies are limited because they focus only on one of the levels of leptin resistance. Thus, new studies considering the activity of a particular food compound at all levels of leptin resistance and using validated markers of leptin sensitivity, such as pSTAT3, are indispensable to clearly ascribe the property of leptin sensitizer.

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II. HYPOTHESIS AND OBJECTIVES

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This PhD thesis has been performed in the Nutrigenomics Group of the Universitat Rovira i Virgili, within the frame of two research project carried out in this group: “Development of an integrated food to maintain body weight and to prevent the risk of obesity related pathologies (AGL2013-40707-R)” and “Illegitimate signaling of fruit consumption and obesity pathogenesis (AGL2013-49500-EXP).

Dietary polyphenols are recognized for their health promoting effects. Several studies report their anti-inflammatory, antioxidant, cardio- and neuroprotective properties. Moreover, great attention is being paid to these compounds since they are present in multitude of plant-derived foods. The Nutrigenomics Group focus on the potential health benefits of proanthocyanidins and other polyphenols. In particular, our group has an extensive experience studying the metabolic effects of proanthocyanidins, using a grape seed proanthoycianidin rich extract (GSPE) which was proved to ameliorate the main components of metabolic syndrome in diet-induced obesity models. Furthermore, the next goal after the identification of bioactive compounds will be to the design functional foods that prevent or treat metabolic risk factors.

To date, strategies to counteract obesity epidemic have been unsuccessful. Leptin has been a research focus since it is the key hormone that regulates central energy homeostasis and, although controverted, it is suggested that obesity coexists with ‘leptin resistance’ .In recent years, potent leptin sensitizers, such as betulinic acid, celastrol and withaferin A, have been identified. They increase leptin sensitivity either suppressing PTP1B, a negative regulator of leptin signaling, or decreasing endoplasmic reticulum stress.

Data about the ability of polyphenols to modulate leptin signaling pathway is scarce. However, the wide protective effects exerted by polyphenols on obesity

models, targeting several of the multifactorial disorders associated to obesity, allows us to **hypothesize that polyphenols or polyphenol-rich fruits might have the potential to modulate central leptin signaling in the hypothalamus and, thereby, restore leptin sensitivity.**

The **aim** of this thesis was **to assess whether polyphenols can improve central leptin sensitivity in obesity and determine the mechanisms by which these compounds affect leptin signaling pathway**. For this aim to be achieved, the following **goals and objectives** were proposed:

1. **To evaluate the ability of different polyphenols to modulate leptin signaling pathway in the hypothalamus and its downstream neuropeptides.**

   In previous studies our group demonstrated that proanthocyanidins are able to prevent metabolic syndrome except for body weight and hypertension. Proanthocyanidins only showed a tendency to reduce body weight and the reduction of blood pressure was only achieved with supraphysiologic doses. Thus, the main goal of the AGL2013-40707-R project is to elaborate what we designate 2.0 functional food, that will contain more than one ingredient, combining proanthocyanidins with other compounds, that complement their effects, targeting metabolic syndrome from a multifactorial point of view.

   To prevent hypertension, researchers in our group already found a peptide hydrolysate that could be effective at low doses. Then, two approaches were taken to find bioactive compounds with the ability of reducing body weight: compounds able to induce the browning process and compounds effective modulating the **leptin signaling pathway,** which appears to be altered in obesity. In this situation, leptin is not exerting its anorectic effects as in

normal conditions. The last approach was investigated in this thesis. Therefore, the objectives were:

a. **To assess the effect of GSPE on central and peripheral leptin signaling pathway on a diet-induced obesity model.** The effect of GSPE on the hypothalamic leptin system had not been investigated yet, but moderate doses of proanthocyanidins tend to reduce body weight. This suggests that GSPE could potentially modulate leptin signaling or downstream factors **[Chapter 1].**
b. **To evaluate the ability of anthocyanins and resveratrol to increase leptin sensitivity.** In order to find other ingredients that could complement GSPE reducing obesity, we opt for the use of an anthocyanin rich extract and resveratrol. The selection was based on the reported anti-obesity effects of both classes of polyphenols in different animal models. The capacity of these polyphenols to modulate the central leptin system was evaluated first by studying the effects of these compounds on the gene expression of leptin signaling pathway in the hypothalamus in healthy mice, as a preliminary study. The most effective compound, resveratrol, was selected and tested on a diet-induced obesity model **[Chapter 2].**

2. **To determine whether seasonal fruits rich in polyphenols are able to modulate the hypothalamic leptin system in rats placed to long-day or short-day photoperiod [Chapter 3].**

The xenohormesis theory states that polyphenols synthesized by plants, as a response to environmental stress, provide a chemical signature of the surrounding conditions. This mark may be recognized by heterotrophs

consuming these plants, to detect changes when conditions are still favorable, such as the need of food, which allows animal survival. Polyphenol structures and content on plants depend on genetic aspects but also on photoperiod, temperature, harvest season and crops conditions. Numerous studies correlate photoperiod alterations with obesity. The hypothesis of the project AGL2013-49500-EXP is that the composition and concentration of polyphenols in fruits inform animals about the season, and the consumption of fruits out-of season may stimulate metabolic diseases, such as obesity. Indeed, circulating leptin levels experience seasonal rhythms. For this reason, the out-of season fruit consumption could be involved in the attenuation of leptin signaling present in obesity. Thus, the objectives of this project were:

a. **To assess the effect of seasonal fruits (grape or cherry) on leptin signaling pathway when they are consumed in or out-of season**
b. **To evaluate whether the effect of seasonal fruits on the leptin system is influenced by an obesogenic diet**

3. **To study hypothalamic neurons that could be potential targets of polyphenols [Chapter 4].**

Leptin action in the hypothalamic arcuate nucleus produces the activation of Pomc neurons and suppresses AgRP/Npy neurons, generating satiety signals and increasing energy expenditure thereby maintaining body weight. Interestingly, Olofsson and colleagues showed that AgRP neurons are mainly located outside the blood brain barrier, being more sensitive to metabolic peripheral signals. Although, the polyphenols used in this thesis have been detected in brain samples, we think that AgRP neurons would be more susceptible to polyphenol since they can directly be affected by blood borne substances.

This part of the PhD thesis has been performed in Professor Allison W. Xu laboratory in the Diabetes Center at University of California, San Francisco. This group discovered that AgRP neurons are outside the blood brain barrier and has extensive knowledge and expertise on the study of hypothalamic neurons and how AgRP neurons sense and integrate peripheral signals, such as leptin, This allowed me to get fully involved in one of their projects that aimed to investigate new mechanisms by which AgRP neurons are activated in both physiological conditions (chapter 4) and after ethanol consumption (data not shown).

Recent data gives evidences that AgRP neurons are activated by a class of G protein-coupled receptors (GPCRs). According to this and other studies, the group of Professor Xu hypothesizes that adenosine receptor 2B could play a role on the activation of AgRP neurons and that this receptor has metabolic implications in normal or pathological conditions.

The research work performed in this Ph.D. has been supported by the grants AGL2013-40707-R and AGL2013-49500-EXP from the Spanish government. This thesis was carried out in the Nutrigenomics Research Group laboratories of the Universitat Rovira i Virgili under a FPI predoctoral fellowship from the Spanish Government. An international phase has been completed in the Diabetes Center at University of California San Francisco under the supervision of Prof. Allison W. Xu to obtain the International Doctorate. Mention. This phase was supported by a personal grant from the Spanish Government.

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III. RESULTS

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CHAPTER 1

Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and *Pomc* gene expression in rats with diet-induced obesity

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Abstract

Dietary obesity is usually linked with hypothalamic leptin resistance, in which the primary impact is interference in the homeostatic control of body weight and appetite. Notably, proanthocyanidins (PACs), which are the most abundant phenolic compounds present in human diet, modulate adiposity and food intake. The aim of this study was to assess whether PACs could re-establish appropriate leptin signalling in both the hypothalamus and peripheral tissues. Male Wistar rats were fed either a standard chow diet (STD group, n=7) or a cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either grape-seed PAC extract (GSPE) at 25 mg per kg of body weight per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) for the last 21 days of the study period. Specific markers for intracellular leptin signalling, inflammation and endoplasmic reticulum stress in the hypothalamus, liver, mesenteric white adipose tissue and skeletal muscle were analyzed using immunoblotting and quantitative PCR. GSPE treatment significantly reduced the food intake but did not reverse the hyperleptinemia and body weight gain assessed. However, the animals treated with GSPE exhibited greater hypothalamic activation of STAT3, which was associated with a rise in the *Pomc* mRNA levels compared to the CD group. In addition, this restoration of leptin responsiveness was accompanied by lower local inflammation and increased *Sirt1* gene expression. The effects of the GSPE treatment in the peripheral tissues were not as evident as those in the hypothalamus, although the GSPE treatment significantly restored the mRNA levels of *Socs3* and *Ptp1b* in the skeletal muscle. The use of GSPE reduces hyperphagia and improves the central and peripheral leptin resistance associated with diet-induced obesity. Our results suggest that GSPE could exert these effects partially by increasing Sirt1 expression and preventing hypothalamic inflammation.

1. Introduction

Obesity has reached truly epidemic proportions worldwide and has become one of the most prevalent health problems that our world currently faces.[1] In mammals, energy balance is regulated by controlling food intake and energy expenditure by means of the interactions of peripheral nutrients and hormones with different neuronal subpopulations. The critical cell populations include the anorexigenic proopiomelanocortin (POMC)- and orexigenic Agouti-related protein (AgRP)-expressing neurons, both located in the arcuate nucleus of the hypothalamus.[2]

Leptin, a hormone secreted mainly from white adipose tissue, is the main molecule that transmits information regarding the energy stores of the periphery to the hypothalamus.[3] The interaction of leptin with its longest receptor isoform (Obrb) in the POMC- and AgRP-expressing neurons promotes the phosphorylation of the signal transducer and activator of transcription-3 (STAT3). Then, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to the POMC and AgRP promoters. This stimulates the expression of POMC and inhibits that of AgRP reducing food intake and increasing energy expenditure.[4,5]

However, leptin is completely ineffective in decreasing food intake and suppressing body weight gain in subjects with diet-induced obesity. In this condition, instead of a leptin deficiency, high circulating levels of the hormone are observed, but the high levels are associated with a loss of responsiveness.[6–8] Although the basis of leptin resistance is not completely understood, it has been generally related to several mechanisms[9–11]including reduced leptin transport across the blood-brain barrier and the enhancement of intracellular processes

that attenuate OBRB signalling. These process include hypothalamic inflammation and endoplasmic reticulum (ER) stress, which, in turn, up-regulate the expression of negative regulatory molecules, including suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B).[12] Moreover, the hypothalamic NAD^+-dependent deacetylase sirtuin 1 (SIRT1) has been confirmed to be a mediator of leptin action in POMC- and AgRP-expressing neurons in which it suppresses nuclear factor-κB (NF-κB) signalling and/or regulates ER stress reactions through the deacetylation of the active spliced form of X-box binding protein 1 (XBP1s).[13] Therefore, hypothalamic SIRT1 activity may be an additional mechanism that is involved in the regulation of leptin signal transduction.[14]

Within this context, because pharmacologic methods to restore the leptin levels and sensitivity have not yet been found, the use of bioactive food compounds may be a useful approach that could complement the existing therapeutic strategies.[15] In this sense, natural dietary polyphenols, specifically proanthocyanidins (PACs), which are a class of flavonoids structurally complex, are bioactive food compounds present in fruits and vegetables and are significantly implicated in health promotion.[16] In particular, our group and others have reported many beneficial effects of grape-seed PACs on various obesity-associated diseases including insulin resistance, dyslipidemia, hypertension and local and systemic inflammation.[17–21] Moreover, although there have been some questionable results regarding the potential effects of these compounds on the control of body weight in diet-induced obesity,[22] latest studies have shown that grape-seed PACs have the potential to significantly modulate food intake and adiposity.[19,23,24] Thus, we hypothesized that the chronic consumption of dietary PACs could rescue the anorexigenic actions of leptin by interfering with those metabolic abnormalities that attenuate leptin

signalling in diet-induced obesity. Accordingly, the aim of the present study was to evaluate the effects of a grape-seed PAC extract (GSPE), administered for 21 days to rats previously fed a cafeteria diet (CD), on the central and peripheral leptin resistance induced by the CD.

2. Materials and methods

2.1 Grape-seed PAC extract

The grape-seed PAC extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract is mainly composed of phenolic compounds (total content higher that 96 %) including procyanidin monomers or flavan-3-ols (21.3%), and dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of procyanidins. The phenolic composition of this extract was further analyzed by Quiñones M *et al*.[25]

2.2 Animals and diet

The investigation was carried out in accordance with the ethical standards and according to the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249 by Generalitat de Catalunya).

Male Wistar rats of 200±50 g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were singly housed in a 12 h light-dark cycle at 22ºC, fed a standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum* and were provided access to tap water during the adaptation week. After the adaptation week, animals were distributed into three equivalent groups of 7 animals and housed individually in cages to permit control of their food intake. One group was fed with standard chow diet (STD) with a calorie breakdown of 14% protein, 8% fat and 73%

carbohydrates, and the other groups were fed with STD plus cafeteria diet (CD) composed by 14% protein, 35% fat and 51% carbohydrates. The animals had access during the dark phase to STD and water and to CD *ad libitum.* CD consisted of bacon, carrots, cookies, foie-gras, cupcakes, cheese and sugary milk. Ten weeks later, an oral treatment was administered in combination with the CD diet for 21 days. The treated group (CD+GSPE) received 25 mg of GSPE/kg body wt dissolved in 5% gum arabic (G9752, Sigma-Aldrich, Madrid, Spain), and the CD group received 1 mL of gum arabic. The rats received the treatment in the afternoon and then were allowed *ad libitum* access to STD and CD at night. On day 21 of treatment, the rats were fasted for 3 hours before anesthesia with 50 mg/kg body wt of sodium pentobarbital *(*Fagron Iberica, Barcelona, Spain) and euthanized by abdominal aorta exsanguination. Blood was collected using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. The plasma was obtained by centrifugation (1 500 x *g*, 4ºC, 15 min) and stored at -80ºC. The hypothalamus, liver, muscle and mesenteric white adipose tissue (mWAT) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80ºC until analysis.

2.3 Adiposity index

The adiposity index was determined by the sum of the mesenteric white adipose tissue (mWAT), perirenal white adipose tissue (pWAT) and epididymal white adipose tissue (eWAT) depot weights. Results were expressed as percentage of the total body wt.

2.4 Plasma leptin levels

The plasma leptin levels were determined using an Enzyme Immunoassay Kit according to the manufacturer's instructions (Biosource International, Inc., San Diego, CA, USA).

2.5 Indirect calorimetry

Indirect calorimetry analyses were performed on day 20 of the treatment using a Oxylet Pro System (PANLAB, Barcelona, Spain). Food was removed at 09:00 a.m., and the animals were fasted for 7 hours (from 09:00 am to 04:00 pm). After an initial acclimatization period of 1 hour, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 6 h. The respiratory quotient (RQ), energy expenditure (EE) and substrate oxidation were calculated as previously described.[26] Briefly, the RQ was calculated as the VCO2/VO2 ratio and the EE in kcal/day/kg0.75 as VO2×1.44×[3.815+(1.232×RQ)]. The rate of carbohydrate and fat oxidation were calculated in g/min as 4.55 × VCO2 – 3.21 × VO2 – 2.87 n and as 1.67 × VO2 – 1.67 × VCO2 – 1.92 n, respectively. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed. Finally, to obtain the values of fat and carbohydrate oxidation in kJ/min, the fat and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor.

2.6 Total RNA isolation

Total RNA from the hypothalamus, liver, muscle and mWAT was extracted using the TRIzol LS Reagent (Life Technologies, Uppsala, Sweden) and RNeasy Mini Kit (Qiagen) according to the manufacturers' protocols. The quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA integrity was evaluated on denaturing electrophoretic gels stained with SYBR Green dye (Bio-Rad, Barcelona, Spain), and only samples with an adequate RNA concentration (A260/A280 ≥1.8) and purity (A230/A260 ≥2.0) were selected for reverse transcription.

2.7 Gene expression analysis

The cDNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Life Technologies and was subjected to quantitative PCR (qPCR) using the CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad)

with SYBR Green PCR Master Mix (Bio-Rad). The forward and reverse primers for the various genes used in this study are shown in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). A cycle threshold (Ct) value was defined by setting the threshold during the geometric phase of the cDNA sample amplification. The fold change in expression of each mRNA was calculated with respect to the STD group using the ΔΔCt method corrected for the primer efficiency and converted to relative expression ratio with *Ppia* as the reference gene.[27]

2.8 Western blot analysis

Activated STAT3 in the hypothalamus, liver, mWAT and skeletal muscle was visualized using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). Additionally, the protein levels of the ObRb leptin receptor isoform as well as total and phosphorylated eIF2α in the hypothalamus were also determined by western blot analysis. Tissues were homogenized at 4°C in 0.5 mL of Radio-Immunoprecipitation Assay lysis buffer containing protease and phosphatase inhibitor cocktails using a TissueLyser LT (Qiagen). The homogenate was incubated for 30 minutes at 4°C and then centrifuged at 20 000 × *g* for 15 min at 4°C. The supernatant was used for total protein and western blot analyses. The total protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific).

A total of 100 μg of protein was solubilized and boiled for 10 min in a loading buffer solution containing Tris HCl 0.5 M, pH 6.8; glycerol, SDS, β-mercaptoethanol and Bromophenol Blue. The total protein extracts were separated using SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and electrotransferred onto supported PVDF membranes (Trans-Blot Turbo Mini PVDF Transfer Packs from Bio-Rad). After blocking, the membranes were incubated with agitation overnight at 4ºC with antibodies specific for ObRb (Abcam, Cambridge, UK), p-STAT3 (Abcam) or total or phosphorylated eIF2α (Cell Signalling, Izasa SA, Barcelona, Spain), diluted 1:1 000 and then with the goat anti-rabbit secondary antibodies (Sigma-Aldrich), diluted 1:10 000. For β-actin analysis, the membranes were incubated with a rabbit anti-actin

primary antibody (Abcam) and then with a goat anti-rabbit secondary antibody (GE Healthcare, Barcelona, Spain), using the same dilutions specified above. The protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Finally, protein band quantification was performed using ImageJ (W.S Rasband, NIH, MD, USA).

2.9 Statistical analysis

The data are expressed as the means ± s.e.m. A two-tailed Student *t* test was used to evaluate the differences between two groups. Multiple independent groups were compared with a one-way ANOVA followed by Tukey or Dunnett's T3 *post hoc* test when necessary. Outliers were determined by Grubbs' test. The statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

GSPE treatment ameliorated food intake but did not reverse the obesity and hyperleptinemia induced by the CD

As shown in Table 1, CD for 13 weeks consistently resulted in obesity and loss of leptin sensitivity in our experimental model as indicated by significant increases in the body wt and circulating leptin levels. Moreover, CD increased the adiposity index and the wt of all of the WAT depots studied including the mWAT, pWAT and eWAT depots, which confirmed the robust metabolic correlation between leptin and fat mass (rho=0.901, P <0.001). However, the administration of GSPE to the CD-fed rats for 21 days did not significantly exert anti-obesity effects, indicating that GSPE consumption during 21 days did

not prevent the total body wt gain measured at the end of the experimental period and did not significantly reduce the adiposity index and the weights of all the WAT depots studied (Table 1). A very similar pattern was also observed for the leptin levels, which exhibited a slight decrease (9.4% lower) in CD+GSPE group, but the difference from that of the CD group was not statistically significant (P=0.41).

Table 1. Body and adipose tissue weights, adiposity index and plasma concentrations of leptin in rats fed the STD or CD and treated with GSPE or the vehicle

	STD	CD	CD+GSPE
Initial body wt (g)	288±9	289±14	292±17
Final body wt (g)	443±11*	531±15	511±17
Body wt gain (g)	155±23*	242±31	219±43
Mesenteric WAT (g)	6.26±0.6*	14.00±1.5	13.33±0.9
Perirenal WAT (g)	8.96±0.4*	20.17±2.0	19.51±0.6
Epididymal WAT (g)	10.02±0.6*	21.61±2.5	18.10±0.8
Adiposity index (%)	5.80±0.3*	10.42±0.8	9.89±0.2
Leptin (ng/mL)	10.09±1.4*	27.85±2.5	25.24±2.4

Abbreviations: GSPE, grape-seed proanthocyanidin extract; WAT, white adipose tissue; wt, weight. The rats were fed a standard chow diet (STD group) or a cafeteria diet for 10 weeks. After 10 weeks, the rats fed the cafeteria diet were treated orally with GSPE (25 mg/kg of body wt) (CD-GSPE group) or the vehicle (CD group) for 21 days. The adiposity index was computed for each animal as the sum of the different WAT weights, expressed as a percentage of body weight. The values are the means ± s.e.m. of seven samples from each group. * denotes significant differences ($P < 0.05$) with respect to both CD and CD+GSPE groups assessed using one-way ANOVA and Tukey's *post hoc* test.

Notably, the energy intake in the GSPE-treated group was significantly lower than that of the CD rats (Table 2). In addition, because leptin has been reported to maintain high-energy expenditure even though it reduces energy intake, we reasoned that the GSPE treatment, despite its effects on energy intake, could also enhance energy expenditure and lead to the utilization of fat as the main energy source. Thus, we measured the energy expenditure and the respiratory quotient (RQ) in both CD- and GSPE-treated rats. However, no significant differences were observed between the groups in the RQ values, energy expenditure or substrate oxidation (Table 2).

Table 2. Food intake, substrate oxidation and energy expenditure in in rats fed with a CD and supplemented with GSPE or vehicle

	CD	CD+GSPE
Energy intake (KJ/day/animal)	1197.32±31.1	1105.83±45.4*
Respiratory quotient	0.89±0.1	0.88±0.1
Energy expenditure (Kcal/day/kg$^{0.75}$)	68.28±2.2	66.73±5.3
Carbohydrate oxidation (kJ/min/kg$^{0.75}$)	127.39±17.9	152.59±34.9
Fat oxidation (kJ/min/kg$^{0.75}$)	51.76±11.4	61.33±9.5

Abbreviations: GSPE, grape-seed proanthocyanidins extract. Rats were fed with a cafeteria diet for 10 weeks. After 10 weeks rats fed a cafeteria diet (CD) received oral treatment of GSPE (25 mg/kg of body wt) (CD-GSPE group) or vehicle (CD group) for 21 days. Values are mean ±s.e.m. of seven samples from each group. * denotes significant differences *(P* < 0.05) with respect to STD group assessed by Student's *t* test.

GSPE treatment activated the hypothalamic leptin receptor-STAT3 pathway in the CD-fed rats.

To determine whether the observed decrease in energy intake indicated that the GSPE treatment affected the functions of the POMC- and AgRP-expressing neurons, we initially assessed the leptin signalling pathway by measuring the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). Indeed, CD did lead to a significant decrease in the basal levels of p-STAT3 in the hypothalamus, and when GSPE was administered to the CD group, the levels of p-STAT3 increased significantly to basal levels. This showed that the GSPE treatment reversed the phenomenon observed in CD rats (Figure 1A) and indicated that treatment of the CD rats with GSPE for 21 days was sufficient to rescue the leptin signalling in this tissue.

Next, we determined whether the modulation of hypothalamic p-STAT3 was directly mediated by enhanced cell surface content of the long leptin receptor isoform Obrb. However, the CD group showed only a slight decrease in the mRNA levels of O*brb* compared to the STD group, and no statistically significant difference between these groups were detected. In addition, the mRNA levels of *Obrb* were not enhanced after the administration of GSPE to the CD rats (Figure 1B). This result indicated that if GSPE is, indeed, a true leptin sensitizer, its effects are not mediated by a rise in the *Obrb* content of the hypothalamic cells. Accordingly, the Obrb protein levels as assessed by western blotting were also not affected by the GSPE treatment. Finally, the gene expression of both the short leptin receptor isoform Obra and low density lipoprotein-related protein 2 (Lrp2), also called megalin, was assessed to determine whether the modulation of hypothalamic p-STAT3 was a result of an enhanced transcellular transport of leptin into hypothalamus. However, the

mRNA levels of both *Obra* and *Lrp2* were similar in all three groups of animals, and the differences among the groups did not reach statistical significance (Figure 1C).

To further investigate the effects of GSPE treatment on the regulation of the leptin signalling pathway, the gene expression of negative feedback regulatory molecules, namely suppressor of cytokine signalling 3 (*Socs3*) and protein-tyrosine phosphatase 1B (*Ptp1b*), was also assessed by qPCR. Notably, the *Socs3* mRNA levels were decreased in the CD-fed rats compared with the STD group, and the GSPE treatment significantly increased its transcript levels relative to the CD-fed rats. In addition, the mRNA expression of *Ptp1b* was reduced in the CD-fed rats compared with STD group, but the GSPE treatment did not induce any significant change after the treatment period compared to the CD-fed rats (Figure 1D).

Figure 1. Effect of GSPE treatment on the hypothalamic leptin signalling. The leptin signalling pathway was primarily assessed by evaluating the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3) (A) and by the determination of the cell surface content of the long leptin receptor isoform b (Obrb) (B). Additionally, qPCR was used to investigate the gene expression of both the short leptin receptor isoform a (Obra) and low density lipoprotein-related protein 2 (Lrp2) (C). Finally, the gene expression levels of the negative feedback regulatory molecules suppressor of cytokine signalling 3 (Socs3) and protein-tyrosine phosphatase 1B (Ptp1b) were also determined in this tissue (D). The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment selectively regulated the expression of hypothalamic peptides involved in appetite regulation

Then we evaluated the hypothalamic mRNA levels of proopiomelanocortin (*Pomc*), agouti-related peptide (*Agrp*), and neuropeptide Y (*Npy*). Interestingly, the *Pomc* mRNA levels were significantly increased in the GSPE-treated rats compared with both the STD group and CD group (Figure 2A). Furthermore, and in contrast to our expectations, we found that the *Agrp* gene expression levels were also significantly increased although to a much lower degree than the *Pomc* levels (Figure 2B). The *Npy* mRNA levels were not statistically altered by either the CD or GSPE treatment (Figure 2C).

Figure 2. Effect of GSPE treatment on the regulation of hypothalamic neuropeptide gene expression. The mRNA levels of hypothalamic proopiomelanocortin (Pomc) (A), agouti-related peptide (Agrp) (B) and neuropeptide Y (Npy) (C) were assessed using qPCR. The mRNA levels of the

selected neuropeptides were normalized to those of Ppia. The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment significantly potentiated the gene expression of hypothalamic *Sirt1* in a manner consistent with an attenuation of local inflammation

To elucidate the intracellular effects by which GSPE treatment potentially rescues leptin signalling in the hypothalamus, we assessed the impact of these compounds on the molecular processes associated with leptin signalling disruption, including local inflammation, ER stress and loss of SIRT1 activity in this tissue.

The contributions of these processes to leptin resistance were initially investigated using the gene expression of inducible nitric oxide synthase (*inos*), which is an important marker of neuroinflammation. Indeed, although the CD did not induce local inflammation in this tissue, as indicated by the similar mRNA levels in the STD and CD groups, the GSPE treatment significantly down-regulated the *inos* gene expression (Figure 3A), which confirmed the ability of these compounds to prevent local inflammation. Notably, GSPE treatment resulted in a significant up-regulation (3-fold higher) of the *Sirt1* mRNA levels in a manner consistent with reduced hypothalamic inflammation (Figure 3B).

Finally, to address the molecular implications of ER stress in leptin resistance, hypothalamic ER stress markers, including the spliced form of X-box binding

protein-1 (XBP1s) and the levels of ATF4 and CHOP mRNA, were also determined by qPCR. However, our results indicated that GSPE treatment did not modify the gene expression levels of any of these indicators (Figure 3C). Additionally, other ER stress markers were assessed by western blotting. However, the total eIF2α protein levels did not display significant differences among the groups (Figure 3D), and the phosphorylated form of eIF2α was not detected in any group of animals. These results indicate not only that the CD did not induce ER stress in this tissue but that these markers of ER stress were also not significantly affected by the GSPE treatment.

Figure 3. Effect of GSPE treatment on hypothalamic inflammation, sirtuin expression and ER stress. To evaluate the possible mechanisms responsible for the GSPE effects on hypothalamic leptin signalling, the gene expression of inducible nitric oxide synthase (inos) (A) and sirtuin 1 (Sirt1) (B) was investigated in this tissue. Additionally, the hypothalamic mRNA levels of the ER stress markers X-box binding protein-1 (XBP1) spliced form, and ATF4 and CHOP were determined using qPCR (C). The mRNA levels of these selected genes were normalized to those of Ppia. In addition, the protein levels of total and phosphorylated eIF2α were also assessed by immunoblotting (D). The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment distinctively modulated leptin signalling in the liver, mWAT and skeletal muscle of the CD-fed rats

Alternatively, to assess the contribution of the metabolic signals derived from peripheral tissues to the regulation of energy intake and energy homeostasis, we next investigated the leptin signal transduction in the liver, mesenteric WAT (mWAT) and skeletal muscle. Indeed, a decrease in the level of p-STAT3 was observed in the livers of the CD and GSPE groups compared to STD group but the differences did not reach statistical significance (Figure 4A). Furthermore, no significant differences were observed in the mRNA levels of *Obrb*, *Socs3* and *Ptp1b* among the three groups of animals indicating that GSPE had no effect on restoring leptin sensitivity in this tissue.

In the mWAT (Figure 4B), the CD-fed rats showed a significant increase in p-STAT3 compared to the STD-fed rats, whereas the GSPE treatment significantly restored the p-STAT3 values to those of the STD-fed rats. Furthermore, the mRNA levels of *Obrb*, *Socs3* and *Ptp1b* in mWAT were also similar in all three groups of animals.

Finally, a slight increase of p-STAT3 was observed in the skeletal muscles of the GSPE-treated rats compared to STD and CD groups (Figure 4C). Importantly, in contrast to the liver and mWAT, the *Socs3* and *Ptp1b* gene expression levels were significantly lower in the CD-fed rats than in the STD group; furthermore, the GSPE treatment induced a robust increase of their expression levels compared to the CD-fed rats. These results indicated that the skeletal muscle is the tissue most sensitive to the GSPE treatment with respect to leptin signalling. In addition, as observed in the hypothalamus, the *Obrb* mRNA levels were not altered by the GSPE treatment.

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Figure 4. Effect of GSPE treatment on the peripheral leptin signalling. The leptin signalling pathway was investigated in the liver (A), mesenteric WAT (mWAT) (B) and skeletal muscle (C). STAT3 phosphorylation (p-STAT3) was assessed using western blotting, and the mRNA levels of the long leptin receptor isoform b (Obrb), suppressor of cytokine signalling 3 (Socs3) and protein-tyrosine phosphatase 1B (Ptp1b) were determined using qPCR. The mRNA levels of these selected genes were normalized to those of Ppia. The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

4. Discussion

Previous results from our group have indicated that chronic consumption of grape-seed PACs are unable to counteract the body weight gain and the hyperleptinemia induced by a CD in rats, but consumption of these compounds significantly reduces the food intake.[23] The hyperphagia observed in animals fed a highly palatable diet has been related to a dysfunctional melanocortin system[28]. We therefore determined whether chronic ingestion of GSPE was able to reverse this dysfunction and normalize the leptin signalling in the hypothalamus of rats fed a CD.

Importantly, our CD model exhibited central leptin resistance as indicated by the decrease in the hypothalamic p-STAT3 levels in rats fed the CD for 13 weeks. Notably, the impairment of leptin-induced STAT3 phosphorylation in the hypothalamus has been considered to be one of the leading markers for cellular leptin signal attenuation in hyperleptinemic rats with diet-induced obesity.[29] Conversely, the CD-fed rats in this study did not display altered gene expression of *Obrb*, *Pomc*, *Agrp* or *Npy* in the hypothalamus. Contradictory

results have been published regarding the effect of a CD on *Pomc* expression in the hypothalamus, with both repression[30] and overexpression[31] having been reported. Thus, the duration of the CD and the grade of obesity achieved can affect the severity of the melanocortin system dysfunction in rats.

Remarkably, 21 days of GSPE treatment normalized the level of p-STAT3 and the gene expression of *Socs3* and *Ptp1b* in the hypothalamus, all of which had been repressed by the CD. Interestingly, this normalization was associated with a high overexpression of *Pomc*, suggesting that the up-regulation of *Pomc* could be mediated by the increase in p-STAT3 induced by the GSPE treatment. Together, these results indicate that chronic consumption of GSPE clearly improved the central leptin signalling in the CD-fed rats. Notably, POMC is an anorexigenic neuropeptide.[2] Thus, the overexpression of POMC induced by GSPE treatment could mediate the significant reduction of food intake and adiposity observed in the CD+GSPE group. Other polyphenols and polyphenol extracts modulate the neuropeptides involved in food intake and energy expenditure (reviewed in [32]). For instance, resveratrol reduces *Npy* and *Agrp* expression[33], and apigenin increases *Pomc* expression[34] in neuronal cell lines. These results reinforce the idea that specific polyphenols could improve central leptin signalling.

The induction of central leptin resistance in diet-obesity models has been mainly attributed to hypothalamic inflammation[35,36] as a result of the induction of the pro-inflammatory signalling molecules JNK and NF-κB and ER stress resulting from over-nutrition. Remarkably, our results showed that GSPE treatment reduced the hypothalamic inflammation, as indicated by the *inos* gene expression levels, which suggested that the local anti-inflammatory activity of proanthocyanidins in this tissue could be one of the mechanisms by which GSPE treatment re-established normal central leptin sensitivity. In addition,

SIRT1activity has been highlighted as a mediator of central leptin action.[13,14] Thus, the hypothalamic overexpression of *Sirt1* induced by GSPE treatment could be another part of the mechanism by which GSPE treatment reduced the central leptin resistance.

The improvement of central leptin signalling could be secondary to the peripheral actions of GSPE on metabolism and hormones or the communication by the afferent nervous system to the brain. However, the capacity of GSPE to modulate *Sirt1* gene expression levels and inflammation in the hypothalamus itself, together with the facts that GSPE compounds can cross the brain-blood barrier [37] and that their metabolites have been found in the rat brain,[38] suggest a direct action of the PACs at hypothalamic level.

In addition to the hypothalamus, peripheral tissues such as the liver, skeletal muscle and adipose tissue are targets of leptin, and peripheral leptin resistance has been associated with obesity.[39] Notably, the GSPE treatment normalized the leptin cascade disruptions caused by the CD in the mWAT and skeletal muscle. Remarkably, leptin resistance in WAT has been associated with the excessive fat mass accumulation that characterizes obesity.[39] Accordingly, the normalization of leptin signalling observed in the mWAT of rats supplemented with GSPE was associated with a decline in the body adiposity. Moreover, we have demonstrated in a previous study that CD-fed rats supplemented with GSPE at the same dose and time period used in this study show an activation of muscle β-oxidation and an improvement in mitochondrial function.[24] These effects of GSPE in muscle are also consistent with the up-regulation of fatty acid oxidation, which is an insulin-sensitizing effect of leptin in this tissue.[40]

Therefore, these results clearly indicate that GSPE treatment was also effective in improving peripheral leptin sensitivity in CD-fed rats.

Despite this improvement of leptin sensitivity, rats treated with GSPE did not displayed a significant body weight reduction indicating that GSPE, at the dose and time used in this experiment, was not sufficient to totally reverse leptin dysfunction induced by a high-fat diet. However, body weight gain and epididymal fat mass of rats treated with GSPE were 10% and 16% lesser that those of rats not treated, respectively. Therefore, the improvement of leptin sensitivity induced by GSPE could behind a body mass rearrangement that, in turn, can improve obesity outcomes.

Intriguingly, GSPE did not reduce significantly the body weight, despite GSPE decreasing energy intake and not affecting energy expenditure nor substrate utilization. However, measurements of energy expenditure and substrate utilization were performed in the fasted state rather than the fed state. Thus, the lack of effects of GSPE on body weight combined with a decrease in energy intake suggest that energy expenditure in the fed state may be reduced by GSPE.

Importantly, translation of the daily dose of PACs (25 mg per kg of body wt per day) used in this study to the human doses[41] estimated for a 70 kg human indicates that the equivalent intake would be between 250 and 280 mg of GSPE/day. Humans consuming a polyphenol-rich diet can easily achieve or even exceed this PAC intake.[42] Therefore, the inclusion of PAC-rich foods in diets of obese people could be a good strategy to reduce the appetite and improve central and peripheral leptin sensitivity, thus complementing dietary therapies intended to promote weight loss in obese subjects. However, the effect of an obesogenic diet on leptin resistance have been described to be sex-

dependent.[43] Therefore, as this study has been performed in males, further studies are warranted in order to determine the potential sex differences of PACs in relation to energy intake and body weight.

In conclusion, 21 days of GSPE treatment normalized the CD-induced leptin signalling disorders observed mainly in the hypothalamus and in the skeletal muscle of rats with diet-induced obesity. This improvement in leptin signalling resulted in part from neuroprotection against diet-induced inflammation and from the increase in hypothalamic sirtuin expression. Together, these results strongly suggest that PACs could reduce energy intake and adiposity by re-establishing central and peripheral leptin sensitivity.

Conflict of interest

The authors declare no conflicts of interest.

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Supplementary information is available at International Journal of Obesity's website

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Supplementary material

Table S1. A summary of the rat-specific primer sequences used for qRT-PCR analysis.

Primer Name (Rat)	Primer sequences (5'-3')	Product size (bp)	Gen Bank accession no/reference
Agrp	GAG AAC TCT GGG AAC AGG GC CAA GCA AAG GCC ATG CTG AC	140	NM_033650.1
Atf4	TAT GGA TGG GTT GGT CAG TG CTC ATC TGG CAT GGT TTC C	145	NM_024403.2
Chop	AAG ATG AGC GGG TGG CAG CG CCG GTT TCT GCT TTC AGG TGT GGT	112	NM_001109986.1
Lpr2	GGA GCC AGT CAG TAG CCA AG CCT GGG AGG ACA GCC AAT TT	136	NM_030827.1
iNos	GGA TCT TCC CAG GCA ACC A AAT CCA CAA CTC GCT CCA AGA TT	60	Martínez-Micaelo N *et al.* [1]
Npy	CTA TCC CTG CTC GTG TGT TTG G TGG TGA TGA GAT TGA TGT AGT GTC G	136	Sun B *et al.* [2]
ObRa	CAC TGT TAA TTT CAC ACC AGA G GTC ATT CAA ACC ATA GTT TAG	235	AF304191.1
ObRb	CCA GTA CCC AGA GCC AAA GT GGA TCG GGC TTC ACA ACA AGC	122	NM_012596.1
Pomc	CAT AGA CGT GTG GAG CTG GT TCA AGG GCT GTT CAT CTC CG	149	NM_139326.2
Ppia	CTT CGA GC TGT TTG CAG ACA A AAG TCA CCA CCC TGG CAC ATG	138	NM_017101.1
Ptp1b	CCC TTT TGA CCA CAG TCG GA TTG GTA AAG GGC CCT GGG TG	119	NM_012637.2
Sirt1	TTG GCA CCG ATC CTC GAA ACA GAA ACC CCA GCT CCA	217	XM_006223877.1
Socs3	CTG GAC CCA TTC GGG AGT TC CTG GGA GCT ACC GAC CAT TG	148	NM_053565.1
Xbp1	GCT GAA GAG GAG GCG GAA G GTC CAG AAT GCC CAA CAG G	172	Mulero M *et al.* [3]

Abbreviations: Agrp, Agouti related protein; Atf4, activating transcriptor factor 4; Chop, C/EBP homologous protein; Lpr2, Low density lipoprotein-related protein 2; iNos, inducible nitric oxide synthase; Npy, neuropeptide Y; Obra, leptin receptor isoform a; Obrb, leptin receptor isoform b; Pomc, proopiomelanocortin; Ppia, peptidylprolyl isomerise A; Ptp1b, protein tyrosine phosphate 1B; Sirt1, sirtuin 1; Socs3, suppressor of cytokine signaling 3; Xbp1, X-box binding protein 1.

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UNIVERSITAT ROVIRA I VIRGILI
POLYPHENOL EFFECTS ON CENTRAL LEPTIN SENSITIVITY IN OBESITY
Maria Ibars Serra

CHAPTER 2

Resveratrol, but not anthocyanins, improves hypothalamic leptin sensitivity and potentially contributes to body weight loss in obesity

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In preparation

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Abstract

Changes in diet composition and increased calorie intake have a big impact in most common forms of obesity. Concomitant to the excess body fat, obesity also causes hyperleptinemia. In this condition leptin sensitivity is decreased in the hypothalamus and the ability to regulate energy balance is deeply blunted. Phenolic compounds may have a role in body weight control and metabolic regulation. Therefore, the aim of this study was to determinate whether resveratrol or anthocyanins could individually increase leptin sensitivity in the hypothalamus and reduce body weight and total body fat mass under obesogenic conditions.

We initially performed a preliminary study in healthy mice fed standard chow diet and supplemented with 100 mg/kg/day of either anthocyanin-rich extract (ARE) or resveratrol (RSV) for 15 days. RSV consumption, but not ARE, enhanced leptin signalling and 24h-energy expenditure by increasing both hypothalamic phosphorylation of STAT3 (pSTAT3) and *Obrb* gene expression. Then, we designed a second study in order to assess whether RSV could re-establish appropriate leptin sensitivity in hyperleptinemic obese animals. Accordingly, male Wistar rats were fed a cafeteria diet (CD) during 8 weeks, afterwards animals were supplemented with a daily dose of 50, 100 or 200 mg/kg of RSV for 22 days together with de CD. We observed that the consumption of 200 mg/kg/day of RSV was significantly associated with both reduced leptin levels and increased hypothalamic leptin sensitivity. Notably, this restoration of leptin action at this dose was accompanied by lower body weight and higher 24h-energy expenditure and fat oxidation compared to untreated obese rats, but not by reduced food intake. Although, these metabolic effects on body weight and energy balance were not so evident at lower doses of RSV, 100 mg/kg/day significantly showed higher levels of hypothalamic

protein content of pSTAT3, ObRb and SOCS3, indicating that RSV at this intermediate dose could be also effective in improving leptin sensitivity in this tissue. In contrast, no significant changes on leptin system were observed at dose of 50 mg/kg/day.

Therefore, these results suggest that the anti-obesity effects of RSV could be at least partly through the modulation of leptin sensitivity in the hypothalamus. Future studies should focus on analyzing more extensively the molecular mechanism by which RSV directly modulates leptin signalling and affects body weight.

1. Introduction

Obesity and overweight are associated with higher all-cause mortality and it is increasing nowadays. Strategies to counteract obesity are a main focus for global health[1]. The hypothalamus is a brain area involved in the regulation of energy homeostasis. Specifically, the arcuate nucleus (ARC) receives signals from peripheral organs that inform about energy status in the body[2]. One of this chemical signals is leptin, the key hormone in energy regulation. It is mainly produced in the adipocytes and is proportional to body fat stores[3]. Circulating leptin reaches the ARC in the hypothalamus and binds to the long form of leptin receptor (ObRb). This interaction activates the transduction pathway through the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) in different neurons to supress food intake and allow energy expenditure[4]. Specifically, leptin activates anorexigenic proopiomelanocortin (POMC) neurons that produce α-MSH peptide responsible to bind melanocortin receptor 4 (MC4R) and produce satiety signals. Concomitantly, leptin also

inhibits orexigenic neurons producing agouti-related protein (AgRP) and neuropeptide Y (NPY)[5].

However, in obesity, leptin is not able to promote energy expenditure and satiety despite plasmatic levels of this hormone appears to be highly increased. This lost in leptin sensitivity is known as 'leptin resistance', although recent data postulate the term should be redefined[6]. Several mechanisms could reduce leptin sensitivity. These include defective transport across the blood brain barrier (BBB) and attenuation of ObRb signalling through increased endoplasmic reticulum stress and/or inflammation, impaired NAD^+-dependent deacetylase sirtuin 1 (SIRT1) function and the overexpression of inhibitory factors such as suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase (PTP1B)[7,8].

As current pharmacological treatments to improve leptinemia and leptin sensitivity in diet-induced obesity did not succeed, several authors, among whom we find ourselves, support that several phenolic compounds present in plants may target hypothalamic leptin system, which makes them a promising strategy to complement the existing therapies against obesity[9]. In particular, we have recently reported that a grape-seed PACs extract is able to reduce circulating plasmatic leptin levels and improve hypothalamic leptin signalling by increasing *Sirt1* gene expression and preventing inflammation[31]. This capacity has been also observed in other polyphenolic-rich extracts obtained from natural sources as well as in several pure phenolic compounds such as oleuropein, quercetin, curcumin and apigenin[9].

In this context, both resveratrol and anthocyanins have consistently demonstrated their anti-obesity effects by lowering total body fat mass and leptin serum levels[10–17]. However, only a few studies have focused on the mechanism by which these phenolic compounds could primarily alleviate

obesity and there is not enough evidence about their effects on leptin signalling. Therefore, as these compounds or its derived-metabolites can cross the BBB and interact with different neuronal subpopulations in the ARC[10], the aim of the present study was to examine whether either anthocyanins or resveratrol could exert part of their anti-obesity effects by modulating leptin sensitivity in these tissue. Initially, we evaluated hypothalamic leptin signalling only in non-obese animals in order to investigate the effects of these compounds under normal physiological conditions. We reasoned that if the consumption of resveratrol or anthocyanins is able to exert a minimal effect in healthy animals, it could give the indication that this effect could potentially be higher under pathological conditions. Thus, after testing them under physiological conditions, we also investigated the impact of these compounds in diet-induced obese animals with hyperleptinemia and impaired central leptin signalling in order to confirm if they could restore leptin sensitivity appropriately in these unfavorable metabolic conditions.

2. Materials and methods

2.1 Natural compounds

Resveratrol (RSV) of >98% purity degree was purchased from Fagron (Fagron Iberica, S.A.U, Barcelona, Spain). MEDOX®, an anthocyanin-rich extract (ARE) was provided by (MedPalett AS, Sandnes, Norway) which contained purified anthocyanins isolated from bilberries (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) (a mixture of 3-*O*-rutinosides of cyanidin and delphinidin, and 3-*O*-*β*-galactosides, 3-*O*-*β*-glucosides, and 3-*O*-*β*-arabinosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin). The 3-*O*-*β*-

glucosides of cyanidin and delphinidin constituted at least 40–50% of the total anthocyanins.

2.2 Animals and diet

The investigation was conducted in accordance with the ethical standards and according to the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the University Rovira i Virgili.

2.2.1 Study 1: Healthy mice

Male NMRI mice aged 8 weeks and of 40.14±0.47g body weight were purchased from Charles River Laboratoires (Bercelona, Spain). Animals were housed in a 12h light-dark cycle at 22ºC and fed with a standard rodent diet (Panlab 04, Barcelona, Spain) with a calorie breakdown of 20% protein, 8% fat and 72% carbohydrate and water *ad libitum*. After one week of adaptation, mice were trained for another week to lick low-fat condensed milk diluted in water 1:1 which was used as vehicle. Then, the animals were divided in 3 groups (n=8). The ARE group received 100 mg/kg of the anthocyanin rich-extract and the RSV group received 100 mg/kg of resveratrol every day. The control animals (STD group) received the same volume of the vehicle. The treatment lasted for 15 days. Weight was monitored every two days and indirect calorimetry was performed at the beginning and end of the experiment. Food intake was recorded 48h before sacrifice. On day 15th of treatment animals were fasted after treatment administration and sacrificed three hours later. Animals were anesthetized with Pentobarbital (60 mg/kg), cardiac puncture for plasma collection was performed and finally cervical dislocation. Blood was collected

using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. The plasma was obtained by centrifugation (1,500 x *g*, 4°C, 15 min) and stored at -80°C. The hypothalamus was excised and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Visceral fat depots were weighed and adiposity was calculated with the absolute weight of epididymal, mesenteric and retroperitoneal adipose tissues. Data was expressed as percentage of total body weight.

2.2.2 Study 2: Diet-induced obese rats

Male Wistar rats aged 5 weeks and of 122.56±3.11g body weight were purchased from Charles River Laboratories (Barcelona, Spain). Animals were housed in a 12h light-dark cycle at 22ºC, fed with a standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum* and provided access to tap water during 10 days of adaptation. After the adaptation period animals were divided into five equal groups composed by 6 rats. One group was fed with standard chow diet (STD group) with a calorie breakdown of 20% protein, 8% fat and 72% carbohydrate and the other groups were fed with STD plus cafeteria diet (CD groups) composed by 15% protein, 25% fat and 60% carbohydrate *ad libitium*. Animals had free access to fresh STD and cafeteria diet every day at 11:00 am. CD diet consisted of cookies, cheese, bacon, foie-gras, sugary milk, ensaïmada (a typical Majorcan pastry) and carrots. Weight gain and food consumption were monitored every week for 9 weeks until animals gained more than 15% body weight compared to STD group. Then, oral treatment was administered together with CD diet for 22 days. Treatment groups were daily supplemented with 50, 100 or 200 mg/kg body weight of resveratrol dissolved in low-fat condensed milk diluted in water 1:1. STD and CD diet group were supplemented with the same quantity of vehicle. Body weight and food intake

was weekly monitored until the end of the experiment. The day before sacrifice body composition was analysed from rats using magnetic resonance imaging system EchoMRI-700 (Echo Medical Systems, LLC., TX, USA). On day 22 of treatment rats were sacrificed by decapitation. Blood was collected and plasma was obtained as previously mentioned. Hypothalamus was excised and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.3 Circulating leptin levels

Leptin concentrations were measured in plasma using a specific Enzyme Immunoassay kit according to the manufacturer's instructions (Millipore, Madrid, Spain).

2.4 Respiratory exchange ratio and energy expenditure

Animals were acclimated in the respirometry system cages for a period of 2h. After this time they were subject to 16h of indirect calorimetry analyses using Oxylet Pro System (Panlab, Cornellà, Spain). Measurements were taken for a 8:8 hours light:dark cycle .The oxygen consumption (VO2) and carbon dioxide production (VCO2) measures were used by the software Metabolism 2.1.02 (Panlab) to calculate Respiratory Quotient (RQ) as VCO2/VO2 and energy expenditure as VO2x1.44x[3.815+(1.232xRQ] (Kcal/day/Kg^0.75) according to Weir equation[15]. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed[16]. Total activity was measured by recording infrared beam breaks (Oxylet Pro System, Panlab).

2.5 Gene expression analyses

Mice hypothalamus (n=4) was homogenized to extract total RNA using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) followed by RNeasy Mini Kit (Qiagen, Barcelona, Spain) according to manufacturer's instructions. Quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only samples with A260/A280 ratio ≥1.8 and A230/A260 ratio ≥2 were chosen to perform reverse transcription. RNA was converted to cDNA using High-Capacity complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain). Gene expression was determined by Real-Time PCR using the iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain) in the CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad) using primers obtained from Biomers.net (Ulm, Germany) (**Supplementary Table 1**). Relative expression of each gene was calculated referring to *Ppia* and normalized to the STD group. ΔΔCt method was used and corrected for primer efficiency [17].

2.6 Immunoblot analysis

Leptin signalling in the hypothalamus was assessed by calculating the activation of STAT3 using a phospho-specific antibody that identifies Tyr705phosphorylated STAT3 (pSTAT3). The assessment of pSTAT3 levels is the gold standard experimental marker for cellular leptin signalling[18]. Moreover, the protein levels of the ObRb leptin receptor isoform, SOCS3 and FOXO1 were also determined by Western blot analysis. Both mouse and rat hypothalamus (n=4 and n=6, respectively) were homogenized at 4°C using a Tissue Lyser LT (Qiagen, Barcelona, Spain) in 0.5 mL of Radio-Immunoprecipitation Assay lysis (RIPA) buffer (50mM Tris-HCl, 150mM

NaCl, pH 7.4, 1% Tween, 0.25% Na-deoxycholate, 0.1M phenylmethylsulfonyl fluoride) containing protease and phosphatase inhibitor cocktails (Sigma Aldrich, Madrid, Spain). The homogenates were incubated for 30 min at 4ºC and centrifuged at 20,000 x *g* for 15 min at 4ºC. The supernatant was placed in fresh tubes and was used to determine total protein and for immunoblotting analyses. The total protein content was quantified using the Pierce BCA protein assay kit (Thermo Scientific, Barcelona, Spain). Samples were denatured by mixing with loading buffer solution (Tris HCl 0.5M pH 6.8, glycerol, SDS, β-mercaptoethanol and Bromophenol Blue) and then heated at 99ºC during 5 min using a thermocycler (Multigen Labnet, Barcelona, Spain). Acrylamide gels were prepared using TGX Fast Cast Acrylamide Kit, 10% (Bio-Rad, Barcelona, Spain) and 30µg of protein were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using electrophoresis buffer (glycine 192mM, Tris base 25mM and 1% SDS). Proteins were electrotransfered onto supported polyvinylidene difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad). After blocking with 5% of non-fat dried milk, membranes were incubated with gentle agitation overnight at 4°C with specific antibodies for ObRb (Abcam, Cambridge, UK), diluted 1:1000, pSTAT3 (Abcam), diluted 1:2500, SOCS3 (Cell Signalling, Izasa S.A., Barcelona, Spain), diluted 1:1000 or FoxO1 (Cell Signalling), diluted 1:1000. For β-actin analysis as a loading control, membranes were incubated with a rabbit anti-actin primary antibody (Sigma, Madrid, Spain), diluted 1:1000. Finally, membranes were incubated with anti-rabbit horseradish peroxidase secondary antibody (GE Healthcare, Barcelona, Spain), diluted 1:10000. Protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Lastly, protein bands were quantitated by densitometry using ImageJ software (NIH, Bethesda, MD, USA) and each band was normalized by the

corresponding β-actin band and finally the treatment groups were normalized by the control group (STD).

2.7 Leptin sensitivity assessment

Consistent with hypothalamic pSTAT3 levels are typically attributable to leptin action, leptin sensitivity was estimated as the ratio of hypothalamic pSTAT3 levels to leptin concentration in plasma.

2.8 Statistical analyses

The results are expressed as mean ± standard error of the mean (S.E.M). Two tailed Student *t* -test was used to find differences between two groups. Multiple independent groups were compared with one-way ANOVA followed by LSD or Tukey post-hoc test. Repeated-measures ANOVA was used in indirect calorimetry analyses. GraphPad Prism 6 was used for statistical analyses and graphs (GraphPad Software, La Jolla, CA, USA). A P value ≤ 0.05 was considered statistically significant.

3. Results

Resveratrol, but not anthocyanins, increased hypothalamic leptin signalling and 24h-energy expenditure in healthy mice by modulating *ObRb* expression

Given the multiple line of evidence supporting leptin as a potential target for the energy balance regulation, we specifically tested the impact of resveratrol (RSV) or anthocyanin-rich extract (ARE) in hypothalamic leptin system. Thus, both plasmatic leptin concentrations and hypothalamic leptin signalling were initially evaluated in healthy mice treated for 15 days at dose of 100 mg/kg of either ARE or RSV in order to investigate them under physiological conditions.

At the end of the trial, any of the treated groups showed significant differences in plasma leptin levels compared to the STD group **(Fig. 1A)**. In order to assess the levels of leptin signalling in the hypothalamus, the activation of STAT3 was determined by immunoblotting in this tissue. The consumption of RSV demonstrated a trend to enhance pSTAT3 protein content compared to STD group (P=0.1) **(Fig. 1B)** while ARE consumption did not exert significant changes in pSTAT3 levels. We also determined whether the gene expression of the long leptin receptor isoform Obrb was up-regulated by any of the treatments. Interestingly, both ARE and RSV consumption produced higher mRNA levels of *Obrb* with respect to untreated animals (4- and 5-fold, respectively), but these changes were only statistically significant in RSV group **(Fig. 1C)**.

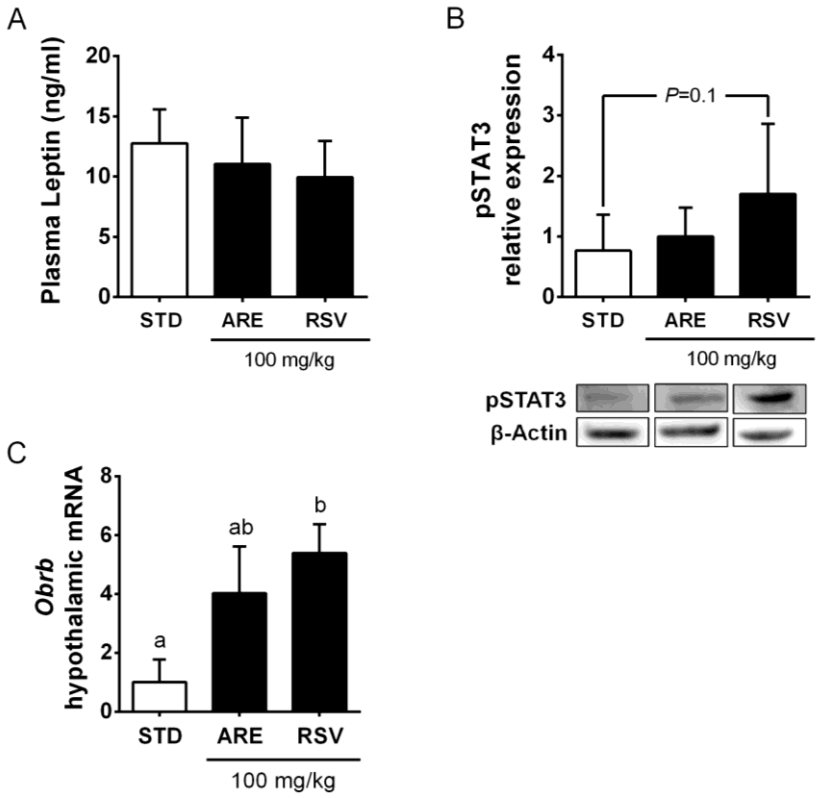

Figure 1. Plasma leptin levels and hypothalamic pSTAT3 and ObRb expression in mice treated with 100 mg/kg of either ARE or RSV. To study leptin signalling we initially determined the plasmatic levels of the hormone after 15 days of treatment with either vehicle (STD), anthocyanins (ARE) or resveratrol (RSV) (A). Additionally, both STAT3 phosphorylation (B) the mRNA levels of the long form of leptin receptor (Obrb) (C) were also assessed by immunoblotting and quantitative PCR, respectively. Values are mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

To further investigate the main regulatory factors involved in the leptin system, the mRNA levels of *Socs3*, *Ptp1b* and *Sirt1* were also analysed by quantitative PCR **(Fig. 2A).** Although, *Ptp1b* and *Sirt1* gene expression were not altered by any of the treatments, RSV consumption, but not ARE, exerted a notably trend to decrease the mRNA levels of *Socs3* with respect to untreated group (*P*=0.09)

Next, we also assessed the hypothalamic mRNA levels of *Pomc*, *Agrp* and *Npy* neuropeptides. However, none of them presented statistically significant changes in their hypothalamic expression after consumption of ARE or RSV **(Fig. 2B)**, although the levels of all of them seemed to be increased in RSV group when compared with both ARE and untreated groups.

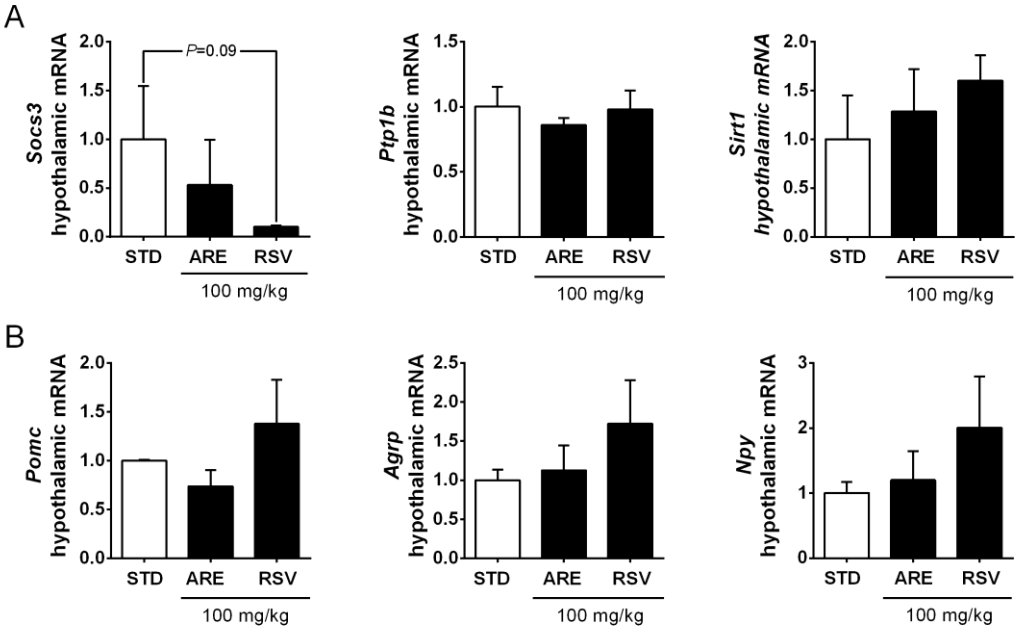

Figure 2. Hypothalamic gene expression in healthy mice. To further investigate leptin signalling, the expression of the genes involved in the signalling pathway was analysed in the hypothalamus of healthy mice. Quantitative PCR was used to determine the mRNA levels of the regulators of the leptin cascade *Socs3*, *Ptp1b* and *Sirt1* **(A)**, followed by the gene expression of anorexigenic (*Pomc*) and orexigenic neuropeptides (*AgRP* and *Npy*) **(B)**. mRNA levels of each gene were normalized to the constitutive gene *Ppia*. Values are mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

Finally, body weight, visceral adiposity, food intake and respiratory quotient (RQ) were not significantly modified after 15 days of treatment with either ARE or RSV (**Table 1**). However, the consumption of RSV significantly

increased 24-hour energy expenditure compared to both ARE-treated group and STD group.

Table 1. Body weight, visceral adiposity, food intake and indirect calorimetry measurements.

	STD	ARE	RSV
Body weight (g)	40.38±0.8	41.75±0.98	41.37±0.91
Visceral adiposity (%)	3.11±0.29	2.86±0.27	3.30±0.50
Food intake (g)	11.27±0.66	9.91±0.72	10.07±0.79
RQ	0.80±0.06	0.79±0.02	0.86±0.06
Energy expenditure (kcal/day/kg^0.75)	125.47±16.58^{ab}	119.94±6.32^{a}	155.13±6.82^{b}

Abbreviations: STD, standard chow diet; ARE, anthocyanin rich extract; RSV, resveratrol; RQ, respiratory quotient. Mice were fed a STD and were orally treated with vehicle (STD group), or either 100 mg/kg of ARE or RSV for 15 days. Visceral adiposity index was estimated for each animal as the sum of mesenteric, epididymal and perirenal fad pads, expressed as percentage of body weight. Values are the mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

High doses of resveratrol reduced circulating leptin levels and improved hypothalamic leptin sensitivity in obese rats

After performing the study in healthy mice, we decided to exclusively confirm the effect of RSV on hypothalamic leptin system using, at this time, diet-induced obese animals that presented both hyperleptinemia and impaired leptin sensitivity. For practical reasons and for similarity to humans, in this study we used Wistar rats fed cafeteria diet (CD) during 12 weeks instead of genetically obese mice[19]. Thus, the main goal was to observe if different doses of RSV could modulate hypothalamic leptin signalling in obesogenic conditions.

Accordingly, rats were divided in a low, intermediate and high dose groups (n=6) and were daily supplemented with 50 mg/kg (CD+50), 100 mg/kg (CD+100) or 200 mg/kg (CD+200) of RSV together with a cafeteria diet for 22 days.

At the end of the trial, circulating leptin levels in plasma showed a significant increase in CD group compared to the STD group **(Fig. 3A)**, indicating that our experimental model developed hyperleptinemia after 12 weeks of CD. Interestingly, at dose of 200 mg/kg/day, RSV consumption significantly reduced leptin levels compared to the CD animals, without any effect at lower doses.

Next, the activation of STAT3 was analysed by immunoblotting **(Supplementary Fig. 1)**. Contrary to our expectations, CD did not lead to a significant decrease in the basal levels of pSTAT3 in the hypothalamus, but when RSV was administered to obese rats, the levels of pSTAT3 significantly increased with respect to CD, although only the dose of 100 mg/kg/day of RSV reached statistical significance **(Fig. 3B).** Considering that hypothalamic pSTAT3 levels are mainly attributable to leptin action, next we performed a ratio between hypothalamic pSTAT3 and plasmatic leptin levels in order to estimate the degree of sensitivity to leptin of this tissue. In particular, the leptin sensitivity of CD animals was significantly reduced when compared to the STD group (**Fig. 3C**). However, we observed that, at dose of 200 mg/kg/day, RSV consumption significantly increased the hypothalamic leptin sensitivity, indicating that the consumption of high doses of this phenolic compound could be a valid tool to rescue leptin action in this tissue. In addition, the consumption of low and intermediate doses also increased the sensitivity to leptin, although no statistically significant changes were detected in comparison with the CD group.

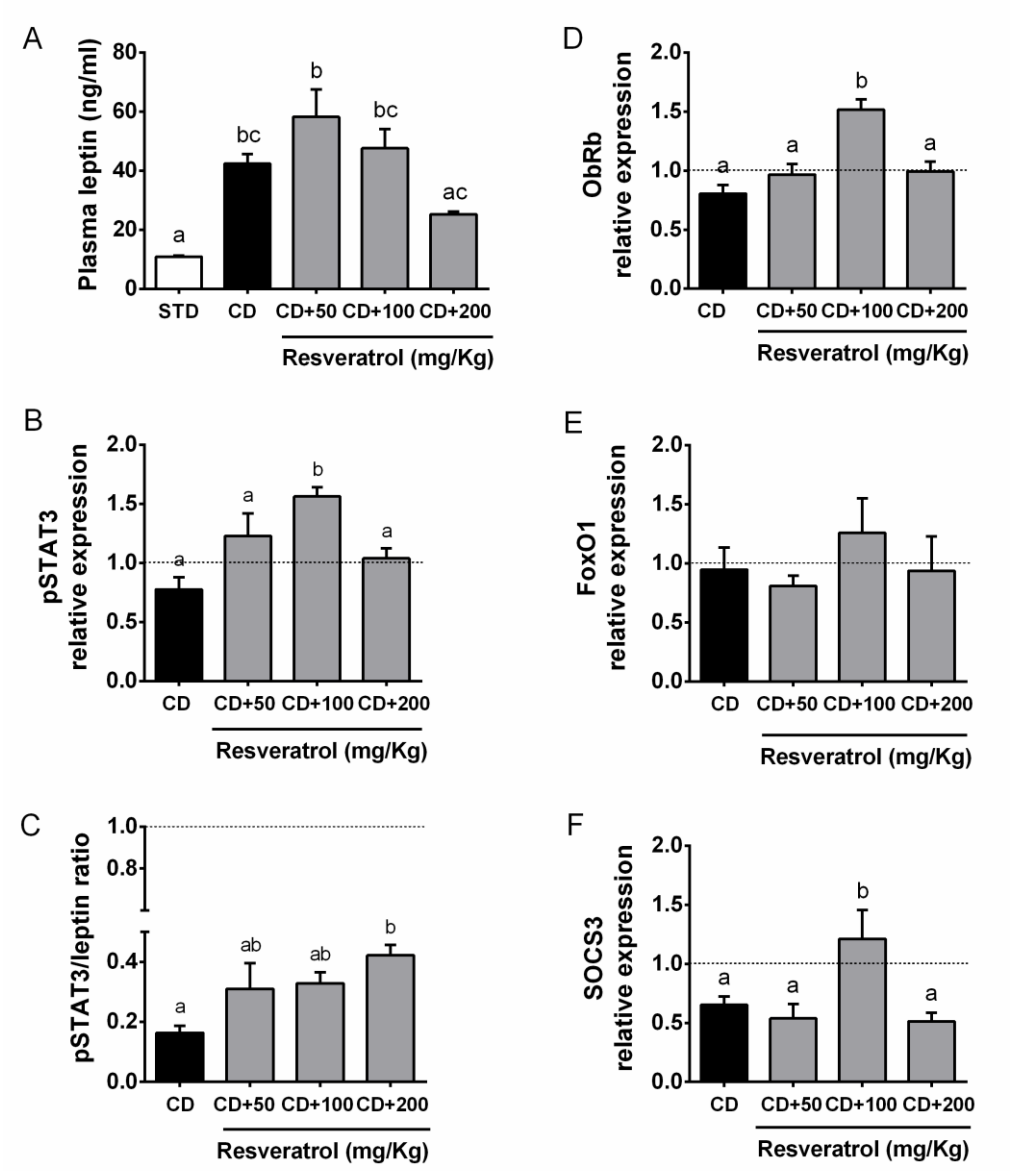

Figure 3. Plasma leptin levels and protein content of regulatory factors involved in leptin signalling in the hypothalamus. Plasma leptin levels of lean (STD) and obese animals treated with resveratrol (CD groups) were analyzed **(A).** Expression levels of pSTAT3 were evaluated by immunoblot in the hypothalamus, as a gold standard of leptin signalling **(B).** To determine the level of leptin sensitivity in this tissue a pSTAT3/leptin ratio was calculated **(C).** In addition the protein content of ObRb **(D),** FOXO1 **(E)** and SOCS3 **(F)** were determined by immunoblotting**.** Intensity signals of each protein were normalized by β-Actin

followed by normalization with the STD group, represented as a dotted line. Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CD+50), a intermediate dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

Next, we determined whether the modulation of hypothalamic leptin sensitivity was directly mediated by enhanced protein content of Obrb. Again, only the consumption of 100 mg/kg/day of RSV resulted in a significant increase in the protein levels of ObRb with respect to the CD group, and no statistically significant differences in the other doses were observed (**Fig. 3D**).

To further assess the effects of RSV on the regulation of the leptin system, the protein content of the negative regulatory factors FOXO1 and SOCS3 were also determined by immunoblotting (**Supplementary Fig. 1**). FOXO1 protein levels were not affected by RSV consumption at any dose (**Fig. 3D**) and only those animals consuming the dose of 100 mg/kg/day showed a significant increase in the protein levels of SOCS3 (**Fig. 3E**).

High doses of resveratrol reduced body weight and fat mass accumulation in obese rats by modulating 24h-energy expenditure but not food intake

RSV consumption increased hypothalamic leptin sensitivity in obese animals. Thus, we next evaluated whether these effects on leptin system could be related to changes in body weight, total body fat content and food intake.

At doses of 50 and 100 mg/kg/day, RSV did not significantly reduce body weight, indicating that the consumption of this phenolic compound for 22 days

did not exert any beneficial effects with respect to body weight at these doses **(Fig. 4A)**. In contrast, when animals were treated with 200 mg/kg/day, the body weight was significantly decreased with respect to CD group, and this reduction was associated with a significant decrease in total body fat mass **(Fig. 4B)**.

In contrast, our results showed that RSV consumption did not modify the levels of food intake in these animals **(Fig. 4C)**. In fact, we could not even detect a statistically significant reduction at dose of 200 mg/kg/day compared to the CD group, indicating that the reduction in body weight and fat mass observed in these animals were not due to a direct decrease in energy intake.

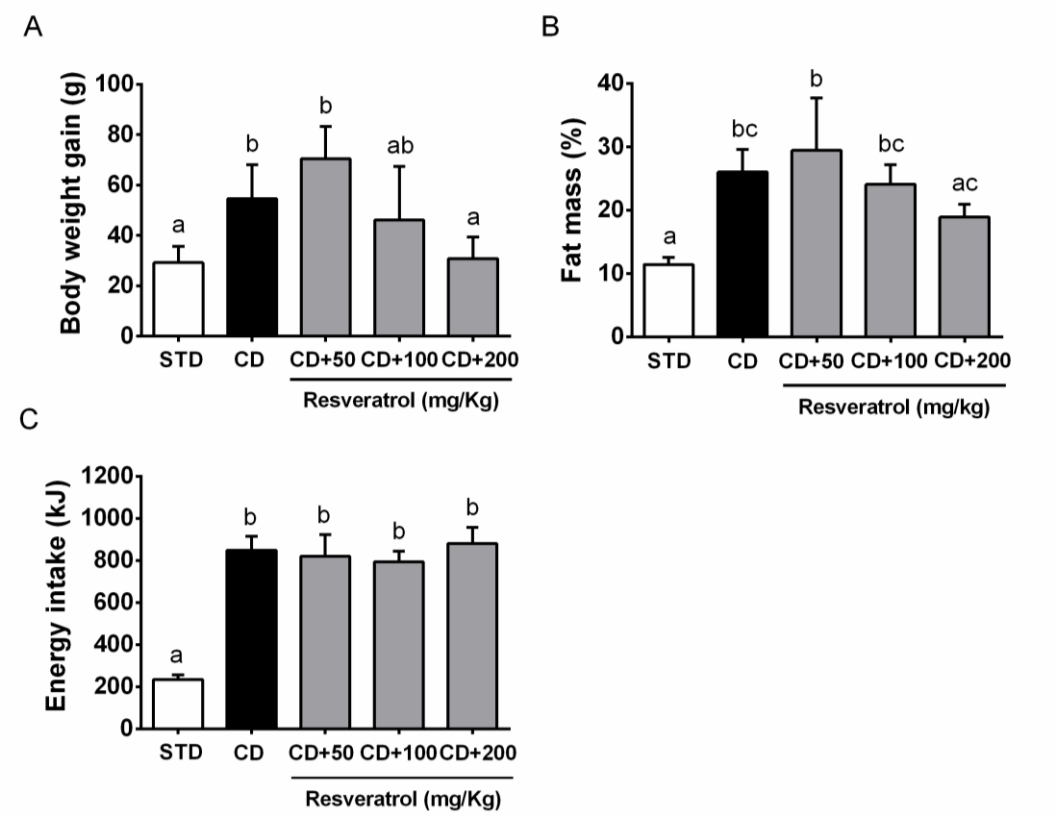

Figure 4. Effect of resveratrol treatment at different doses on body weight, fat mass and energy intake of obese rats. Body weight measurements were

taken the day of the sacrifice **(A),** fat mass was assessed one day before the sacrifice by magnetic resonance and is expressed as percentage of body weight **(B)** and energy intake refers to the last week of treatment **(C).** Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CD+50), a medium dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

Finally, we reasoned that if the consumption of RSV did not have significant effects on food intake, it could enhance 24 hours-energy expenditure and lead to the utilization of fat as the principal energy source. As expected, animals fed with standard chow diet used carbohydrates as a main energetic substrate and, therefore, presented RQ values very close to 1 (**Fig. 5A**). In contrast, the CD group presented lower RQ values compared to the STD group, indicating that they are not fully using carbohydrates since they have a diet rich in fat. Notably, RSV consumption significantly modulated RQ in a dose-dependent manner. Specifically, the consumption of RSV induced a significant decrease in RQ values in comparison to untreated animals, indicating that RSV consumption significantly potentiate the utilization of lipid as the principal energetic substrate. In fact, when the levels of both carbohydrates and lipid oxidation were analyzed by indirect calorimetry (**Fig. 5B and 5C**), RSV consumption at doses of 100 and 200 mg/kg/day significantly favoured 24 hours fat oxidation compared with untreated group. Surprisingly, at dose of 50 mg/kg/day, the consumption of RSV did not enhance lipid oxidation, confirming that, at this dose, RSV did not exert any beneficial effects in our experimental model.

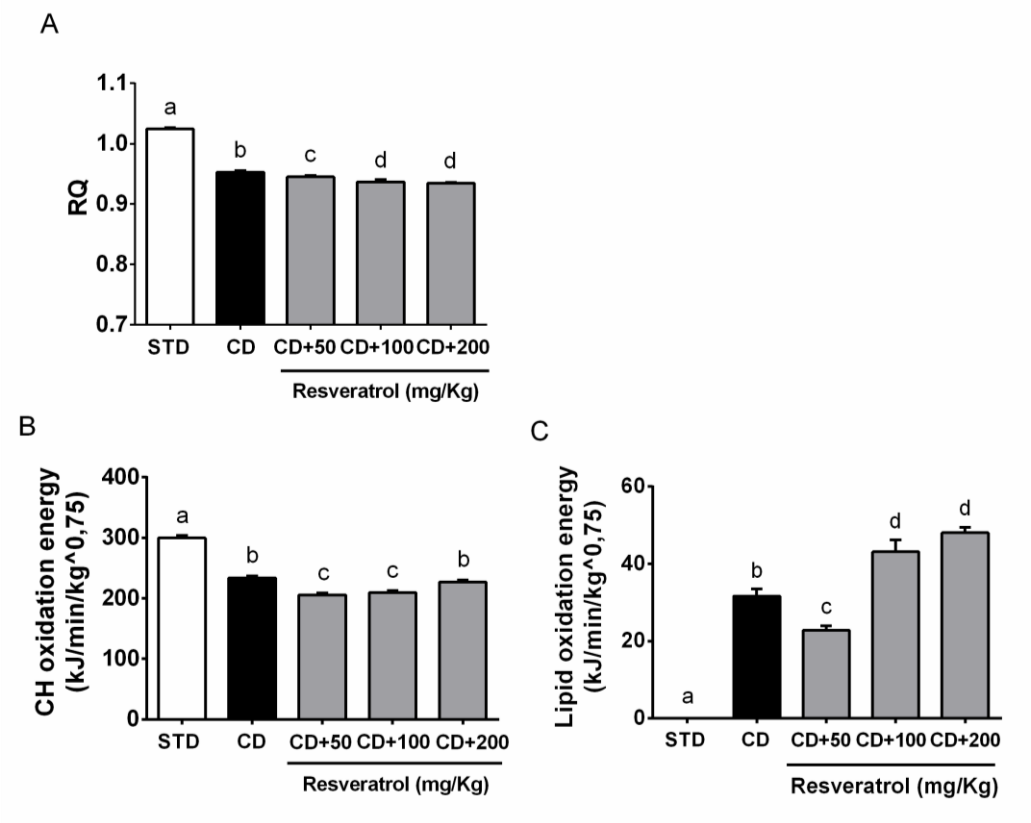

Figure 5. Effect of resveratrol treatment on RQ, carbohydrate oxidation and lipid oxidation of obese rats. In order to know how efficiently animals used the available energy, 24 hours-indirect calorimetry was performed as displayed with the respiratory quotient (RQ) **(A),** carbohydrate **(B)** and lipid **(C)** oxidation energy. Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CD+50), a medium dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

4. Discussion

Phenolic compounds have gained importance over the years for their beneficial effects in promoting health and preventing diseases[20]. Our group has special focus on these compounds and particularly has been investigating the effects of grape-seed proanthocyanidins (PACs) during last ten years. In this context, we have recently reported that a chronic treatment of 25mg/kg/day of PACs for three weeks in obese rats significantly decreased both food intake and circulating plasmatic leptin levels by presumably restoring the hypothalamic leptin signalling[10]. However, it is necessary to keep investigating other compounds with complementary or more powerful effects to fight against obesity and metabolic diseases. Thus, in the present study, we evaluated whether the consumption of other dietary phenolic compounds such as resveratrol (RSV) and anthocyanins are able to decrease body weight, food intake and leptinemia with significant changes in hypothalamic leptin signalling.

The hypothalamus is the metabolic tissue responsible for homeostatic regulation of body weight by modulating both energy expenditure and food (energy) intake. Under physiological conditions, leptin efficiently modulates the activity of hypothalamic POMC neurons to reduce meal size and to increase energy expenditure. However, in obesity, a decreased sensitivity to leptin occurs, resulting in an inability to control energy balance despite high energy stores[10]. For this reason, we investigated the effects of RSV and anthocyanins extract in both non-obese and obese animals in order to evaluate the effects of these compounds under physiological but also pathological conditions.

Despite anthocyanins consumption had demonstrated promising effects on hypothalamic energy balance regulation[12], our results in non-obese animals did

not give any indication that the consumption of 100 mg/kg/day of anthocyanins for 15 days could significantly affect hypothalamic leptin system. In fact, our data suggest that if anthocyanins have the ability to suppress body weight gain and body fat accumulation, their effects are not supported by an increase in hypothalamic leptin sensitivity. Remarkably, some authors have attributed the effects of anthocyanins to a direct interaction with adipose tissue inducing changes in both lipid synthesis and adipocytokines expression levels[10,11]. Moreover, as the bioavailability of anthocyanins is very low (about 0.1%)[14], further studies are needed to confirm the molecular mechanism of action of these compounds and to elucidate which phenolic metabolites derived from anthocyanins are responsible of their bioactivity[21].

Regardless of the current evidence that RSV has anti-obesity effects[22], majority studies have focused on the role of this compound in regulating adipogenesis, lipolysis, mitochondrial function and thermogenesis in peripheral organs such as liver, muscle and adipose tissues[23], but little is known about RSV effects on the modulation of hypothalamic energy homeostasis. In this context, our results show the ability of RSV to normalize plasmatic leptin levels and enhance hypothalamic leptin action in both obese and non-obese animals. Accordingly, it had been previously reported that the offspring of high-fat diet fed mice treated with 30 mg/kg/day of RSV rescued hyperleptinemia and increased pSTAT3 levels in ARC[24]. However, contradictory results have been widely published regarding the effect of this compound on circulating leptin levels. A recent meta-analysis of randomized controlled trials did not find changes on plasma leptin of obese and non-obese subjects supplemented with different doses of RSV. In contrast, other *in vitro*[25] and *in vivo*[26] studies showed a reduction of leptin levels. Possible issues explaining these controversial data could be the low bioavailability of this compound to reach the target tissues, the

different range of doses used and the treatment length[27]. In addition, many animal studies administrate RSV together with obesogenic diet when the animal is still not obese, producing a preventive effect rather than treating obesity once it has been developed[28]. Thus, from now on, human and animal studies should be designed in agreement to be able to compare the data and to obtain similar results.

Concomitant decrease in plasmatic leptin concentration with increasing hypothalamic pSTAT3 levels is consistent with higher leptin sensitivity in this tissue. Thus, we reasoned that if hypothalamic STAT3 phosphorylation is mainly consequence of leptin-induced activation of ObRb signalling, the levels of hypothalamic pSTAT3 (assessed by immunoblotting) referred to the levels of leptin in plasma could be a more reliable estimation of leptin sensitivity in this tissue. Using this estimation, our results showed for the first time that, at dose of 200 mg/kg/day, RSV consumption restored the hypothalamic sensitivity to leptin appropriately in obese rats. Several authors have indicated that the metabolic disturbances observed in animals fed a highly palatable diet are firmly related to a dysfunctional leptin system in the hypothalamus. Accordingly, our results showed that this restoration of leptin action in obese animals was concomitant to body weight loss that, in turn, was associated with increased 24h-energy expenditure and lipid oxidation but not with a reduction in food intake. Although, it has been reported that RSV is able to decrease food intake in rodents fed chow diet via modulation of melanocortinergic system[35], our results indicate that food intake in obese animals presumably is likely to be regulated not only by leptin signal pathway but also by other mechanisms such as ghrelin, cholecystokinin, glucagon-like-peptide-1 and peptide YY (PYY) signal pathways.

The mechanisms by which RSV increases energy expenditure still need to be clarified. Accordingly, Ramadori et al.[29] showed that RSV was able to mediate anti-diabetic effects in the brain after chronic intracerebroventricular infusion by increasing SIRT1 functionality, and we also observed a significant increase in hypothalamic *Sirt1* expression in our previous study with PACs[31]. In addition, its known that differently genetic overexpression of SIRT1 in mice reduces food intake and energy expenditure[30]. However, our results demonstrated that the consumption of RSV improve leptin sensitivity by probably up-regulating hypothalamic *Obrb* expression without any difference in *Sirt1* gene expression levels in non-obese animals, indicating that RSV is able to enhance hypothalamic response to leptin in a SIRT1-independent manner. In fact, the consumption of 100 mg/kg/day of RSV significantly increased the cellular content of ObRb in both obese and non-obese animals. Further studies are needed to confirm the role of SIRT1 in obesity.

As reported in other medicine fields, RSV could also have beneficial effects at lower doses to maintain health[43-45]. However, in regard to the lower doses of RSV tested in this study, only the consumption of 100 mg/kg/day showed a trend to reduce body weight and body fat content with slight but significant changes in hypothalamic leptin signalling as indicated by increased levels of both pSTAT3 and ObRb**.** In addition, at dose of 100 mg/kg/day, the consumption of RSV also produced higher hypothalamic levels of SOCS3 with respect to untreated obese animals. There is a controversial interpretation of SOCS3 in the literature because it negatively regulates leptin signalling**,** but also its transcription is activated by higher levels of pSTAT3, working as a negative feedback mechanism, and high levels of SOCS3 would indicate enhanced leptin signalling. Nevertheless, further studies are needed to confirm the effect 100 mg/kg/day of RSV on hypothalamic leptin system.

Although high doses of RSV have been a matter of concern, the toxicity and tolerability of doses ranging from 5 mg to 5 g has been widely examined in clinical trials[32]. In our study we worked within a dose range that is acceptable for rodents and that would be tolerated by humans. It was based on Body Surface Area (BSA) calculation[33] and human equivalent dose for an obese adult would not exceed 5 g per day. Studies supplementing high doses of RSV, state that it was well tolerated exempting some intestinal discomfort and that high doses may be needed since RSV is greatly metabolized which limits the availability in certain tissues[34,35]. In fact in rats, it has been reported that doses of 300 mg/kg are the maximum tolerated without detrimental effects[36]. The fact that RSV has different effects in a large range of concentration emphasizes that this compound affect different signalling pathways, and, therefore, it is essential to know the suitable dose in each case to achieve beneficial effects[37].

In conclusion, our study shows that the consumption of a high dose of RSV within a tolerable range in rodents is able to produce beneficial effects decreasing circulating leptin levels, body weight and body fat mass but not food intake in obese animals. These metabolic effects could be partly due to increased hypothalamic leptin sensitivity mediated by increased pSTAT3 signalling and ObRb protein content in the hypothalamus and, in consequence, enhanced 24h-energy expenditure and fat oxidation. Mechanisms of action should be investigated thoroughly to provide more accurate information about the anti-obesity effects of both resveratrol and anthocyanins.

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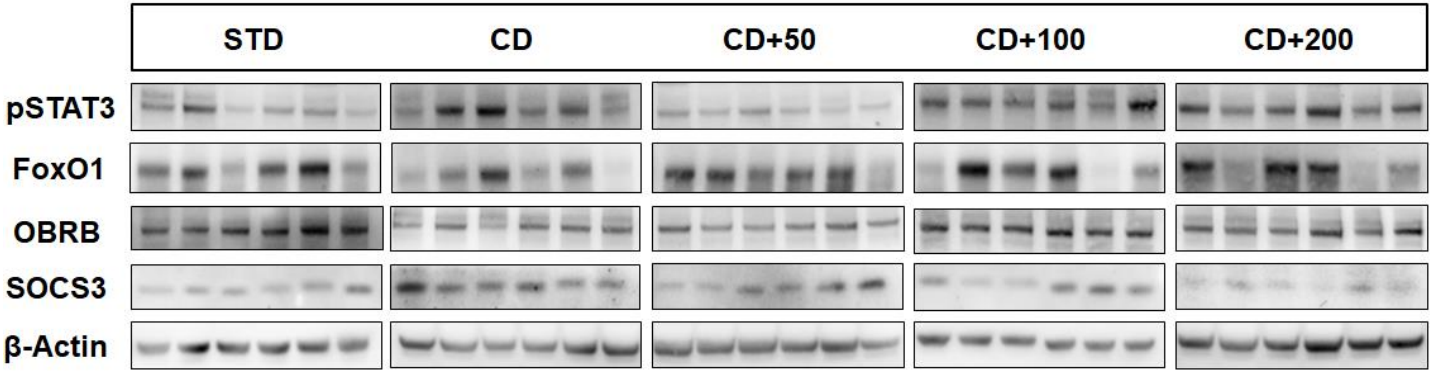
Supplementary materials

Table S1. Primer sequences used in qPCR amplification from mouse genes in hypothalamus.

Primer (Mouse)		Sequence 5'-3'	Product size (bp)	GenBank accession no
Obrb	**Fw**	GGGACGATGTTCCAAACCCC	132	NM_146146.2
	Rw	CAGGCTCCAGAAGAAGAGGAC		
Socs3	**Fw**	ACCAGCGCCACTTCTTCACG	171	NM_007707.3
	Rw	GTGGAGCATCATACTGATCC		
Ptp1b	**Fw**	GTCACCGGCTTCTTTCCTCA	130	NM_011201.3
	Rw	GTCAGCCAGACAGAAGGTCC		
Pomc	**Fw**	CCATAGATGTGTGGAGCTGG	134	NM_008895.3
	Rw	CCAGCGAGAGGTCGAGTT		
AgRP	**Fw**	AGTTGTGTTCTGCTGTTGGC	149	NM_007427.2
	Rw	CTGATGCCCTTCAGTGGAG		
Npy	**Fw**	ATACTACTCCGCTCTGCGAC	143	NM_023456.2
	Rw	GTGTCTCAGGGCTGGATCT		
Sirt1	**Fw**	GATGACAGAACGTCACACGC	110	NM_019812.3
	Rw	ATTGTTCGAGGATCGGTGCC		
Ppia	**Fw**	CTTCTGTAGCTCAGGAGAGCG	117	NM_008907.1
	Rw	CCAGCTAGACTTGAAGGGGAA		

Abbreviations: *Obrb*, leptin receptor isoform b; *Socs3*, suppressor of cytokine signaling 3; *Ptp1b*, protein tyrosine phosphate 1B; *Pomc*, proopiomelanocortin; *Agrp*, agouti-related protein; *Npy*, neuropeptide Y; *Sirt1*, NAD-dependen deacetylase sirtuin 1; *Ppia*, peptidylprolyl isomerase A; Fw, Forward; Rw, Reverse.

Figure. S1. Epression of proteins involved in leptin signalling cascade in the hypothalamus.



Abbreviations: STD, standard diet; CD, cafeteria diet; CD+50; cafeteria diet+50 mg/kg resveratrol; CD+100; cafeteria diet+100 mg/kg resveratrol; CD+200; cafeteria diet+200 mg/kg resveratrol pSTAT3, phosphorylated signal transducer and activator of transcription 3; FoxO1, forkhead box protein O1, OBRB, long form of leptin receptor; SOCS3, suppressor of cytokine signalling 3; β-Actin, beta actin isoform. Effect of different doses of resveratrol treatment on the hypothalamic leptin signalling pathway in animals fed a cafeteria diet. Leptin signalling pathway was assessed by evaluating the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3) and by the determination of the cell surface content of OBRB. Negative regulation of leptin signalling pathway was assessed by determining the expression of FoxO1 and SOCS3. β-Actin expression was used as a loading control.

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CHAPTER 3

Seasonal fruits consumption affects hypothalamic leptin signaling system in a photoperiod dependent mode

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In preparation

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Abstract

Leptin has a central role on the maintenance of energy homeostasis in the hypothalamus by which produces satiety and decreases energy expenditure through the modulation of neuropeptides including POMC, AgRP and NPY. Leptin secretion is influenced by photoperiod which is able to produce a switch on energy balance. For instance, seasonal animals exposed to long-day (LD) photoperiod show a decrease on leptin sensitivity. Similarly, changes in lifestyle in modern society are associated with altered eating patterns and the consumption of energy rich food that contributes to the obesity epidemic and metabolic diseases, in which cases leptin signaling is also attenuated. Dietary strategies to prevent metabolic diseases and promote a healthy life style include the consumption of fruits, since they are a rich source of nutrients and phytochemicals. However, the health implications of consuming fruits out of season have not been evaluated. The goal of this study is to investigate the effect of consuming seasonal fruits in different photoperiods on hypothalamic leptin signaling pathway and overall energy balance in animals fed a balanced or high-energy diet. Grapes and cherries are a rich source of polyphenols and are harvested in autumn (SD) and spring (LD) respectively. These fruits present similar phenolic composition with some differences that in turn can be highly influenced by environmental signals specific of each season. This fact is related to the Xenohormesis Hypothesis and how molecules produced by a mild environmental stress in plants can affect the animals that consume them. Therefore, the potential of this two fruits in modulating hypothalamic leptin signaling in different photoperiods could bring useful information on the regulation of energy balance and how to increase leptin sensitivity to prevent obesity.

1. Introduction

Leptin is a hormone produced by adipose tissue that has a key role in the central regulation of energy homeostasis[1]. The main target is the hypothalamic arcuate nucleus (ARC) where it activates anorexigenic neurons (proopiomelanocortin, Pomc) and inhibits orexigenic neurons (agouti related peptide, AgRP and neuropeptide Y, Npy), modulating second order neuron activity through melanocortin 4 receptor (MC4R)[2] and NPY1 receptor (NPY1R) [3]. As a result, leptin produces a satiating effect as well as an increase on energy expenditure in order to maintain body weight[4].

Remarkably, Leptin secretion follows circadian[5,6] as well as circannual [7] rhythms. Studies with mammals sensitive to photoperiods have shown that animals develop an adaptive leptin resistance in long-day periods to overcome periods of food scarcity[8,9]. This means that, despite presenting high leptin levels in blood, leptin is unable to produce anorectic effects, providing animals with a sufficient energy stores on the upcoming short-day seasons[8]. This has been considered an evolutionary mechanism for survival[7].

Westernized society developed inappropriate dietary patterns which contribute to the obesity epidemic[10] and fruit consumption is advised because fruits are a valuable source of nutrients with health promoting properties which supports their daily consumption[11]. Furthermore, fruit contains phytochemicals that despite not being essential for life can exert long-term beneficial effects. Among them, polyphenols are an important group of compounds present in fruits[12] and some studies demonstrate that specific polyphenols increase leptin sensitivity in obese animals[13–15]. Each fruit has a distinctive polyphenol

composition [16,17] that could determine its capacity to modulate the leptin system.

Notably, nowadays there is a broad fruit offer and people can choose to consume either seasonal or out-of-season fruits. However, the effects regarding the consumption of fruits in- or out-season on leptin sensitivity have not been studied yet. Accordingly, our goal was to mimic seasons by submitting animals to different photoperiods, short-day for autumn and long-day for spring, to investigate the effects of seasonal fruit intake on the leptin system. Because of the importance of leptin maintaining body weight, this study was performed in both lean and dietary-induced obese rats. Numerous studies report the metabolic protective effects of grape, grape by-products, cherries or their pure compounds[18–23], thus we have chosen red grape and cherry as representative fruits of autumn and spring, respectively. This approach might provide valuable information to design strategies of fruit consumption to counteract positive energy balance through the modulation of central leptin signaling pathway and downstream effectors.

2. Materials and Methods

2.1 Fruit characteristics and preparation

Royal Down sweet cherries (*Prunus avium* L.) were original from Argentina and were purchased in Mercabarna (Barcelona, Spain). Grapes (*Vitis vinifera* L.), Grenache variety were from Ribera de l'Ebre (Catalonia, Spain) and were gently gift by the producer. Cherry pits were removed whereas grapes were kept intact. Fruits were frozen in liquid nitrogen and later grinded to achieve homogeneity. Afterwards, the homogenates were lyophilized until reaching

dryness in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Barcelona, Spain) at -55 ºC. Lyophilized fruits were further grinded to obtain a fine powder. Cherry and grape powder was aliquoted and protected from humidity and light.

2.2 Animals

The study was approved by the Animal Ethics Committee of University Rovira i Virgili (reference number 4249 by Generalitat de Catalunya) and carried out according to ethical standards comprised in the Declaration of Helsinki.

2.2.1 Experimental design in lean animals

Male Fisher 344 rats of 8 weeks of age and 186±17g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were paired-housed, distributed in two different rooms, according to photoperiod, and fed a standard chow diet (Panlab 04, Barcelona, Spain) composed by a 72% carbohydrate, 8% lipid and 20% protein and water *ad libitum*. Photoperiod groups consisted in long-day (LD) (18:6h light:dark cycle) and short day (SD) (6:18h light:dark cycle) and kept at 22ºC. After an adaptation period of 4 weeks, animals in each photoperiod were weight-matched and distributed into 3 subgroups of 6 animals, the control group, and 2 groups supplemented with an oral dose of 100mg/Kg (diluted in water) of lyophilized grapes or cherries, for a period of 10 weeks. The control group was supplemented with the same volume of a sugar solution (10mg fructose/10mg glucose) to much the sugar present in lyophilized fruits.

2.2.2 Experimental design in diet-induced obese animals

Male Fisher 344 rats of 8 weeks of age and 216±15g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were paired-housed and distributed in two different rooms, according to the photoperiod, and fed a standard chow diet (Panlab 04, Barcelona, Spain) plus a cafeteria diet, composed by a 65% carbohydrate, 20% lipid and 14% protein and water *ad libitum*. Cafeteria diet consisted of bacon, carrots, cookies, foie-gras, muffins, cheese and milk with 22% sucrose (w/v). Animals were distributed in two photoperiod groups, LD and SD and kept at 22ºC. After an adaptation period of 4 weeks, animals in each photoperiod were weight-matched and distributed into 3 subgroups of 10 animals, the control group, and 2 groups supplemented with an oral dose of 100mg/Kg (diluted in water) of lyophilized grapes or cherries, for a period of 7 weeks. The control group was supplemented with the same volume of a sugar solution (10mg fructose/10mg glucose) to much the sugar present in lyophilized fruits.

In the two experiments, the treatment was administered at 09:00 am every day. The animals were sacrificed by decapitation at the start of light cycle (lights on at 09:00 am) taking no more than two hours to complete the sacrifice. Blood was collected and allowed to clot at room temperature. Serum was obtained after centrifugation (2000g, 15min, 4ºC), aliquoted and stored at -80ºC. The hypothalamus was dissected, weighted and frozen immediately in liquid nitrogen. Body weight and food intake were weekly measured.

2.3 Body composition analysis

The last week of the study, animals were echoed by magnetic resonance imaging (MRI) using EchoMRI-700 (Echo Medical Systems, LLC., TX, USA) to determine the lean and fat mass composition. Data is expressed as a percentage of total body weight.

2.4 Indirect calorimetry

After an acclimation period of 3h animals underwent 20h of indirect calorimetry analyses using Oxylet Pro System (Panlab, Cornellà, Spain) performed during the 7th week of treatment. The oxygen consumption (VO2) and carbon dioxide production (VCO2) measures provided information about the energy expenditure. The program software Metabolism 2.1.02 (Panlab) automatically calculated respiratory quotient (RQ) as VCO2/VO2 and energy expenditure as VO2x1.44x[3.815+(1.232xRQ] (Kcal/day/Kg^0.75) according to Weir equation[28]. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed[29].

2.5 Serum leptin levels

Leptin concentration in serum samples was measured using a rat specific Enzyme Immunoassay kit (Millipore, Madrid, Spain), following manufacturer's protocol

2.6 Gene expression analyses

The hypothalamus was processed to extract total RNA using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) followed by RNeasy Mini Kit

(Qiagen, Barcelona, Spain) according to manufacturer's instructions. RNA quantity and purity was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only RNA samples with A260/A280 ratio ≥1.8 and A230/A260 ratio ≥2 were included in the study. Afterwards, RNA quality was assessed on a denaturing agarose gel stained with SYBR Green dye (Bio-Rad, Barcelona, Spain). Reverse transcription was performed to convert RNA to cDNA using the High-Capacity complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain). Gene expression was analyzed by Real-Time PCR, using the iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain), in the ABI prism 7900HT Real-Time PCR system (Applied Biosystems) using primers obtained from Biomers.net (Ulm, Germany) (Supplementary Table 1). Relative expression of each gene was calculated referring to *Ppia* and *Rplp0* housekeeping genes and normalized to Long-day vehicle (LD-VH) control group. □□Ct method was used and corrected for primer efficiency [30].

2.7 Statistical Analysis

The effect of fruit supplementation (F), photoperiod (P) or the interaction between the two variables (F*P) was evaluated by Two-way ANOVA. If a main effect was significant, differences between groups were further assessed using one-way ANOVA Tukey post-hoc, unless specified. In the absence of main effect but with a significant interaction between fruit and photoperiod, pairwise comparisons were calculated among photoperiod groups and fruit groups using a Student's *t* test. GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. The values are expressed as the means ± SEM. Grubb's test was used to determine outliers. $P \leq 0.05$ was considered significant.

3. Results

Photoperiod and fruit consumption modulated energy balance by altering energy expenditure in lean rats

First, we focused on the effect of photoperiod and fruit consumption on body weight, fat mass and energetic homeostasis.

Supplementing animals with 100mg/kg of grape or cherry for 10 weeks did not produce significant changes on body weight in any photoperiod, and the photoperiod itself did not affect this feature in control group (**Table 1**). However, control animals placed at SD had a significant lower fat mass than those placed at LD. Cherry consumption kept this photoperiod effect whereas grape consumption abolished it.

In order to know how efficiently animals used the available energy, cumulative food intake (**Table 1**) and energy expenditure (**Figure 1**) were analyzed. Photoperiod and cherry consumption did not alter food intake. In contrast, grape consumption significantly decreased food intake at SD.

Energy expenditure is expressed as oxygen inspired (VO2) throughout 20h. **Fig. 1A** shows the effect of photoperiod on the VO2 inspired, showing that animals placed in SD significantly increased VO2 inspired at all the times studied, indicating that animals spent more energy in SD than in LD**.** Grape consumption did not alter this pattern (**Fig. 1B and 1C**)**.** In contrast, cherry intake significantly increased the VO2 inspired in the animals placed at LD **(Fig. 1D),** without any alteration at SD (**Fig. 1E**). Thus, cherry consumption modulated energy expenditure in a dependent way of the photoperiod, increasing it only at LD.

Table 1. Effect of different photoperiod and fruits consumption on body weight gain, fat mass, cumulative food intake, energy balance and respiratory quotient of animals fed a standard chow diet.

	Photoperiod	Control	Grape	ANOVA[1]	Cherry	ANOVA[1]
Body weight gain (g)	**LD**	89.17±4.1	91.33±4.7	ns	83.80±5.0	ns
	SD	84.17±4.8	80.67±1.7		90.00±7.0	
Fat mass (%)	**LD**	14.38±0.7	14.40±1.2	ns	15.17±0.7	P
	SD	12.52±0.3	14.98±0.3		13.43±0.6	
Cumulative food intake (MJ)	**LD**	2.26±0.1	2.20±0.0	F	2.21±0.0	ns
	SD	2.26±0.0	2.14±0.0		2.23±0.1	
24h Energy balance (MJ)	**LD**	0.10±0.0	0.09±0.0	F, P	0.07±0.01	F, P
	SD	0.04±0.0	0.01±0.0		0.00±0.01	
24h RQ	**LD**	0.86±0.0	0.86±0.0	P	0.83±0.01	P
	SD	0.80±0.0	0.82±0.0		0.78±0.01	

Abbreviations: LD, long day; SD, short day; RQ, respiratory quotient; Energy balance; (energy intake-energy expenditure); ns, nonsignificant. Animals in each photoperiod were fed with standard chow diet and supplemented with vehicle (Control group), grape or cherry at 100mg/kg for a 10-week-period. Body weight and food intake was monitored weekly. Fat mass was assessed by MRI during the last week together with the indirect calorimetry analyses to determine RQ and energy expenditure. Values are presented as mean±SEM of six animals per group. [1]Denotes two-way ANOVA analysis. P, photoperiod effect; F, fruit effect, assessed by two-way ANOVA ($P \leq 0.05$).

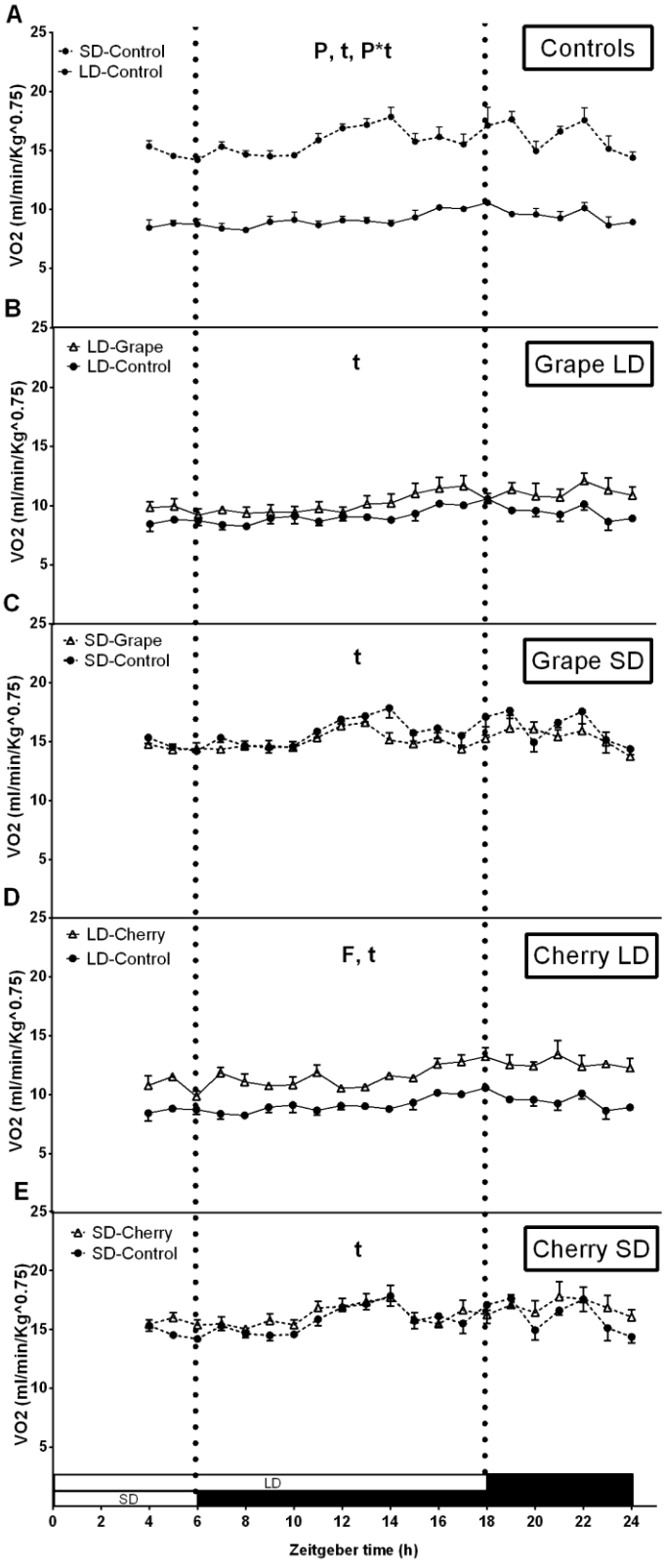
A
SD-Control
LD-Control
P, t, P*t
Controls
B
LD-Grape
LD-Control
t
Grape LD
C
SD-Grape
SD-Control
t
Grape SD
D
LD-Cherry
LD-Control
F, t
Cherry LD
E
SD-Cherry
SD-Control
t
Cherry SD
VO2 (ml/min/Kg^0.75)
LD
SD
Zeitgeber time (h)

Figure 1. Oxygen consumption (VO2) measures assessed by indirect calorimetry analyses. 20 hours of indirect calorimetry were measured in animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A-E).** First, VO2 of the controls in SD and LD are displayed **(A).** Grape effect on VO2 during LD **(B);** Grape effect on VO2 during SD **(C);** Cherry effect on VO2 during LD **(D)** and Cherry effect on VO2 during LD **(E).** Values are mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*t, interaction of fruit treatment and time; P*t, interaction of photoperiod and time, assessed by two-way ANOVA ($P \leq 0.05$).

24-hour energy balance was estimated from the values of 24 hours energy expenditure and food intake (**Table 1**). Animals placed at SD presented a significant reduction in its energy balance, and this effect was magnified by grape or cherry consumption. These results indicate that SD directed energy homeostasis towards a zero balance whereas LD did towards a positive one. Furthermore, animals placed in SD presented a significant reduction on RQ (**Table 1**) meaning that the contribution of carbohydrates and lipids, as energetic substrates, depended of the photoperiod in the sense that LD favored carbohydrate whereas SD favored lipids use.

Altogether, these results indicate that SD, compared to LD, put healthy lean animals on a higher energy expenditure and favoured lipid use as energetic substrate, thus resulting in reduced energy balance and lower fat mass. Fruit consumption kept this global photoperiod pattern. However, cherry consumption also increased energy expenditure when was consumed at LD whereas grape consumption decreased food intake and blocked the fat mass drop when was consumed at SD.

Photoperiod and fruit consumption modulated serum leptin levels and *Pomc* expression in the hypothalamus of lean rats

Leptin controls body fat mass, food intake and energy expenditure. Thus, the next goal was to investigate whether photoperiod and/or fruit consumption could change these parameters through the modulation of leptin levels and/or central leptin sensitivity.

Photoperiod significantly affected serum leptin concentration, increasing its levels at LD (Figure 2A and 2C). Despite no significant effects were observed on leptin levels by fruit intake, grape and cherry consumption magnified the increase induced by the LD photoperiod.

Central leptin sensitivity was predicted by analyzing the expression of the leptin receptor (*Obrb*), the negative regulators *Socs3* and *Ptp1b*, and the neuropeptides regulated by leptin in the hypothalamus.

The expression of *Obrb*, *Socs3* and *Ptp1b* was not modified by photoperiod or grape consumption (Fig. 2B). In contrast, cherry significantly affected *Obrb* expression in a way that was dependent of the photoperiod, increasing or decreasing it at SD or LD, respectively (Fig. 2D). Furthermore, cherry consumption resulted in a significant overexpression of *Socs3* in both LD and SD photoperiods.

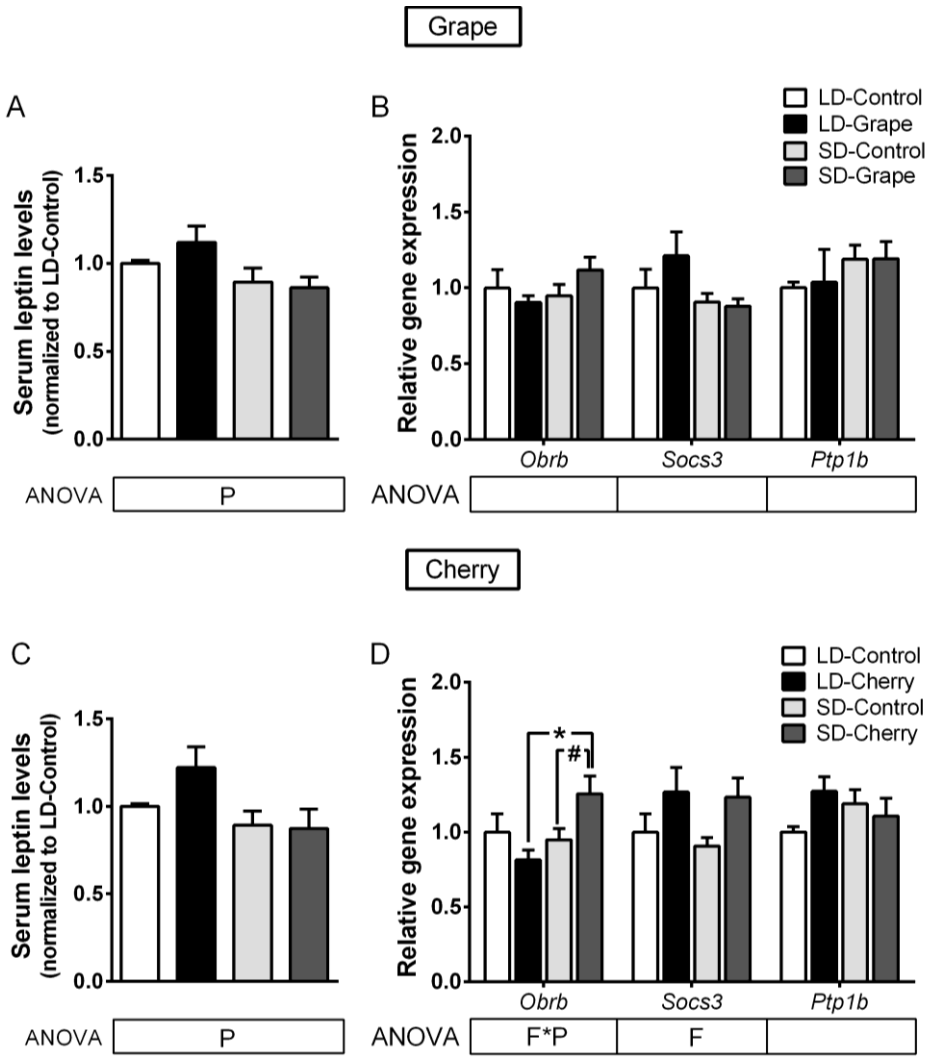

Figure 2. Effect of photoperiod and seasonal fruit supplementation on leptin signaling pathway. Serum leptin was measured in animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle. **(A, C).** Afterwards, gene expression of the long form of leptin receptor (*Obrb*), negative regulator molecules *Socs3* and *Ptp1b* were analysed by qPCR**(B, D).** Values are normalized against the LD-Control group for leptin concentration and gene expression. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA (*P* ≤0.05). *****Effect of photoperiod in the fruit treated groups; **#**effect of fruit in the photoperiod group determined by Student's *t* test (*P*≤0.05).

Further, we determined the expression of *Pomc*, the precursor of the anorexigenic neuropeptide α-MSH, and orexigenic neuroppetides *Agrp* and *Npy*, all of them produced by first order neurons in the ARC under leptin regulation. In addition, the expression of *Mc4r* and *Npy1r* receptors was examined as main targets of α-MSH, AgRP and NPY in second order neurons. Interestingly, grape and cherry intake remarkably increased *Pomc* expression (20 times) only at SD (**Fig. 3A and 3B**). Thus, the effect of grape and cherry was clearly dependent on the photoperiod where they were consumed. In addition, cherry consumption significantly increased *Agrp* expression in both photoperiods (**Fig. 3B**)**.** Photoperiod or fruit consumption did not modify the expression of Npy, *Mc4r* and *Npy1r*.

From these results stand out that SD, compared to LD, was associated to lower serum leptin. Remarkably, the consumption of each fruit strongly increased this photoperiod *Pomc* expression pattern, inducing *Pomc* overexpression when fruits were consumed at SD. In addition, cherry consumption increased *Agrp* expression at both photoperiods. Only cherry consumption was able to modulate the expression of some leptin-signalling components, such as *Obrb* and *Socs3*. Thus, the consumption of cherry modulated the leptin system with higher intensity than grape.

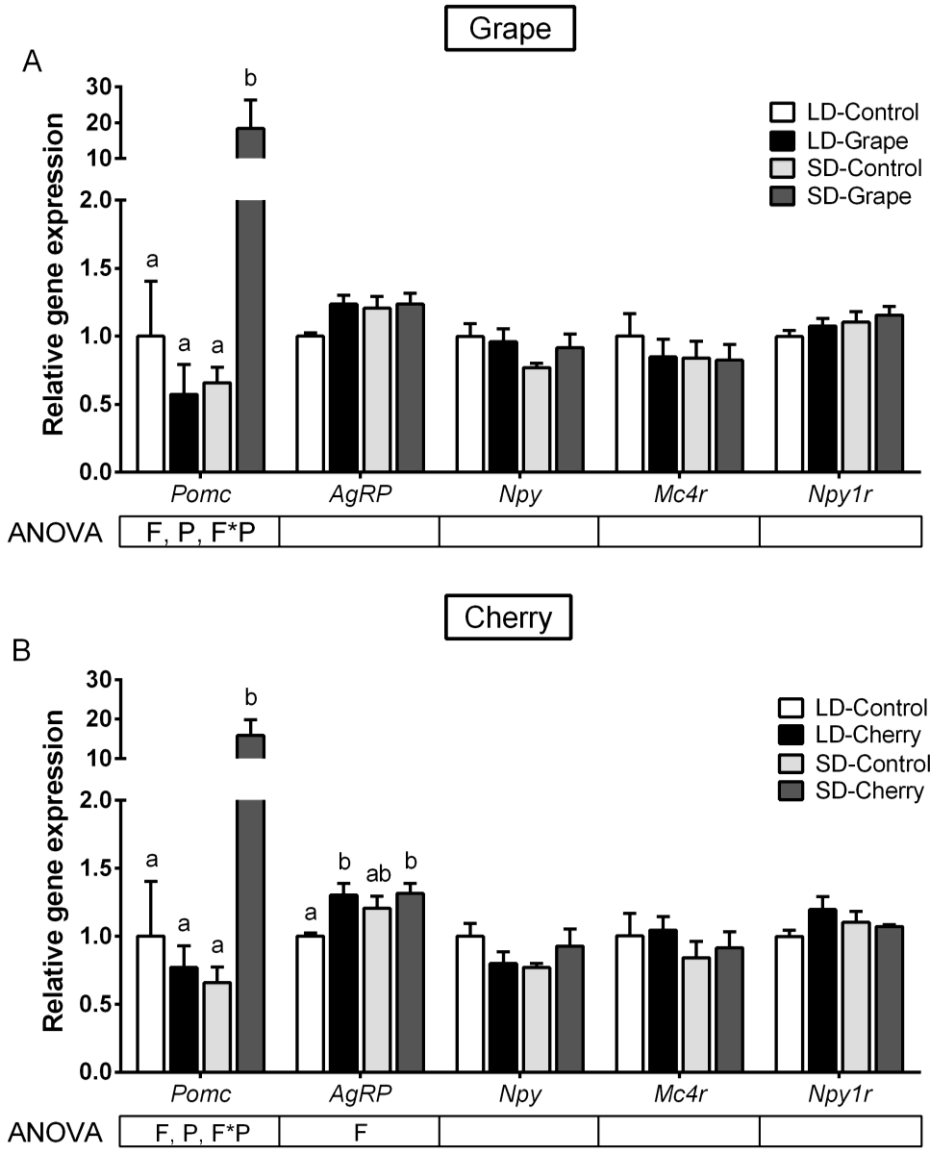

Figure 3. Effect of photoperiod and seasonal fruit supplementation on hypothalamic neuropeptides regulated by leptin. Hypothalamic gene expression of neuropeptides was evaluated by qPCR on animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, B).** Anorexigenic *Pomc* and orexigenic *Agrp* and *Npy* mRNA levels were determined, together with the mRNA's of receptors involved in energy balance *Mc4r* and *Npy1r* . Values are normalized against the LD-Control group. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of

photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$).

Photoperiod and fruit consumption modulated energy balance by altering energy intake in diet-induced obese rats

The observation that photoperiod and fruit consumption conditioned fat mass and energy balance in healthy and lean rats, prompted us to investigate how fruit and photoperiod could affect energy homeostasis in obesity. Rats were fed a cafeteria diet for 7 weeks together with 100 mg/kg of lyophilized grape or cherry.

Table 2 shows the effect of photoperiod and fruit consumption on body weight, fat mass, cumulative energy intake, energy balance and RQ. Photoperiod or fruit consumption did not modulate body weight, like in lean animals. In addition, body fat mass, which was reduced by SD photoperiod in lean animals, was neither affected by photoperiod or fruit consumption.

Obese rats significantly decreased their cumulative energy intake **(Table 2**) when placed at SD and grape consumption did not modify this photoperiod pattern. Remarkably, cherry consumption even reduced the cumulative energy intake when it was consumed at LD. Furthermore, photoperiod or fruit consumption did not significantly altered energy expenditure, measured as VO2 inspired (**Figure 4**). As expected, 24-hour energy balance **(Table 2)** in obese rats was positive in all conditions, but was lower in animals placed at SD, like in lean animals. Interestingly, cherry also significantly reduced energy balance when was consumed at LD. All these data indicate that the lower 24h-energy balance experimented by obese rats placed at SD was consequence of a reduced

energy intake. This fact totally diverges of that observed in lean animals, in which energy balance was lower at SD as consequence of an increase of energy expenditure.

Table 2. Effect of different photoperiod and fruit consumption on body weight gain, fat mass, cumulative food intake, energy balance and respiratory quotient of animals fed cafeteria diet.

	Photoperiod	Control	Grape	ANOVA[1]	Cherry	ANOVA[1]
Body weight gain (g)	**LD**	113.31±5.4	128.36±4.3	ns	121.09±3.2	ns
	SD	116.74±6.6	117.39±4.3		119.70±5.9	
Fat mass (%)	**LD**	22.03±0.6	22.57±0.6	ns	21.70±0.7	ns
	SD	21.53±0.8	21.10±1.3		21.82±1.0	
Cumulative food intake (MJ)	**LD**	5.79±0.2	5.72±0.2	P	5.13±0.2	F
	SD	5.43±0.2	5.20±0.1		5.01±0.1	
24h Energy balance (MJ)	**LD**	0.83±0.1	0.79±0.0	P	0.40±0.1[#]	F, F*P
	SD	0.60±0.1	0.46±0.1		0.55±0.1	
24h RQ	**LD**	0.78±0.0	0.78±0.0	P	0.79±0.0	F*P
	SD	0.81±0.0	0.80±0.0		0.78±0.0[#]	

Abbreviations: LD, long day; SD, short day; RQ, respiratory quotient; Energy balance; (energy intake-energy expenditure); ns, nonsignificant. Values are presented as mean±SEM of six animals per group. [1]Denotes two-way ANOVA analysis. P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Pairwise comparisons for interactions were determined by Student's *t* test. #effect of fruit in the photoperiod group determined by Student's *t* test ($P \leq 0.05$).

In addition, photoperiod also modulated RQ in obese animals. Specifically, obese animals placed at SD presented a significant increase on RQ (**Table 2**), indicating that LD photoperiod favored lipid whereas SD favored carbohydrate use as energetic substrates in obesity, a situation totally opposed as those observed in lean rats. In addition, in obese animals, cherry consumption at SD significantly decreased RQ.

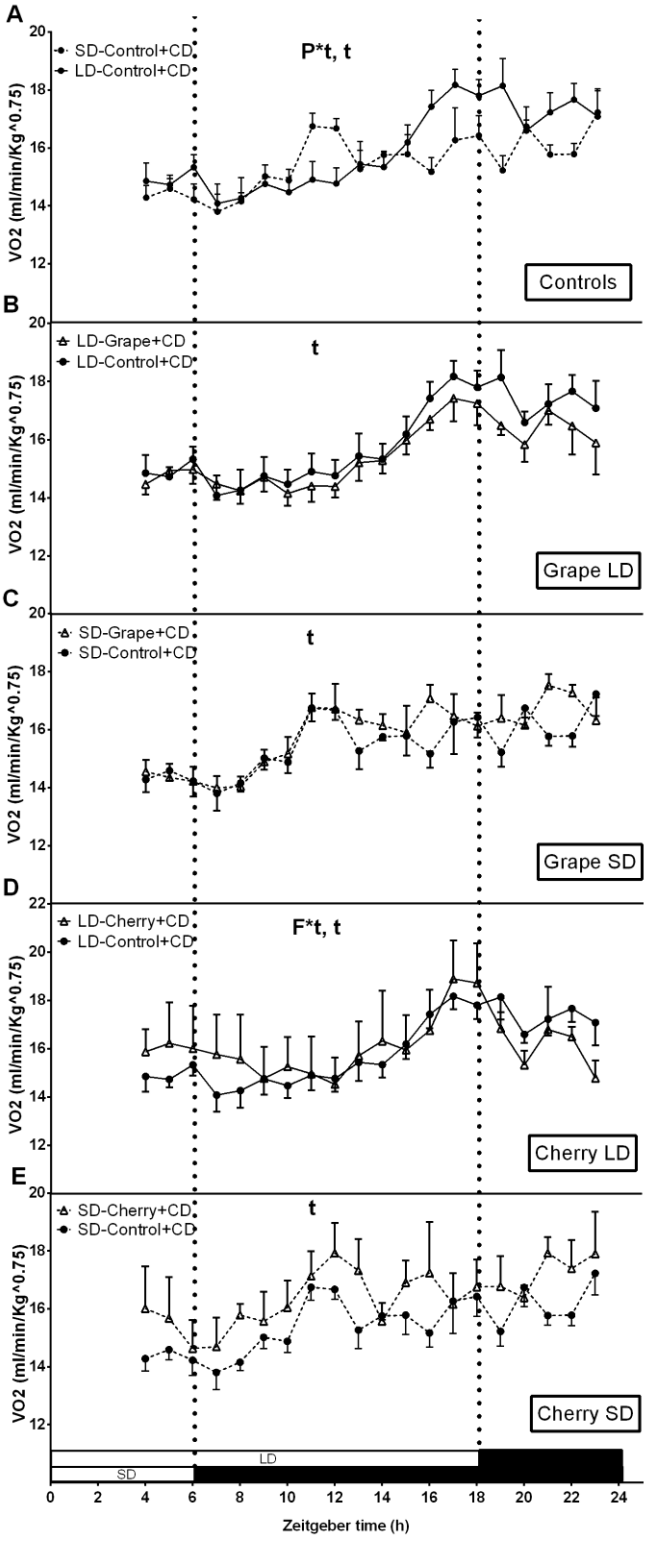
A
SD-Control+CD
LD-Control+CD
P*t, t
Controls
B
LD-Grape+CD
LD-Control+CD
t
Grape LD
C
SD-Grape+CD
SD-Control+CD
t
Grape SD
D
LD-Cherry+CD
LD-Control+CD
F*t, t
Cherry LD
E
SD-Cherry+CD
SD-Control+CD
t
Cherry SD
VO2 (ml/min/Kg^0.75)
SD
LD
Zeitgeber time (h)

Figure 4. Oxygen consumption (VO2) measures assessed by indirect calorimetry analyses. 20 hours of indirect calorimetry were measured in animals fed cafeteria diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A-E).** First, VO2 of the controls in SD and LD are displayed **(A).** Grape effect on VO2 during LD **(B);** Grape effect on VO2 during SD **(C);** Cherry effect on VO2 during LD **(D)** and Cherry effect on VO2 during LD **(E).** Values are mean±SEM (n=6). F*t, interaction of fruit treatment and time; P*t, interaction of photoperiod and time, assessed by two-way ANOVA ($P \leq 0.05$).

All these results indicate that SD, compared to LD, put obese rats on a lower energy intake and favored carbohydrate use as energetic substrate, resulting in reduced energy balance that was not reflected on body weight or fat mass. Grape consumption kept this photoperiod pattern, but cherry consumption decreased RQ at SD and even reduced the cumulative energy intake when it was consumed at LD. Comparing these data with that of lean animals, it is evident that the response of obese animals to photoperiod and fruit consumption strongly diverged from that of lean animals.

Photoperiod and food consumption modulated Socs3 and *Agpr* expression in the hypothalamus of diet-induced obese rats

Next, we studied whether photoperiod and/or fruit consumption modulated leptin system in obesity, and thereby the cumulative food intake.

First, we analyzed serum leptin. All obese rats showed the same levels. Thus, remarkably, feeding animals with the cafeteria diet eliminated the photoperiod serum leptin response observed in lean animals **(Fig. 5A and 5C).**

The potential of leptin signaling pathway in the hypothalamus of obese rats was evaluated by determining the expression of *Obrb* as well that of *Socs3* and *Ptp1b*, the negative regulators. Interestingly, *Socs3* was sensitive to photoperiod, increasing its expression in obese rats placed at SD (**Fig. 5B and D**). Grape consumption magnified (**Fig. 5B**) whereas cherry consumption softened (**Fig. 5D**) this overexpression of *Socs3* when consumed at SD. Furthermore, cherry consumption significantly repressed *Ptp1b* expression when it was consumed at SD. Thus, the gene expression of the leptin-signalling cascade was more sensitive to photoperiod and fruit consumption in obese than in lean rats.

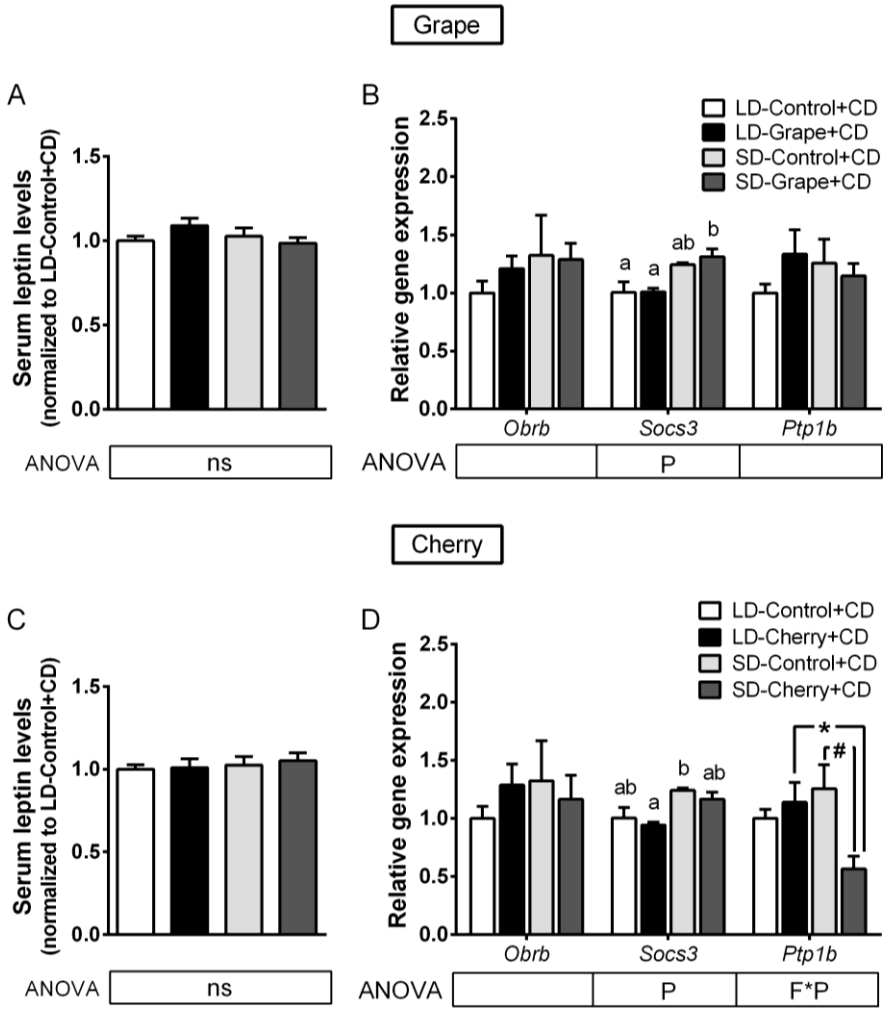

Figure 5. Effect of photoperiod and seasonal fruit supplementation on leptin signaling pathway in DIO rats. Serum leptin was measured in animals fed a cafeteria diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, C).** Afterwards, gene expression of the long form of leptin receptor (*Obrb*), negative regulator molecules *Socs3* and *Ptp1b* were analysed **(B, D).** Values are normalized against the LD-Control group for leptin concentration and gene expression. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$). *****Effect of photoperiod in the fruit treated groups; **#**effect of fruit in the photoperiod group determined by Student's *t* test ($P \leq 0.05$).

After analyzing the expression of the main factors involved in leptin signaling, we determined the hypothalamic mRNA levels of downstream neuropeptides and their receptors. Placing rats at SD induced a marked overexpression of the orexigenic *Agrp* neuropeptide (**Figure 6A and B**). However, the consumption of both fruits at SD prevented this *Agrp* overexpression. Besides, cherry consumption also modulated the expression of Mc4r and Npy1r, the neuropeptide receptors in second order neurons, repressing their expression when cherry was consumed at SD (**Fig. 6B**).

These results point out that SD, compared to LD, was linked to the overexpression of *Socs3* and *Agrp* in the hypothalamus. In contrast, the consumption of each fruit strongly reversed this photoperiod expression pattern of *Agrp*, repressing its overexpression when fruits were consumed at SD. The comparison of these figures with those of lean animals evidences that photoperiod and fruit consumption modulated the leptin system in both obese and lean states, but in a different way that was by altering *Agrp* expression in obesity whereas by modulating *Pomc* expression in lean animals. Like in lean

animals, cherry consumption was more powerful than grape consumption modulating leptin system in the hypothalamus.

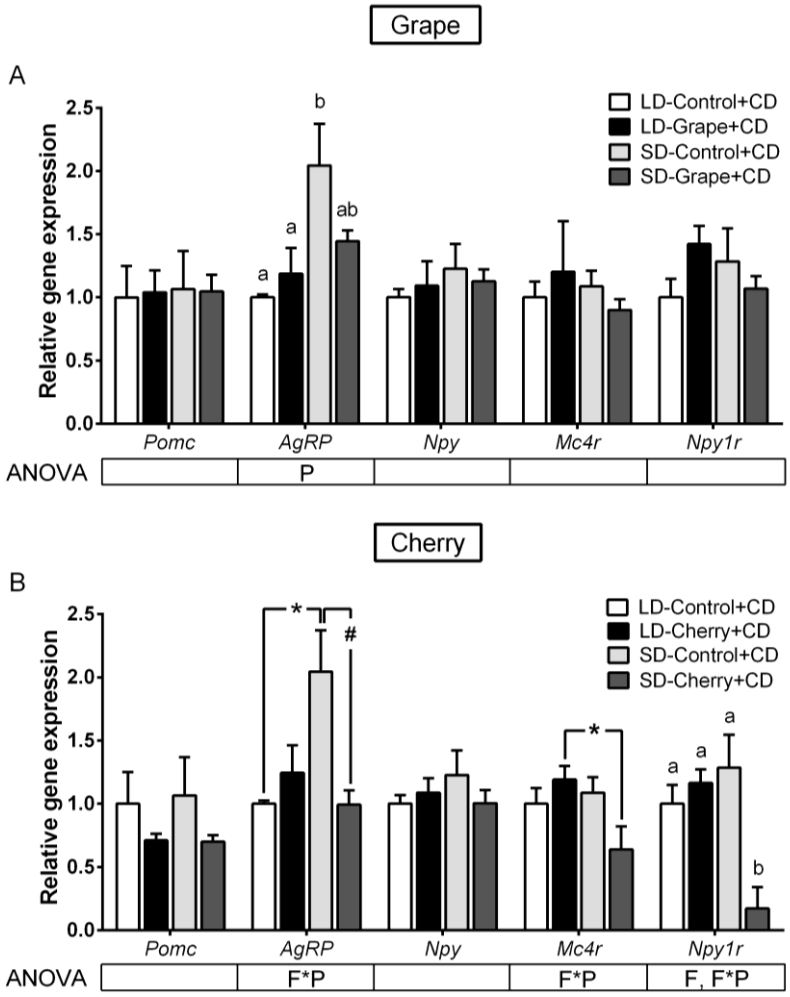

Figure 6. Effect of photoperiod and seasonal fruit supplementation on hypothalamic neuropeptides regulated by leptin. Hypothalamic gene expression of neuropeptides was evaluated by qPCR on animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, B).** Anorexigenic *Pomc* and orexigenic *Agrp* and *Npy* mRNA levels were determined, together with the mRNA's of receptors involved in energy balance *Mc4r* and *Npy1r* . Values are normalized against the LD-Control group. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of

photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$). ***** Effect of photoperiod in the fruit treated groups; **#** effect of fruit in the photoperiod groups determined by Student's *t* test ($P \leq 0.05$).

4. Discussion

Great attention has been paid to calorie consumption and diet composition in order to improve metabolic health and decrease obesity[24]. Hitherto, there is a lack of studies focusing on seasonal foods and their detrimental or beneficial effects when they are consumed in or out-of-season. Seasonality plays an important role in all the organisms and the environment to maintain the life balance[25]. Here, we present the results of this novel approach showing that seasonal fruits can modulate the leptin system depending on the photoperiod where they are consumed, both in healthy and obese animals.

Siberian Hamster (*Phodopus sungorus*) is the most widely used animal model to study photoperiod responses[26]. However, in this study we have opted for the Fisher 344 strain rat because rat metabolism is closer to humans[27] and this strain is sensitive to photoperiods[28].

Lean animals presented the characteristic phenotype described for Fisher344 rats placed at LD or SD photoperiods, displaying lower fat mass[29,30] and leptin serum[29,31] levels at SD. However, these rats did not present the significant reduction of body weight described as typical for the SD photoperiod[29,30]. But, remarkably, animals can become refractory to SD after a long exposure to this photoperiod in order to attain reproduction[32]. Specifically, this time is considered 10 weeks for Fisher344 rats[33] and rats in our experiment were kept 14 weeks at SD, thus they could be in a refractory state. However, we selected 4

weeks of adaptation to the photoperiod plus 10 weeks of fruit treatment because we aimed to find out the long-term effect of seasonal fruit consumption in animals already adapted to a specific photoperiod.

The fat mass reduction observed in lean rats placed at SD was associated with a lower energy balance (near to zero) that can be ascribed to increased energy expenditure rather than reduced food intake, such as has been described by other authors[29,33]. Studies analyzing energy expenditure in response to photoperiod exposure are scarce and mainly in Siberian hamster. In this rodent spices, energy expenditure increases during the first 2 weeks whereas decreases after 8 weeks of exposure to SD [34]. Leptin is essential on the regulation of central energy homeostasis[35]. However, any of the leptin-signaling components studied, the neuropeptides regulated by leptin or their receptors, in second order neurons, altered their expression in the hypothalamus of lean animals at SD. Thus, other hormones, such as prolactin[36], or an increase of leptin sensitivity[37,38], induced by other leptin signaling components not determined in this study, could participate in this higher energy expenditure observed in SD. In this sense, small rodents, such as hamster or the field vole (*Microtus agrestis*), develop leptin resistance in LD by decreased pSTAT3[38] that has not been quantified in the present study.

Our objective is not to directly compare lean versus obese animals rather than describe fruit and photoperiod effects in the two models separately. However, it is important to stand up that the response of obese animals to photoperiod quite diverged from that of lean animals. Few studies have focused on the photoperiod effects on animals fed an obesogenic diet. However, a study using Fisher 344 rats fed with a high-fat diet during 4 weeks[29] agrees with our findings referring to both, the loss of photoperiod regulation of fat mass and the photperiod regulation of food intake, decreasing it in SD. Thus, obese rats in the

present study reduced their energy balance at SD as consequence of a reduced cumulative energy intake, without any modification on energy expenditure. In addition, the photoperiod effect on serum leptin levels was blunted when animals were on the obesogenic diet despite the leptin system was sensitive to photoperiods in obese animals, in the sense that SD was associated to *Socs3* and *Agrp* overexpression in the hypothalamus. Remarkably, it has been described that leptin resistance associated to LD is caused by high serum leptin levels together with Socs3 overexpression[38] whereas leptin sensitivity accompanying to SD is associated to *Agrp* downregulation in the arcuate nucleus[36]. Thus, the pattern of the leptin system in rats fed the cafeteria diet, together with the high serum leptin levels in SD, indicate that obese rats have lost the capacity to modulate their leptin sensitivity according to photoperiods and strongly suggest that obese rats were even more leptin resistant in SD that in LD.

From all seasonal fruits, red grape was selected as representative fruit of autumn (SD) and cherry as representative of fruit of spring (LD) because their high content on polyphenols[39–41]. Red grapes contain mainly flavonoids including anthocyanins, flavanols (monomers and proanthocyanidins), flavonols and non-flavonoids such as phenolic acids and stilbenes (mainly resveratrol)[40,41]. Cherries also contain flavonoids which include anthocyanins, flavanols (monomers and proanthocyanidins) and non-flavonoids such as phenolic acids (mainly hydroxycinnamates)[42,43]. Evidently, grape and cherry contain similar classes of polyphenols; however cherries contain larger amounts of anthocyanins and hydroxycinnamic acids compared to grapes, but lack the flavonols and stilbenes[17]. Moreover, red grape and cherry also diverge in the individual polyphenol compounds of each polyphenol class[17].

The consumption of both fruits induced a lower energy balance in lean animals placed both at SD or LD, indicating that this fruit attribute is photoperiod

independent. Remarkably, grape and cherry consumption reduced the energy balance through different ways, grape reducing food intake whereas cherry increasing energy expenditure. The anorexigenic effect of grape consumption agrees with previous reported results demonstrating that a grape seed proanthocyanidin extract reduces food intake in obese animals[13].

Interestingly, both cherry and grape intake resulted in the overexpression of *Pomc in* the hypothalamus when they were consumed at SD. Thus, this enhanced anorexigenic effect of both fruits could account, at least partially, for the lower energy balance observed in rats consuming fruits at SD. However, the fact that *Pomc* was only modulated in SD points out the photoperiod dependent capacity of these fruits to modulate leptin signaling. In addition, cherry consumption also modulated hypothalamic *Obrb* expression in a photoperiod dependent manner, increasing its expression in SD. Thus, cherry clearly increased central leptin sensitivity out of season in lean animals. In contradiction, cherry intake was associated with *Socs3* and *Agrp* overexpression in the hypothalamus at both photoperiods, both markers of leptin resistance. The increase of the last could be due to higher sensitivity to cherry polyphenols since the majority of AgRP neurons are found outside of the blood brain barrier[44].

Remarkably, neither the energy balance reduction nor the leptin sensitivity modulation induced by fruits were reflected in the body weight or fat mass accretion, which were not affected or even increased such as fat mass after grape consumption at SD. Thus, cherry and mainly grape should modulate other mechanisms that, in turn, counteract the expected fat mass reduction. In this sense, grape seed proantocyanidins have adipogenic activity, overexpressing PPARγ, increase adipocyte number and reduce adipocyte hypertrophy in visceral and subcutaneous fat pads[45]. Thus, grape could act at adipocyte level,

counteracting the expected response of white adipose tissue to a lower energy balance sate. In addition, grape seed proanthocyanidins increase mitochondrial oxidative capacity in skeletal muscle and brown adipose tissue[46]. Thus, the increase of energy expenditure in these organs also could contribute to the lower energy balance observed in rats fed grapes. There are not studies about the effects of cherry extracts on energy expenditure, but cherry effect increasing energy expenditure by activating brown adipose tissue thermogenesis, and/or the mitochondrial activity in other organs, cannot be ruled out.

In obese rats, grape seed proanthocyanidins also stimulate thermogenesis in brown adipose tissue[47] and increase hypothalamic leptin sensitivity[13], associated with the overexpression of *Pomc*. However, no effects of grape intake were observed in obese animals in either photoperiod. In contrast, cherries were very effective modulating the leptin system in obese rats in a photoperiod dependent mode. Specifically, cherry repressed the expression of *Agrp* and *Ptp1B* in SD. Interestingly, *Ptp1b* is a negative regulator of the leptin signaling pathway and its inhibition increases leptin sensitivity in obese animals[48], thus cherry consumption at SD clearly increased leptin sensitivity in obese animals. Furthermore, *Mc4r* and *Npy1r* were also repressed in SD, indicating that cherry was also effective modulating the response in second order neurons. Altogether these results indicate that cherry intake modulated central leptin system out of season in obese animals, like did in lean animals. Moreover, these results strongly suggest that cherry has anorexigenic effects in a photoperiod dependent mode, but independently of the fat stores and body weight. This reduction of the orexigenic signal *Agrp* by cherry agrees with the decreased cumulative food intake in obese rats that consumed cherry in SD. In contrast, the photoperiod dependent effect of cherry on leptin sensitivity, increasing it in SD, disagrees with the lower energy balance and RQ in obese

rats consuming cherry at LD. Thus, like in lean animals, a cherry effect increasing energy expenditure in brown adipose tissue when it is consumed in-season cannot be excluded.

The xenohormesis hypothesis[49], defined by Howitz and Sinclair, proposes that animal uses chemical signals from plants, mainly polyphenols, to know about the environmental status or food supply. This acknowledgment would allow animals to respond in advance to environmental alterations, thus increasing their probability of survival. Therefore, the specific polyphenol content in grape and cherry could act as a distinctive mark, informing rats of the different environmental conditions such as photoperiod. Reinforcing this idea, previous studies of our group have demonstrated that grape proanthocyanidins modulate circadian rhythms at hypothalamic level[50]. Nevertheless, cherries are also rich on melatonin[51] a hormone that informs about day length, controlling seasonal phenotypic adjustments[52]. Interestingly, melatonin reduces the energy expenditure induced by cold exposition in the Siberian hamster placed at LD[53] and stimulates *Pomc* expression in mice[54]. Therefore, the high melatonin contained in cherries could significantly contribute to the effects of induced by cherry intake observed in this study.

Summing up, fruit consumption decreased energy balance in lean animals in a photoperiod independent manner. Grape reduced cumulative food intake whereas cherry increased energy expenditure. However, rats fed cherry did not display any fat mass reduction and rats fed grape even increased it when placed at SD. In contrast, both fruits modulated the leptin system in a photoperiod dependent way, increasing leptin sensitivity in SD. In obese animals, only cherry intake modulated the leptin system, repressing *Agrp* and *Ptp1B* in SD. Thus, cherry intake modulated central leptin system when was consumed out of season both in obese animals and lean animals.

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Supplementary materials

Table S1. Primer sequences used in qPCR amplification from rat genes in hypothalamus.

Primer (Rat)		Sequence 5'-3'	Product size (bp)	GenBank accession no/reference
Obrb	**Fw**	CCAGTACCCAGAGCCAAAGT	122	NM_012596.1
	Rw	GGATCGGGCTTCACAACAAGC		
Socs3	**Fw**	CTGGACCCATTCGGGAGTTC	148	NM_053565.1
	Rw	CTGGGAGCTACCGACCATTG		
Ptp1b	**Fw**	CCCTTTTGACCACAGTCGGA	119	NM_012637.2
	Rw	TTGGTAAAGGGCCCTGGGTG		
Pomc	**Fw**	GAGGCGACGGAGGAGAAAAG	98	NM_139326.2
	Rw	TGAGGCTCTGTCGCGGAAA		
AgRP	**Fw**	GAGAACTCTGGGAACAGGGC	140	NM_033650.1
	Rw	CAAGCAAAGGCCATGCTGAC		
Npy	**Fw**	CTATCCCTGCTCGTGTGTTTGG	136	Sun B et al. 2014
	Rw	TGGTGATGAGATTGATGTAGTGTCG		
Mc4r	**Fw**	CAACTCCTTTGCAAGCTCCG	129	NM_013099.3
	Rw	TCCAACCTCCTAGGTCAGGG		
Npy1r	**Fw**	TCTTCTCTGCCCTTCGTGATC	73	NM_001113357.1
	Rw	TGAACGCCGCAAGTGATACA		
Ppia	**Fw**	CTTCGAGCTGTTTGCAGACAA	138	NM_017101.1
	Rw	AAGTCACCACCCTGGCACATG		
Rplp0	**Fw**	GGACCTCACCGAGATTAGGG	225	NM_022402.2
	Rw	CCCACCTTGTCTCCAGTCTT		

Abbreviations: Obrb, leptin receptor isoform b; Socs3, suppressor of cytokine signaling 3; Ptp1b, protein tyrosine phosphate 1B; Pomc, proopiomelanocortin; Agrp, agouti-related protein; Npy, neuropeptide Y; Mc4r, melanocortin 4 receptor; Npy1r, neuropeptide Y receptor Y1; Ppia, peptidylprolyl isomerase A; Rplp0, ribosomal protein lateral stalk subunit P0.

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CHAPTER 4

The adenosine A2B receptor contributes to AgRP neuronal activation

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Abstract

Adenosine signaling has an important role in the central nervous system modulating neuronal activity. However, little is known about the function of the adenosine A_{2B} (A2B) receptor. Recently, it has been reported that AgRP neurons can be activated by G protein-coupled receptors (GPCR) linked to G stimulatory protein (Gs-GPCR), leading to a maintained increase in feeding. A2B receptors are linked to Gs proteins that activate intracellular signaling pathways. These evidences suggest a link between adenosine signaling and AgRP neurons that has not been investigated in physiological conditions. First, we show that mice that lack the A2B receptor in the nervous system exhibit reduced food intake after a 24-hour fast-refeed test, consistent with a decrease in *AgRP*/*Npy* and *AgRP/Pomc* mRNA levels. Further, mice that lack A2B receptors specifically in AgRP neurons do not display a feeding phenotype. However, after a short fast they show a decrease in the activation of AgRP neurons in the arcuate nucleus. In summary, we report that the A2B receptor signaling plays a role in the nervous system food intake regulation and in AgRP neurons under physiological conditions.

1. Introduction

Adenosine is a purine nucleoside that acts as a constituent of nucleic acids and when phosphorylated is the metabolic energy currency ATP[1]. Adenosine is found in the majority of mammalian cells and its levels depend on the export and uptake or on the release of adenine nucleotides that are hydrolyzed to adenosine in the extracellular space. The production of extracellular adenosine from ATP, ADP and AMP is performed by ectonucleotidases such as CD39 (nucleoside triphosphate diphosphohydrolase) and CD37 (5'-ectonuclotidase)[2]. Similarly when intracellular adenosine concentrations are high due to ATP degradation it can be released through the equilibrative nucleoside transporters (ENT1 and ENT2)[3].

Adenosine signaling plays an important role in the central nervous system (CNS) and it is considered a modulator of neurotransmitters and neuronal activity[4]. Extracellular adenosine plays a variety of physiological roles by binding different adenosine receptors which are G protein-coupled receptors (GPCR): $A_{1,}$ A_{2A}, A_{2B}, A_3. Each receptor has specific tissue distribution, distinct abundance and affinity[5]. Particularly, A2B receptors are stimulatory G proteins (Gs), that increase adenylate cyclase activity, triggering a variety of effects depending on the cell type[6].

The role of A2B receptors in the brain and nervous system is still not well understood because of the low abundance and low affinity of A2B for adenosine and most agonists[4,7]. However, in the periphery, it has been shown to be involved in metabolic effects such us the regulation of glucose homeostasis and lipid metabolism[8]. Metabolism is centrally regulated by the brain which receives and integrates peripheral signals. In particular, the hypothalamus is a brain area of key importance in metabolic regulation[9]. Proopiomelanocortin

(POMC) and AgRP neurons are found in the arcuate nucleus (ARC) of the hypothalamus and are key regulators of energy balance, promoting satiety and food intake respectively[10]. It is remarkable that the majority of AgRP neurons are found outside the blood-brain barrier (BBB) and are more sensitive to peripheral signals and susceptible to changes[11]. Recently, it has been reported that AgRP neurons express Gs-GPCR and the activation of this receptors increases food intake[12]. We hypothesize that AgRP neurons express A2B receptors and have physiological effects in the modulation of AgRP activity.

To address this we determined the gene expression profile of neuropeptides found in the ARC in mice with A2B (*Adora2b*) deletion in the nervous system. We also studied AgRP neuronal activation in the arcuate nucleus (ARC) and dorsomedial hypothalamic nucleus (DMH) of mice with A2B deletion specifically in AgRP neurons.

2. Materials & Methods

2.1 Animals

Mice were housed in barrier facility under a 12-hour light cycle (lights on from 7 AM to 7 PM) with ad libitum access to water and a standard mouse chow (21.6%, 23.2% and 55.2% kcal from fat, protein and carbohydrate, respectively; Purina mouse diet #5058). Transgenic mice expressing Cre recombinase under the *Nestin* promoter [B6.Cg-Tg(Nes-cre)1Kln/J] or the *AgRP* promoter [Agrptm1(cre)Lowl/J] were purchased from Jackson Laboratory. To generate mice lacking the A2B receptor in the central and peripheral nervous system (*Nestin-Cre/+; Adora2b*$^{fl/fl}$ mutants) and their littermate controls (*Nestin-Cre/+; Adora2b*$^{fl/+}$*), Adora2b*$^{fl/fl}$ females were mated with *Nestin-Cre/+; Adora2b*$^{fl/+}$

males. Since expression of *Nestin-Cre* alone presents a body weight phenotype[13,14], control mice expressing Nestin-Cre were chosen. Mice of 23 weeks of age under were sacrificed under *ad libitum* conditions before the onset of the dark cycle. Only male mice are used for experiments.

To generate mice lacking the A2B receptor in AgRP neurons, *Adora2b*$^{fl/fl}$ females were mated with *Adora2b*$^{fl/+}$*; Agrp-Cre* males. This breeding pair resulted in control (*Agrp*+/+*; Adora2b*$^{fl/fl}$ or *Agrp*+/+; *Adora2*$^{fl/+}$*)* and AgRP-specific A2B receptor mutant (*Agrp-Cre*/+; *Adora2b*$^{fl/fl}$) animals. Mice of 23 weeks of age were fasted at 10:00 and sacrificed at the onset of the dark phase at 19:00, at a time when AgRP neurons show high activity levels[15]. Only male mice are used for experiments.

All experiments were carried out under a protocol approved by the University of California at San Francisco Institutional Animal Care and Use Committee.

2.2 Immunofluorescence

AgRP-Cre; Adora2b mice were anesthetized with ketamine/xylazine solution and perfused with 4% (wt/vol) paraformaldehyde (PFA). Brain was excised and post-fixed in 4% PFA solution for 2 hours at 4 ºC. After this, brain samples were transferred to a 30% sucrose solution for cryoprotection and kept overnight at 4 ºC. Samples were embedded in Shandon M-1 embedding matrix (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and stored at -80 ºC. Brain samples were cut using a cryostat to obtain 10 µm-thick coronal sections. Double staining was performed by simultaneous incubation with primary antibodies, rabbit anti-cFos (1:4000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-AgRP (1:1000, Neuromics, Minneapolis, MN,

USA) overnight at 4 ºC. Sections were then washed and incubated at room temperature for 1 hour with secondary donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488 (1:200, Thermo Fisher Scientific Inc.) Nuclear counterstain was performed by 4',6-diamidino-2-phenylindole (DAPI, 1:2000) incubation together with the secondary antibody. Sections were prepared using Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting media.

2.3 Images quantification

Images were captured using an Olympus BX51WI microscope equipped with a QImaging Retiga 2000R digital camera. Images were taken with the same exposure, avoiding pixel saturation and merged using QCapture Pro 6 (Qimaging, Surrey, BC, Canada). Only sections processed in the same experiments were compared for analysis. Images were viewed using Adobe Photoshop (San Jose, CA, USA) and Image, (NIH, Bethesda, MD, USA) was used for the quantification of cFos-positive cells. Images were blinded for the cell counting analysis. Sections were matched by anatomical landmarks according to Bregma (anterior, Bregma: -1.84 mm; medial, Bregma -1.94 mm; posterior, Bregma -2.06 mm) using the Mouse Brain Atlas[16]. At least two sections per mouse per region were quantified and then averaged. Quantification of cFos-positive cells was performed normalizing the background intensity with ImageJ. Only signals clearly positioned overlapping the cell nuclei, which is visible by DAPI staining, is taken as true cFos signal and quantified. In the ARC, only cFos-positive cells located together with the pre-synaptic AgRP fibers were counted, and in the DMH only cFos-positive cells located together with the AgRP projections in post-synaptic neurons.

2.4 Gene expression studies

Hypothalamic and liver RNA isolation from *Nestin-Cre; Adora2b* mice was performed using the RNeasy Lipid Tissue Mini Kit and RNeasy plus mini kit respectively (Qiagen, Valencia, CA, USA). Gene expression was assessed by qPCR using the following Taqman gene expression assay probes (Thermo Fisher Scientific Inc.): *Pomc*, mm00435874_m1; *Agrp*, Mm00475829_g1; *Npy*, Mm00445771_m1 for hypothalamus and *Fasn*, mm00662319_m1*; Acaca*, mm01308023_m1for liver. Relative expression was referred to *Rn18S*, mm03928990_g1.

2.5 Liver triglyceride levels

Homogenization of 40-50 mg of liver was done in 500 µl buffer (250 mM sucrose, 50 mM Tris-HCL pH 7.4) and determined using an enzymatic (Serum) Triglyceride Determination Kit (TR0100, Sigma-Aldrich, St. Louis, MO, USA). Samples were diluted 1:5. Serial dilutions of the standard known concentrations were placed in the same microplate and all samples were incubated with Free Glycerol Reagent and Triglyceride reagent 1:4 mixture for 15 minutes at room temperature. Absorbance was measured at 540 nm. Triglyceride levels of each sample were normalized to total protein content. Protein quantification was measured with Bradford Reagent (B6916, Sigma-Aldrich, St. Louis, MO, USA).

2.6 Body composition and metabolic analysis

Body composition was measured using EchoMRI-700 (Echo Medical Systems, LLC., HoustonTX, USA). Data is expressed as grams of total body weight. After sacrifice, gonadal white adipose tissue was excised for weight determination. Indirect calorimetry, locomotor activity and food intake were measured over 5 days at room temperature (23.5 ºC) using a comprehensive lab animal monitoring system (CLAMS) (Columbus instruments, Inc., Columbus, OH, USA). Mice were allowed to acclimate in the first day and data from this period were excluded from analysis. Respiratory exchange ratio (RER) was calculated as (VCO_2/VO_2) and energy expenditure calculation was performed using the following formula: (3.815 + 1.232 x RER) x VO_2.

2.7 Statistical analysis

Data is expressed as mean ± SEM. Two-tailed Student's *t*-test was used to compare two independent groups of mice. Under conditions where the same animals were analyzed over time, repeated-measures two-way ANOVA was used. Statistical analyses were performed using Prism software (GraphPad Software, Inc, La Jolla, CA, USA). Differences were considered as statistically significant when $P \leq 0.05$.

3. Results

Mice lacking the A2B receptor in the nervous system maintain normal body weight, body composition and hepatic metabolism.

To investigate the role of the A2B receptor in the nervous system, we studied the effect of A2B deletion using *Nestin-Cre/+; Adora2b*$^{fl/fl}$ (*Nestin-Cre;A2B*$^{fl/fl}$) mice and their littermate controls (*Nestin-Cre/+; Adora2b*$^{fl/+}$). Since expression of Nestin-Cre alone presents a body weight phenotype[13,14], heterozygous mice expressing Nestin-Cre were chosen as controls. Both groups of mice were fed a standard chow diet. Body weight, whole-body lean and fat masses as well as gonadal white adipose tissue (WAT) at 23 weeks of age **(Fig. 1A, B)** were not significantly different between mutants and control animals, indicating that there is not a major role for the A2B receptor under physiological conditions.

It has been reported that the A2B receptor has a role on the development of fatty liver[17]. Specifically, this receptor promotes the accumulation of hepatic triglyceride through the decrease in peroxisome proliferator-activated receptor alpha (PPARα) and AMP-activated protein kinase (AMPK) phosphorylation, factors involved in fatty acid oxidation[5]. AMPK regulates lipid metabolism by inhibiting acetyl CoA carboxylase (ACC)[18,19], the rate limiting enzyme for *de novo* synthesis of fatty acids in liver[20]. Furthermore, the A2B receptor also regulates sterol regulatory element-binding protein 1 (SREBP1), a fundamental transcription factor on the regulation of fatty acid synthesis genes in the liver which include fatty acid synthase (*Fasn)* and acetyl-CoA carboxylase (*Acaca*)[21,22]. Further, It has been shown that there is a neural regulation of liver metabolism through the autonomic nervous system[23,24] Together, these findings suggests that the A2B receptor may play a role in the brain which could

influence hepatic metabolism. To investigate this, we measured the liver triglyceride content and the gene expression of *Fasn* and *Acaca* under baseline conditions in mutant and control mice. Triglyceride levels in the mutants are comparable to the ones in the controls **(Fig. 1C)**. The mRNA expression of *Fasn* and *Acaca*, did not reach statistical significance although the mutants show a trend to reduction of its levels **(Fig. 1D, E)**.

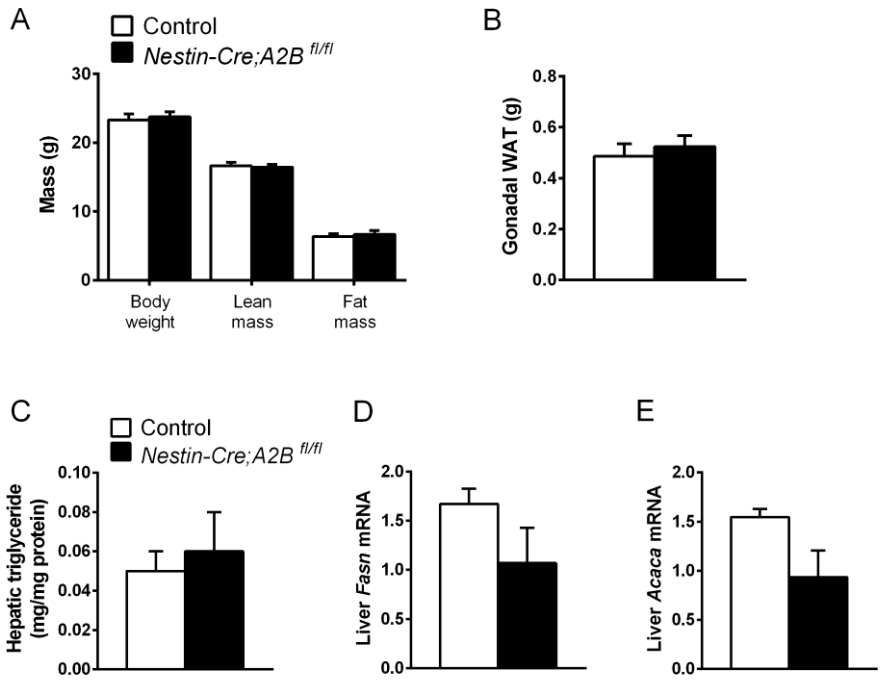

Figure 1. Mice that lack A2B in central and peripheral nervous system present similar body weight, fat mass and hepatic triglyceride compared to control mice. *Nestin-Cre; A2B*$^{fl/fl}$ mice show similar body weight and body composition **(A)** compared to control mice. Gonadal WAT remains unchanged between groups **(B).** Triglyceride levels **(C)** and mRNA expression of *Fasn* **(D)** and *Acaca* **(E)** in the liver are not significantly different in *Nestin-Cre; A2B*$^{fl/fl}$ mice relative to the controls. However, gene expression in mutant mice shows a trend to reduction. Mice were weighed at 20-23 weeks of age and body composition was assessed using EchoMRI one day before the sacrifice. Fat pads and liver were excised and weighted after the sacrifice**.** Data are mean ± SEM of n=5-7/group. *P≤0.05 as determined by Student's *t*-tests.

Deletion of the A2B receptor in nervous system promotes an anorectic effect after food deprivation

To determine if deletion of A2B receptors from the nervous system has an impact on metabolism and energy utilization, animals were placed in CLAMS. Following one day of acclimation period, we measured respiratory exchange ratio (RER), energy expenditure and food intake under baseline condition and following a 24-hour fast **(Fig. 2A-F).** Both RER **(Fig. 2A)** and energy expenditure **(Fig. 2C)** were comparable in control and mutant mice within the four-day period of measurement. Further, no difference was observed in these parameters during the day and night **(Fig. 2B, D)**. Under baseline condition, food intake under baseline condition is not different in control versus *Nestin-Cre; A2B*$^{fl/fl}$ animals **(Fig. 2E, F).** However, following a 24-hour fast, *Nestin-Cre; A2B*$^{f/f}$ mutant animals showed a marked reduction in hyperphagia relative to control mice **(Fig. 2G).** This change in food intake after food deprivation is consistent with a decrease in *Agrp/Npy* mRNA ratio, since *Agrp* expression in the mutants is decreased compared with *Npy* expression which is a component of the same cells. Furthermore, there is a trend on the reduction of *Agrp/Pomc* mRNA ratio in the mutants which indicates that the overall melanocortin tone is different, with higher *Pomc* expression compared to *Agrp.* AgRP is an antagonist of melanocortin-4 receptor (MC4R), whereas POMC acts as agonist activating the same receptors and producing an anorexigenic effect[25]**(Fig. 2H, I)**. This result suggests that the reduction of food intake could be driven by a decrease in *Agrp* expression, leading to a reduction in its orexigenic effect that is exerted through MC4R signaling pathways.

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Figure 2. Food intake is decreased in *Nestin-Cre; A2B$^{fl/fl}$* after a fast-refeed challenge consistent with the modulation of gene expression of orexigenic and anorexigenic neuropeptides. Metabolic analysis of RER **(A)**, energy expenditure **(C)** and food intake **(E)** during four full days measurement in CLAMS are similar between controls and mutant mice except for the 6-hour refeeding period were mutants show a decrease on food intake **(E).** Shaded areas in A, C and E represent dark cycle (19:00-07:00). The average of hourly daily (07:00-07:00h), day (07:00-19:00) and night (19:00-07:00) of RER **(B)**, energy expenditure **(D)** and food intake **(F)** CLAMS analysis do not show differences between groups. The average of 6 hours refeeding measurements after 24-hour fast **(G)** further confirms the reduced fasting-induced feeding of mutant mice compared to controls. Accordingly, the mutant mice showed decreased *Agrp* ratio compared to *Npy* and *Pomc* mRNA levels in the hypothalamus **(H, I).** Animals were fasted towards the dark phase (18:00) and food was introduced again after 24 hours. (n=5-7, 12-15 weeks of age). Hypothalamus was excised at the end of the experiment (n=5-7, 20-23 weeks of age). Data are mean ± SEM *$P \leq 0.05$ as determined by two-way repeated measures ANOVA (A, C, E) and Student *t*-tests (B, D, F, G).

Physiological parameters are maintained after deletion of A2B in AgRP neurons

To determine if the reduction in fasting-induced food intake in *Nestin-Cre; A2B$^{fl/fl}$* animals could be attributed to AgRP neurons, an important group of neurons that regulate feeding[26], we next investigated the effect of AgRP-specific A2B deletion. *AgRP-Cre; A2B$^{fl/fl}$* mice show similar body weight, body composition and hepatic triglyceride levels relative to control animals **(Fig. 3A-C).** Further, analysis of RER, energy expenditure and food intake measured in CLAMS shows no difference between controls and *AgRP-Cre; A2B$^{fl/fl}$* mice **(Fig. 4A-F)**. In contrast to the *Nestin-Cre; A2B$^{fl/fl}$* mutant, *AgRP-Cre; A2B$^{fl/fl}$* mice do not show a defect in mounting a hyperphagic response to fasting **(Fig. 4G).** Indeed, the amount of food consumed in the first 6 hours following the reintroduction of food was similar between *AgRP-Cre; A2B$^{fl/fl}$* mice and

controls. Together, these data suggest that there are no differences in metabolism between control and *AgRP-Cre; A2B*$^{fl/fl}$ animals. We next investigated the possibility that there are alterations at a neuronal level that might underlie a pathophysiological role of A2B receptor in AgRP neurons.

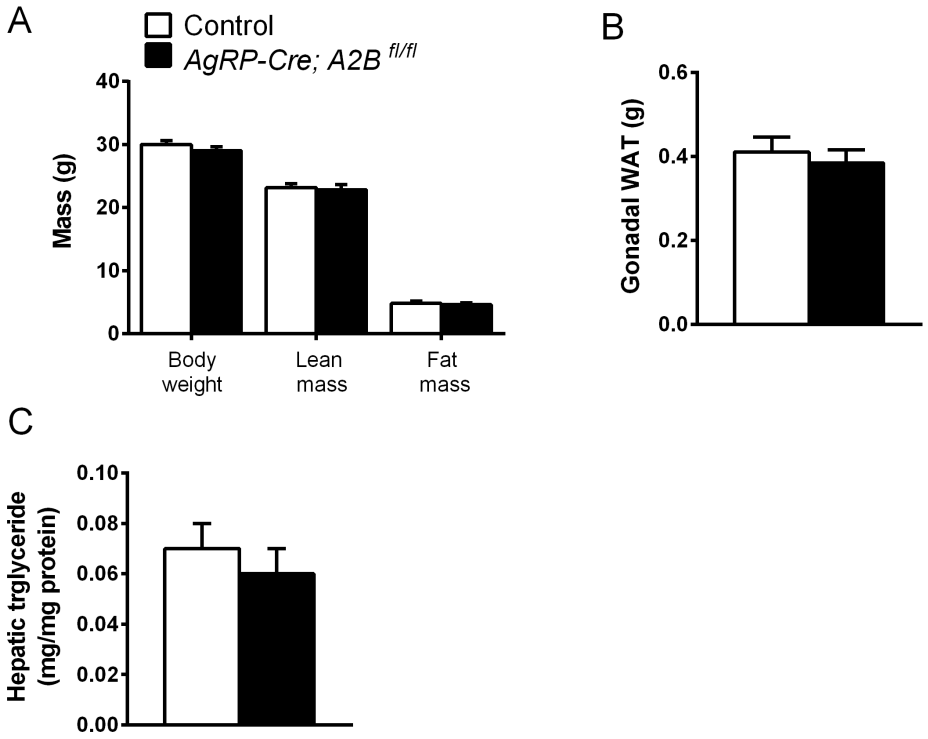

Figure 3. Deletion of the A2B receptors in AgRP neurons does not alter body weight, body composition and hepatic triglyceride levels. *Agrp-Cre; A2B*$^{fl/fl}$ mice show similar body weight and body composition **(A)** compared to control mice. Specifically, gonadal WAT remains unchanged between groups **(B)** as well as the hepatic triglyceride content **(C)**. Mice were weighted and echoed the day before the sacrifice using RMI to assess body composition. Fat pads and liver were excised and weighted after the sacrifice (n=9-10, 20-23 weeks of age). Data are mean ± SEM. **P*≤0.05 as determined by Student's *t*-tests.

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Figure 4. Metabolic measurements of RER, energy expenditure and food intake are similar in both controls and *AgRP-Cre; A2B*$^{fl/fl}$ and fast-refeed challenge does not show a different phenotype. Metabolic analysis of RER **(A)**, energy expenditure **(C)** and food intake **(E)** during four full days measurement in CLAMS are similar between controls and mutant. Shaded areas in A, C and E represent dark cycle (19:00-07:00). The average of hourly daily (07:00-07:00h), day (07:00-19:00) and night (19:00-07:00) of RER **(B)**, energy expenditure **(D)** and food intake **(F)** CLAMS analysis do not show differences between groups. The average of 6 hours refeeding measurements after 24-hour fast **(G)** is not different between mutant and control mice. Animals were fasted towards the dark phase (18:00) and food was introduced again after 24 hours. (n=9-10, 12-15 weeks of age). Data are mean ± SEM *$P \leq 0.05$ as determined by two-way repeated measures ANOVA (A, C, E) and Student *t*-tests (B, D, F, G).

A2B is involved in the activation of AgRP neurons in the arcuate nucleus

We next investigated the neuronal characteristics of these mice in the ARC where AgRP neurons are found. Apart from fasting, AgRP neurons exhibit the highest level of activity towards the dark phase[27]. Activation of AgRP neurons in this case requires a large amount of energy for ATP production, which leads to the generation of adenosine that is then released from the neurons. We hypothesize that extracellular adenosine will bind the A2B receptor to sustain neuronal firing in a positive feedback loop. Further, intracellular adenosine will produce cAMP that will activate intracellular signal transduction pathways. To examine the activity of ARC neurons under a setting of high energy demand, we sacrificed control and *AgRP-Cre; A2B*$^{fl/fl}$ mice at the onset of dark phase following a 10-hour daytime fast (09:00-19:00). AgRP fibers were localized in brain sections by staining with an AgRP antibody. cFos staining in the ARC was used as a marker of neuronal activity[28,29].

Our result demonstrates a typical nuclear cFos staining that overlaps with nuclear marker, DAPI **(Fig. 5A, B).** Merged images demonstrate that cFos-positive cells are found alongside with AgRP fibers. Interestingly, mice that lack A2B in AgRP neurons show a clear reduction in cFos-positive cells than controls in the ARC **(Fig. 5A),** particularly in the mediobasal area where AgRP neurons predominate. AgRP neurons are known to project to downstream neurons, including those in the DMH (dorsomedial hypothalamus)[30], where they exert an inhibitory effect **(Fig. 5B)**. We investigated whether mutant mice show a greater abundance of cFos-positive cells in DMH in association with reduced ARC neuronal activity. Quantification of cFos-positive cells in the DMH reveals that cFos is expressed similarly in both groups **(Fig. 5C).** Together, these data indicate that ARC neurons, likely AgRP neurons, are less active in animals without A2B receptor.

Figure 5. A2B deletion in AgRP neurons decreases the number of cFos-positive cells in the ARC. Immunostaining for AgRP fibers (green), cFos (red) and DAPI (blue) in the arcuate nucleus and merged images **(A, B)**. cFos positive cells in the mediobasal hypothalamus adjacent to the third ventricle in the arcuate nucleus are decreased in mutant mice compared to control mice **(A).** cFos positive cells in the DMH are expressed equally in mutant and control mice **(B)**. Quantification of cFos positive cells in the arcuate and DMH is consistent with the immunofluorescence images, showing a significant decrease in mutant mice compared to the controls in the arcuate nucleus whereas DMH cFos positive cell counting is similar in both groups **(C)**. Mice were fasted 10h before sacrifice to produce a fasting-activation of AgRP. Activation of AgRP neurons is visualized by

the indirect marker of neuronal activity, cFos in the ARC. Mice were sacrificed towards the dark phase (19:00). Data are mean ± SEM of at least 4 mice. 2 sections per mouse per Bregma were quantified. *$P \leq 0.05$ as determined by Student's *t*-tests.

4. Discussion

AgRP neurons play a key role in hypothalamic regulation of food intake and energy expenditure through the mealnocortin system. These neurons produce AgRP, an orexigenic neuropeptide that antagonizes melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R). AgRP cell bodies are found in the ARC and exert their effect via projections to DMH and PVN among other areas[31]. Interestingly, unlike other hypothalamic neurons, the majority of AgRP neurons are located outside the blood-brain barrier, allowing them to sense and respond to circulating metabolic signals[11,32]. Indeed, circulating hormones such as ghrelin, a gut hormone that informs the brain about energy availability is known to activate AgRP neurons by binding to the growth hormone secreatagogue receptor (GHSR) to produce an increase on caloric intake and adiposity[33]. Aside from ghrelin, other factors can also activate AgRP neurons in a different manner. Among them, acute ethanol dose increases AgRP immunoreactivity in the ARC of C57BL/6J mice. Ethanol consumption leads to the production of acetate in neurons and increases extracellular adenosine levels in the brain[34]. The mechanism by which alcohol triggers the increase of AgRP signaling is yet to be determined, but it is plausible that there is a link between adenosine levels and AgRP activity. We hypothesize that extracellular adenosine might bind the A2B, which is known to be a stimulatory GPCR, to sustain the firing in a positive feedback loop and to activate cellular responses

in AgRP neurons. In this study, our goal is to determine the function of A2B in the nervous system and in AgRP neurons.

The use of two genetic mice models, one with a the deletion of A2B in the central and peripheral nervous system and the other with the deletion of A2B only in AgRP neurons allows distinguishing between the effects produced by A2B in the nervous system or in AgRP neurons alone. *Nestin-Cre; A2B$^{fl/fl}$* mice maintained at physiological conditions and fed a normal chow diet did not show changes on body weight, body composition, nor liver triglyceride levels. Fatty acid synthase and acetyl CoA-carboxylase, genes involved in hepatic lipid accumulation showed a trend to be reduced although it did not reach statistical significance**.** Hepatic analyses are relevant since it has been reported that whole body A2B knockout mice are protected from fatty liver after chronic alcohol consumption[5]. Further metabolic analyses were performed in CLAMS and animals were challenged with a 24-hour fast-refeed test to examine metabolic adaptation of the mutant mice. RER and energy expenditure remained the same among groups. Nevertheless, food intake analysis showed a meaningful decrease in refeeding after a 24-hour fast in *Nestin-Cre; A2B$^{fl/fl}$* mice**.** This phenotype indicates that A2B could have a role on food intake regulation. Then, gene expression of the neuropeptides that control food intake was assessed. Consistent with the previous results *Agrp* gene expression is reduced compared to other neuropeptides in mutant mice, meaning that they receive a lower degree of orexigenic signals. Previous studies reported that after consuming high-fat diet (HFD) A2BKO mice show elevated fat to lean ratio and higher leptin levels compared to WT mice[35]. This data further supports a connection with the regulation of energy homeostasis although no mechanisms have been described.

Subsequently, we focused on the specific population of AgRP neurons. In contrast to our previous results with *Nestin-Cre; A2B$^{fl/fl}$* deletion of A2B

receptor in AgRP neurons does not show significant phenotypical changes on the metabolic factors analyzed. Thus, we proceed to analyze more extensively the hypothalamic nucleus where AgRP neurons are localized. The approach we used to study AgRP neuronal activity consisted in achieving a situation where AgRP neurons are activated such in fasting[27]. Furthermore, it has been demonstrated that AgRP stimulates feeding at the onset of dark-phase[31,36]. Therefore, these two stimuli were taken into account to provide the right conditions. To detect AgRP neuronal activity we used cFos as indirect marker. We show that control mice have higher cFos expression in the arcuate nucleus surrounded by AgRP fibers and particularly there are more abundant in the area adjacent to the third ventrical in the medibasal hypothalamus. cFos signal is specific since it colocalizes with nuclear DAPI staining. As reported by Olofsson et al. the predominant neuronal type in the mediobasal hypothalamus situated outside of the blood-brain barrier are AgRP neurons. Accordingly, they are exposed to peripheral signals making them more sensitive to metabolic changes[11]. This data supports that the majority of neurons seen in the images that express cFos are truly AgRP neurons, and this are more active when A2B is present. On the contrary, *AgRP-Cre; A2B*$^{fl/fl}$ mice immunofluorescence shows fewer cFos positive cells in the arcuate which is confirmed with the quantification analysis. Additionally, cFos expression can also be seen in neurons located in the DMH, where AgRP fibers project. In this case, the images show slightly fewer cFos signal in the control mice compared to mutants. However, the posterior quantification indicates that there are no differences between mice groups. Even though ARC neurons are less activated, neurons that receive inhibitory inputs from AgRP neurons such as those in the DMH are not affected. Additional investigations should be carried out to study effects in downstream neurons.

In summary, mice with a deletion of A2B in the nervous system show a reduced refeeding phenotype, whereas deletion of A2B in AgRP neurons does not produce changes during refeeding compared to their controls. This could mean that A2B exerts effects through neurons other than AgRP that also regulate food intake such us neuropeptide Y (NPY) neurons or POMC neurons among others. Relative to AgRP, we suggest that during fasting, which is characterized by a state of energy deficiency, AgRP neurons increase their firing rate and produce ATP which is hydrolyzed to adenosine to obtain energy. Adenosine is transported outside of the cell through ENT1 and subsequently extracellular adenosine levels rise. In this situation adenosine may bind A2B and activate the transcription of AgRP and contribute to a positive feedback. Potentially, the same pathway that modulates AgRP activity through A2B might be high-jacked by ethanol producing AgRP neuron activation. Future approaches include to carry out these experiments using a female cohort and the study of the effects of ethanol in both mice lines using mice of both genders, to better define the A2B signaling and its pathophysiological role.

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IV. GENERAL DISCUSSION

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Dietary polyphenols have been widely studied due to its beneficial effects on health[1,2]. Furthermore, data regarding the effects of polyphenols on metabolism is promising for the prevention and treatment of cardiovascular disease, diabetes type II and obesity[3]. It is of great interest to establish new approaches to decrease obesity since this condition is accompanied by increased health risk factors[4]. In this sense, *in vitro*, *in vivo* and some human clinical trials tested the potential of polyphenols on obesity treatment as compounds with the ability to increase energy expenditure[5]. Up to now, the vast majority of studies have focused on studying the effects of polyphenols improving obesity by its action in peripheral organs such as liver, adipose tissue and muscle. For instance, green tea catechins, resveratrol and curcumin *in vivo* may improve obesity by increasing fat utilization, decreasing plasma triglycerides, improving glucose homeostasis, reducing inflammation and activating Sirt1[5,6]. However, few studies investigated the effects of polyphenols on the regulation of energy homeostasis through their action in the central nervous system.

As mentioned in the introduction section, leptin is a key hormone on the regulation of energy homeostasis by signaling first order neurons in the arcuate nucleus of the hypothalamus to [7–9]. Leptin activates POMC neurons and suppresses AgRP/NPY neurons activity producing satiety and increased energy expenditure[10]. In obesity, leptin action is blunted which is reflected by increased circulating leptin levels that fail to counteract the increase in body weight, which is known as leptin resistance[11–13]. Several mechanisms are suggested to be involved in the development of leptin resistance[14]. Recent data challenged the classic idea that there is no response to leptin in obesity, In fact it has been shown that obese animals are able to respond to endogenous leptin, despite not responding to exogenous administration of this hormone[15]. Therefore, this points out that the so called “leptin resistance” does not occur at the level of

leptin receptor rather than altered downstream signaling, that might involve the overexpression of negative regulators such as SOCS3 among other mechanisms[16]. Therefore, the aim of this thesis was to evaluate the potential of polyphenols to improve leptin sensitivity in the hypothalamus in obesity and determine by which mechanisms these compounds modulate leptin signaling pathway.

Previous research demonstrated that a high, pharmacologic, dose of grape seed proanthocyanidins (GSPE) is able to reduce body weight by inhibiting food intake and boosting energy expenditure. The described mechanisms by which GSPE exerts these effects include the upregulation of β-oxidation and lipolylitic genes, particularly in subcutaneous WAT[17] and the satiating effects of GSPE involving the action of the enterohormones together a delayed gastric emptying[18]. These are promising results for the use of GSPE as anti-obese compounds. However, further studies limiting the dose of GSPE to dietary doses are still necessary. Moreover, the satiating effects may be produced by additional mechanisms that regulate food intake and energy homeostasis such hypothalamic leptin signaling. Hence, we performed an experiment that consisted on the first induction of obesity to animals using a cafeteria diet and after 10 weeks, when animals achieved at least a 10% increase on body weight, were supplemented with 25 mg/kg for a total of 3 weeks **[Chapter 1]**. This dose corresponds to a human intake of 284 mg GSPE/day by converting animal doses to human equivalent doses (HED) using the body surface area (BSA) normalization method and estimating the daily intake for a 60 kg adult[19]. The human intake of proanthocyanidins ranges from 95 to 200 mg/day[20–22]. Thus, GSPE doses administered in this study simulate the human proanthocyanidin intake.

The effect of GSPE on central leptin signaling was evaluated using rat hypothalamus and determining the expression of genes that regulate leptin signaling pathway and downstream effectors. Firstly, hypothalamic pSTAT3 protein levels were determined as one of the gold standard to assess leptin cascade activation *in vivo* (besides responses to food intake and body weight after leptin administration)[23]. The results show that obese animals supplemented with GSPE increased pSTAT3 levels compared to the cafeteria control group reaching similar values to the ones depicted by the lean group. Furthermore, *Socs3* levels were also normalized as well as *Ptp1b*. According to these changes *Pomc* levels were remarkably enhanced by GSPE treatment,. Therefore, the data obtained suggest that GSPE treatment increased leptin signaling in the cafeteria-diet fed rats. Importantly, POMC expression is linked to the induction of anorexigenic signals[24] and animals supplemented with GSPE showed a significant reduction on food intake and a tendency to reduce adiposity after the treatment. This increased of leptin sensitivity in the hypothalamus of obese rats supplemented with GSPE was not explained by higher *Obrb* expression. Therefore other mechanisms were assessed. Diet induced obesity and leptin resistance is characterized by increased hypothalamic inflammation and ER stress[25,26]. Notably, in our experiment GSPE reduced hypothalamic inflammation, depicted by the decrease in *inos mRNA* levels, suggesting this effect could be one of the mechanisms involved in the improvement of leptin signaling induced by GSPE. Furthermore, it has been reported that Sirt1 activity is able to increase leptin sensitivity[27,28] which lead us to evaluate *Sirt1* expression in the hypothalamus. Interestingly, *Sirt1* was overexpressed by GSPE treatment meaning that proanthocyanidins could also improve leptin sensitivity by this mechanism. The fact that proanthocyanidins are able to cross the BBB[29] strengthens the idea that GSPE may have a direct action in the hypothalamus.

Additionally, leptin actions in the periphery play an important role on the development of leptin resistance[30]. Hence, we further investigated the capacity to modulate the leptin signaling cascade in these animals. Remarkably, GSPE supplementation normalized the expression levels of the leptin cascade components in mesenteric white adipose tissue and muscle. Therefore, proantohocyanidins were also able to improve leptin sensitivity in the periphery.

Altogether these results indicate that GSPE increased central leptin sensitivity that was associated to a significant reduction of food intake. However, GSPE supplementation did not correct hyperleptinemia, body weight and adiposity at this dose and time length.

For this reason we next focused on other phenolic compounds that could complement GSPE effects modulating leptin signaling and successfully reducing body weight **[Chapter 2].** Many studies indicate that resveratrol and anthocyanins have the capacity to reduce body weight (cites). Thus, we planned a first preliminary study to test the capacity of these types of polyphenols to modulate the leptin signaling pathway in the hypothalamus using healthy mice. The results showed that the daily treatment of mice with resveratrol, at a dose of 100 mg/kg body weight for 15 days, increased the energy expenditure in mice and overexpressed *Obrb* in the hypothalamus whereas an anthocyanin rich extract did not produce significant changes. According, some *in vivo* studies show that anthocyanins have anti-obesity properties by decreasing circulating leptin levels[31] through the modulation of adipocytokine secretion and lipid metabolism in the adipose tissue[32,33].

These results indicate a higher potential of resveratrol over the anthocyanins to improve leptin signaling in the hypothalamus and to reduce energy

homeostasis. Moreover, resveratrol was an interesting candidate since, besides some reported effects on the reduction of body weight and fat mass through the modulation of lipid metabolism in the periphery[34], one study[35] also indicate its ability to modulate hypothalamic leptin signaling in . Specifically, Franco et al. [35] reported an increase of pSTAT3 levels in the hypothalamus and a reduction of hyperleptinemia in maternally programed HFD animals at 150 days of age after consuming a high-fat diet supplemented with 30 mg resveratrol /kg body weight for 30 days. Despite these effects, only adiposity was reduced due to resveratrol treatment whereas body weight and food intake remained unaltered[35].

Due to the wide range of resveratrol doses used in animal studies studies[36], we decided to test the effect of a low, moderate and high dose of resveratrol using a diet-induced obesity model. Therefore, obese animals were supplemented with 50, 100 and 200 mg/kg of resveratrol which in HED correspond approximately to 486 mg, 970mg and 1.95 g resveratrol/day for an individual of 60 kg[19]. These doses have previously been tested in human clinical trials and were generally well tolerated[37,38]. The results demonstrated that the highest dose of resveratrol effectively corrects hyperleptinemia produced by the cafeteria diet and increased hypothalamic pSTAT3, suggesting that leptin sensitivity was improved. Taking into account that in normal conditions leptin signaling through ObRb produces the phosphorylation of STAT3[39], we found out that pSTAT3 levels in the hypothalamus referred to plasma leptin levels is a good ratio for leptin sensitivity estimation . Interestingly, the highest dose of resveratrol produced a significant reduction of body weight, increased 24h energy expenditure and lipid oxidation without decreasing food consumption. Therefore, the high dose of resveratrol clearly exerted anti-obesity effects that were mediated, at least partly, by the restoration of leptin sensitivity in the

hypothalamus of obese rats. Although *Sirt1* expression in lean mice was not affected by resveratrol supplementation, further investigations in this obesity model are needed to clarify the mechanism by which leptin sensitivity is improved by this polyphenol.

The findings obtained in chapter 1 and 2 suggest that a mixture of 25 mg GSPE with 200 mg resveratrol by kg of body weight has the potential to successfully reverse obesity in obese animals since both types of polyphenols act as leptin sensitizer in the hypothalamus and GSPE modulate satiety whereas resveratrol induced energy expenditure, all of them key factors for body weight regulation.

Fruits are an important polyphenol source in the human diet[20–22] and their consumption is highly recommended to maintain health. Nowadays the consumption of seasonal and out-of season foods has generated some debate related to health effects and ecosystem sustainability[40]. In this sense, the Xenohormesis Hypothesis states that plant molecules, such as polyphenols, are able to modulate mammalian physiology when consumed. These cues warn about environmental conditions where the plant developed allowing animals to adapt to the situation[41].. Thus, we consider relevant to assess the effects produced by seasonal fruits rich in polyphenols when they are consumed out of season regarding their impact in the central leptin system and obesity **[Chapter 3]**.

Leptin secretion is influenced by the light-cycle in seasonal animals which means the levels of this hormone fluctuate in accordance to a circannual rhythm[42]. It is common that seasonal mammals develop leptin resistance as an adaptive response to survive to changes on food availability[43]. Therefore, leptin resistance is not an isolated phenomenon that occurs in obesity, it is rather related to several changes that occur at hypothalamic level in different

situations such as malnutrition, seasonal changes and reproduction, some of them affected by photoperiod[44]. Seasonal animals increase their body stores and develop hyperleptinemia during summer and spring and gain less weight and decrease serum leptin during winter and autumn [43,45].

For this study we used the Fisher-344 rat strain because its sensitivity to photoperiods (cita). Rats were placed in long-day (LD) (18:6h light:dark cycle) or short day (SD) (6:18h light:dark cycle) to simulate spring or autumn, respectively. Numerous studies report the metabolic protective effects of grape, grape by-products, cherries or their pure compounds[33,46–50], thus we have chosen grape and cherry as representative fruits of autumn and spring, respectively. In addition, this study was performed in both lean and dietary induced obesity models.

Our results show that lean animals placed in SD photoperiod displayed decreased fat mass and serum leptin, despite no changes in body weight were observed. This data agrees with other studies using the same rat strain[51,52]. Moreover, animals in SD showed a lower energy balance attributed to increased energy expenditure since no changes in cumulative food intake were detected. The gene expression of the components involved in leptin signaling pathway in the hypothalamus were not affected by photoperiod in lean animals. On the other hand, animals that consumed an obesogenic diet loose the photoperiodic regulation of fat mass but also showed a decreased energy balance in SD, in this case as a consequence of a reduction on the cumulative food intake. Contrarily to lean animals, in the obese model the photoperiod did not affect circulating leptin. However, central leptin system was affected, overexpressing *Socs3* and *Agrp* during SD. Seasonal leptin resistance in seasonal rodents and sheep is characterized by increased SOCS3 during LD[43,44] and some studies indicate a downregulation of AgRP using Fisher 344 rats[51]. Thus, these discrepancies

between our and other results could be mainly due to different time length to photoperiod exposition that was longer in our experiment.

In view of the previous results, we attempted to understand how the consumption of seasonal fruits in different photoperiod could affect central leptin system and energy balance in both lean and obese animals. In lean rats, grape and cherry consumption decreased the energy balance in a photoperiod independent manner. Interestingly, this effect was consequence of a reduced cumulative food intake in rats consuming grape whereas an increased energy expenditure was observed in animals consuming cherry. Furthermore, both fruits increased *Pomc* mRNA levels in SD, which could explain the drop in energy balance observed in animals exposed to SD. Besides, cherry intake increased *Obrb* expression in SD suggesting that cherry consumption increases the central leptin sensitivity. However, Socs3 and *Agrp,* which are markers of attenuated leptin signaling, were overexpressed by cherry consumption in both LD and SD photoperiods. Thus, further studies are needed in order to clarify the exact effect of cheery consumption on leptin sensitivity.

No effects of grape intake were observed in obese animals in either photoperiod. In contrast, cherries were very effective modulating the leptin system in obese rats in a photoperiod dependent mode. Specifically, cherry consumption produced an anorexigenic response in SD depicted by reduced cumulative food intake as well as downregulation of *Agrp* and *Ptp1b* meaning that leptin sensitivity was increased by this fruit in a photoperiod dependent manner. In addition, cherry intake effectively modulated receptors expressed in second order neurons, outside of the arcuate nucleus, *Mc4r* and *Npy1r* when was consumed at SD Therefore, cherry modulated leptin system when it was consumed out of season in both lean and obese animals . Despite cherry consumption did not modulate the central leptin system when was consumed at

LD, its intake also reduced the energy balance and RQ in this photoperiod. Thus, other mechanism, instead leptin system modulation, could be induced when cherry is consumed in season such as enhanced energy expenditure at brown adipose tissue level. Importantly, in this study cherry modulated *Agrp* gene expression in photoperiod independent manner in both lean and obese animals, which could mean that AgRP neurons were more sensitive to cherry polyphenols compared to Pomc neurons.

Interestingly, it has recently been demonstrated by researchers in Xu Laboratory that around 60-70% of AgRP neurons are located outside the blood brain barrier which makes them more susceptible to metabolic changes, since they are expose to blood borne substances[53,54]. Then, we hypothesize that although polyphenols are able to cross the BBB[29], AgRP neurons are a good putative target for polyphenols since polyphenols circulating in systemic blood can directly interact with these neurons. In order to go in depth in the regulation of AgRP neurons, this part of the thesis **[Chapter 4]** was performed in Professor Allison W. Xu laboratory, in the Diabetes Center at University of California, San Francisco.

AgRP neurons are activated by fasting[55,56]. However the specific mechanisms that modulate AgRP activity are not completely clarified. Recent data showed that AgRP neurons are activated by Gs protein-coupled receptors (GPCRs) which triggers a sustained increase in food intake[57]. Adenosine signaling plays an important role in the CNS modulating neuronal activity[58] by specifically binding to its receptors. Interestingly, adenosine receptor 2B (A2B receptor) is a Gs-coupled GPCR, which means that adenosine binding will produce stimulatory effects[59,60]. Little is known about the role of this receptor in the CNS. However, it has been reported that an acute ethanol dose promotes an increase of AgRP immunoreactivity in the ARC of C57BL/6J mice[61] and, at the

same time, ethanol intake raises the extracellular adenosine levels in the brain[62]. The mechanism by which alcohol triggers AgRP activation has not been explained yet, but the data suggest a possible link between adenosine levels and AgRP activity. Hence, we hypothesize that extracellular adenosine might bind the A2B receptor to sustain the firing in a positive feedback loop and to activate cellular responses in AgRP neurons.

For this reason, we aimed to determine the role of A2B receptor in the nervous system and in AgRP neurons. The results obtained show that mice with a deletion of A2B in the nervous system reduced refeeding phenotype after 24-hour fasting, whereas deletion of A2B in AgRP neurons did not produce any change during refeeding. This could mean that A2B receptor exerts its effects through other pathways that also regulate feeding, such as neuropeptide Y (NPY) release or POMC neurons activity because of the reduced *Agrp/Npy* and *Agrp/Pomc* gene expression ratios observed in these mice.

Furthermore, mice with A2B deletion in AgRP neurons and challenged with 8-hour fasting at the beginning of the dark-phase, showed reduced cFos expression. Remarkably, AgRP neurons are activated by fasting and stimulate feeding at the beginning of the dark phase [55,63,64]. Therefore, our results indicate that mutant mice presented a reduced neuronal activity in the ARC nucleus, where AgRP cell bodies are located. In addition, AgRP neurons may exert their effects through inhibitory projections to the dorsomedial hypothalamus (DMH) and the paraventricular nucleus (PVN)[63]. In this study, cFos expression is also observed in neurons located in the DMH. Assuming that the control mice may have increased AgRP activity, since they show increased cFos expression, in turn they could exert a inhibitory effect to DMH neurons. However, quantification of cFos positive neurons in the DMH did not differ between mutant and control mice. Therefore, despite AgRP neurons in the ARC nucleus

are less activated, neurons that receive inhibitory inputs from AgRP neurons are not affected. Further research should be performed to study the effects in downstream neurons such as the ones in the DMH.

Altogether the results obtained in this study allows to suggest that during fasting AgRP neurons increase their firing rate and produce ATP in order to obtain energy. Adenosine is transported outside of the cell through the ENT1 transporter and, subsequently, extracellular adenosine levels rise. In this circumstance adenosine may bind A2B receptor and activate the transcription of AgRP gene which, in turn, leads to a positive feedback loop that keeps these neurons activated. This study brings novel insights about the modulation of AgRP neurons through A2B receptor which is a potential target to prevent or correct metabolic diseases. Future research on the field of bioactive compounds such as polyphenols may take advantage of these findings.

In summary, different classes of polyphenols showed the ability to ameliorate energy homeostasis in obesity partly through the modulation of leptin signaling and improving leptin sensitivity in the hypothalamus. Therefore, these compounds are promising candidates for the design of functional foods that help to reduce obesity and the associated risk factors.

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V. CONCLUSIONS

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POLYPHENOL EFFECTS ON CENTRAL LEPTIN SENSITIVITY IN OBESITY
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1. **Chronic consumption of a dietary dose of proanthocyanidins normalizes pSTAT3 levels and overexpresses *Pomc* in the hypothalamus of obese rats indicating that proanthocyanidins sensitize first-order neurons to leptin in obesity.** This improvement of leptin signaling emerges, at least to a certain extent, from the neuroprotection against inflammation and enhanced *Sirt1* expression induced by proanthocyanidins in the hypothalamus of obese animals.

2. **Chronic consumption of a dietary dose of proanthocyanidins produces an anorexigenic response in obese rats that is associated to the overexpression of *Pomc*.** Thus, the anorexigenic effect of proanthocyanidins is mediated, at least partly, by the activation of POMC neurons in the hypothalamus.

3. **Chronic consumption of a pharmacological dose of resveratrol represses SOCS3 expression in the hypothalamus and increases the ratio pSTAT3/serum-leptin indicating that resveratrol is able to partially recover central leptin sensitivity in obesity.** This improvement of central leptin sensitivity could be behind the decreased body weight and fat mass as well as the increased energy expenditure observed in obese rats treated with this pharmacologic dose of resveratrol.

4. **The consumption of grape, a seasonal fruit representative of autumn, modulates hypothalamic *Pomc* expression in a photoperiod-dependent manner in lean animals, overexpressing *Pomc* in the hypothalamus of rats placed at short-day photoperiod.** Remarkably, this overexpression of

Pomc is associated to lower cumulative food intake and reduced energy balance in lean rats consuming grape in short-day. Thus, grape consumption improves leptin sensitivity in lean rats when it is consumed in season

5. **Grape consumption together with an obesogenic diet prevents the overexpression of AgRP induced by this obesogenic diet in rats placed at short-day photoperiod**. However, this protection exerted by grape consumption was not reflected in the energy balance or food intake.

6. **The consumption of cherry, a seasonal fruit representative of spring, modulate hypothalamic *Pomc* and *Obrb* gene expression in a photoperiod-dependent manner in lean animals, increasing their expression in the hypothalamus of lean rats placed at short-day photoperiod.** Notably, this overexpression of *Pomc* and *Obrb* is associated to higher energy expenditure and reduced energy balance in lean rats consuming cherry in short-day. Thus, cherry consumption improves leptin sensitivity in lean rats when it is consumed out of season.

7. **Cherry consumption together with an obesogenic diet modulates the central leptin system in a dependent-photoperiod manner, decreasing hypothalamic *Socs3, Ptp1b, AgRP, Mc4r* and *Npy1r* gene expression in short-day photoperiod.** Thus, cherry consumption regulates leptin sensitivity in obese rats when it is consumed out of season, like in lean animals. However, cherry consumption decreases energy balance in both long- and short-day photoperiods in rats fed the obesogenic diet, pointing out that other mechanisms contribute to the stimulation of energy expenditure induced by cherry consumption.

8. **Adenosine receptor 2B plays a significant role on the activation of AgRP neurons. This fact can be concluded because:**

   a. **Deletion of A2B receptor in the nervous system reduces the refeeding phenotype in association with the decreased *Agrp/Npy* and *Agrp/Pomc* ratios.**

   b. **Deletion of A2B receptor in AgRP neurons does not produce a refeeding phenotype. However, arcuate neurons in the mediobasal hypothalamus, where AgRP neurons are abundant, are less activated.**

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VI. APPENDICES

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ABBREVIATIONS

A2B	Adenosine receptor 2B
AGRP	Agouti-related protein
ARC	Arcuate nucleus
BBB	Blood brain barrier
CD	Cafeteria diet
CNS	Central nervous system
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamic nucleus
ER	Endoplasmic reticulum
GSPE	Grape seed proanthocyanidin extract
HFD	High-fat diet
LD	Long-day photoperiod
MC4R	Melanocortin 4 receptor
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y receptor Y1
OBRB	Long form of the leptin receptor
PAC	Proanthocyanidin
POMC	Proopiomelanocortin
PTP1B	Protein-tyrosine phosphatase 1B
SD	Short-day photoperiod
SIRT1	NAD-dependent deacetylase sirtuin-1
SOCS3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3

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ABOUT THE AUTHOR

Maria Ibars Serra was born on the 9th of March, 1989, in Sabadell, Catalonia. After completing secondary school studies in 2008, she chose the bachelor's degree in Biology and Biomedical Sciences at Universitat Autònoma de Barcelona (Barcelona). Her main field of interest was the clinical part of biochemistry, endocrinology and neurobiology. During her bachelor's she did three internships. One at CERBA-Keynova (Barcelona) under supervision of Dr. Pilar Grao, one at the Clinical Immunology Department in KCUS in Bosnia and Herzegovina under supervision of Prof. Jasenko Karamehic and the last one at Ulster University in the Northern Ireland Centre for Food and Health (UK) supervised by Dr. Chris Gill . There, she participated for a period of one year in Ileostomy Berry project which was related with the study of polyphenols from raspberries in ileostomy patients and assessment of antigenotoxic activity in colon cancer cells and SWAFAX project which studied the seaweed derived anti-inflammatory agents and antioxidants. In September 2013 she undertook the MSc degree in Nutrition and Metabolism at Universitat Rovira i Virgili (Tarragona) in the Department of Biochemistry and Biotechnology and she was awarded a research grant to study the effect of proanthocyanidins on the modulation of hypothalamic leptin signaling in obesity in the Nutrigenomics Research Group supervised by Prof. Cinta Bladé and Dr. Gerard Aragonès. After completing her Master's thesis she was selected as a PhD student as a continuation of the project started during the Master's and was awarded a FPI predoctoral fellowship by the Spanish Government. During her PhD, she was awarded a Mobility grant by the Spanish Government and she was involved in a project at University of California San Francisco in Professor Allison W. Xu laboratory where she studied the regulation of neurons involved in feeding and energy homeostasis. The results of this research are presented in this thesis.

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Ardid-Ruiz A, **Ibars M**, Bladé C, Aragonès G, Suárez M. Resveratrol rescues hepatic leptin signal transduction via STAT3 pathway in a cellular model of fat accumulation induced by palmitic acid. Presented at XXVIIIth International Conference on Polyphenols. Vienna, Austria (2016).

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RESUM

L'obesitat és un problema de salut en augment i suposa un risc per al desenvolupament de malalties cròniques. Les estratègies per reduir i prevenir l'obesitat no han sigut satisfactòries el que fa necessari el desenvolupament d'alternatives terapèutiques. Nombrosos estudis en animals i humans demostren que els polifenols tenen propietats protectores en front a trastorns metabòlics per la qual cosa aquests compostos bioactius poden ser útils per a reduir l'obesitat i malalties metabòliques associades. La leptina és una hormona encarregada de la regulació del balanç energètic al sistema nerviós central on activa les neurones POMC i inhibeix les AgRP produint sacietat i promovent la despesa energètica. No obstant això, l'acció de la leptina en l'obesitat es troba afectada. L'objectiu principal d'aquesta tesi és identificar polifenols que millorin la sensibilitat a la leptina en situacions d'obesitat i que tingui com a resultat la pèrdua de pes. En aquesta tesi demostrem com el consum crònic d'un extracte de pinyol de raïm ric en proantocianidines millora la senyalització de la leptina a través de l'augment de l'expressió gènica del neuropèptid POMC i redueix la ingesta energètica sense mostrar canvis al pes corporal. A més, s'ha investigat el potencial d'altres polifenols amb efectes complementaris a les proantocianidines per tal d'estimular la pèrdua de pes. Els resultats presentats mostren que el resveratrol és efectiu reduint el pes i el greix corporal i la hiperleptinèmia en animals obesos, actuant com a agent sensibilitzador de la leptina. D'altra banda, es demostra el potencial de fruites estacionals riques en polifenols en la modulació de la senyalització de la leptina en condicions normals i d'obesitat. Finalment, s'explica el rol d'una nova diana per modular l'activitat neuronal de les neurones AgRP. Els resultats d'aquesta recerca aporten nous coneixements pel disseny d'aliments funcionals que combinin diferents compostos bioactius amb el potencial de poder ser utilitzats com a teràpia anti-obesitat.

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Polyphenol effects on central leptin sensitivity in obesity

Doctoral Thesis

Directed by Prof. Maria Cinta Bladé Segarra

And

Dr. Gerard Aragonès Bargalló

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

NUTRIGENOMICS RESEARCH GROUP

UNIVERSITAT ROVIRA i VIRGILI

Tarragona 2017

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FAIG CONSTAR que aquest treball, titulat "Polyphenol effects on central leptin sensitivity in obesity", que presenta Maria Ibars Serra per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a la Menció Internacional de Doctorat .

HAGO CONSTAR que el presente trabajo, titulado "Polyphenol effects on central leptin sensitivity in obesity", que presenta Maria Ibars Serra para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de esta universidad y que cumple los requisitos para poder optar a la Mención Internacional de Doctorado .

I STATE that the present study, entitled "Polyphenol effects on central leptin sensitivity in obesity", presented by Maria Ibars Serra for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university and that this thesis is eligible to apply for the International Doctorate Mention.

Tarragona, 30 de Juny 2017
Tarragona, 30 de Junio 2017
Tarragona, 30th June 2017

El/s director/s de la tesi doctoral
El/los director/es de la tesis doctoral
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La vida és experimentar i aprendre a arriscar-se, tot i no saber com sortirà. Crec que per això vaig endinsar-me en el món de la ciència pel qual tenia molta curiositat sense saber massa com aniria i que m'ha portat a fer un doctorat. Si hagués fet cas a aquells que es pensen que tenen una bola de vidre per poder llegir el futur i que tenen molt clar pel que vals i pel que no, ara no estaria fent el que realment m'agrada. Volia entendre més bé la vida, literalment, i estudiar Biologia. Per aquesta etapa de decisions complicades, que ara m'han conduït fins on sóc, vull agrair als meus pares, el seu suport incondicional, i per recordar-me sempre que un ha de fer el que creu que li agrada sempre que pugui, tenint en compte que la dedicació i l'esforç seran la clau per aconseguir-ho i que si no surt bé sempre hi ha alternatives. Per a guanyar coses sempre hi ha part de sacrifici i per això vaig haver de deixar la música que m'havia acompanyat des dels 6 anys, sortir de la zona de confort i anar a totes. Així va ser, en acabar la carrera vaig buscar un grup especialitzat en compostos naturals i salut i vaig marxar un any a Irlanda del Nord, a comprovar si realment el que havia estat estudiant durant quatre anys era com em pensava.

I would like to mention Dr. Chris Gill from University of Ulster (Northern Ireland Centre for Food and Health), to give me that great opportunity to participate in your research, it was the first time I heard the word polyphenols! Thank you for being so helpful during that year. Those were my first steps towards a PhD. Thanks to Noella, Tanya, Kate, Chris, Kevin and Gary; you were my family and friends thank you for your patience, lab hours doing the comet assay, laughs, Guinness and the good "craic"!! Dr. Francina Baldrick and Dr. Adrian McCann, you inspired me with your hard work, thank you for your help, I learned a lot from you.

Després d'un any de massa pluja i vent i investigant una mica, vaig descobrir el Màster de Nutrició i Metabolisme de la Universitat Rovira i Virgili. Va ser

parlar amb la Dra. Cinta Bladé que gràcies al seu entusiasme ho vaig tenir clar, faria el màster i qui sap? potser després el doctorat. Recordo la primera reunió amb el Dr. Lluís Arola, gràcies per deixar-me formar part del grup de Nutrigenòmica i per proposar-me un projecte tant interessant que he pogut continuar més enllà del màster. A partir d'aquí vaig conèixer els que han sigut els meus tutors de tesi al màster i que he tingut la sort de poder conservar durant el doctorat. Dra. Cinta Bladé i Dr. Gerard Aragonès, m'heu guiat, ensenyat, ajudat, aconsellat, i m'heu fet veure el costat positiu de les coses (dels experiments!) i reconduït en moments de dubte. Moltes gràcies, tot el que he pogut aprendre té un valor incalculable. També vull agraïr-vos la llibertat que m'heu donat a l'hora de decidir i opinar sobre els meus projectes. Gràcies a això vaig poder triar un laboratori als Estats Units per a fer l'estada i poder viure el que en diuen "the american dream" durant set mesos. Mesos de somnis fets realitat i també de "hard-work".

I could have not found a better place to do my PhD stay than University of California San Francisco in Xu Lab. In the first place, I would like to express my gratitude to Professor Allison W. Xu, for the smooth communication and to accept me in her lab with amazing views to the Golden Gate Bridge. The views and all the members of the lab helped me during those months of continuous learning. Moreover, I had a wonderful time in the lab retreat in Tahoe. It was very enriching to be part of your team. Ernie Yulyaningsih, you are an example of dedication and accuracy that is difficult to outpace. Thank you for your patience training me in the lab and the mouse house and for your help and motivation during and after my stay. Besides science, it was always nice to chat during the cookie hour or coffee breaks, and also try some of your food creations. Eirini Vagena, I appreciate your tips and assistance in the lab it was a pleasure to learn from you. Furthermore, thanks for kindly lend me your camping equipment. I cannot forget the stories you told about the bears, luckily

I didn't encounter one! Matt, this American accent was a bit hard to understand at the beginning but I guess now I can be proud to sound a little bit like a Californian. Thanks for the English tips and the entertained lab hours. We also had good moments after work, together with Adélaïde playing music or just hanging out in SF. Talking about Adélaïde, you are the pearl of these months on the other side of the Atlantic! Tuve mucha suerte de encontrarte en UCSF, desde el principio me acogiste, y cuando no estábamos en mouse house, siempre había algo interesante que hacer y es que contigo uno no tiene tiempo de aburrirse o sentirse solo y especialmente gracias, por poner otra vez un piano en mi vida. Mucha suerte con el doctorado! Keep going and...Let's go daaancing ;). Finally, thanks to Vaisse and Kolliwad Lab members, it was very constructive to assist to the Monday Lab Meetings and Journal Club. Muchas gracias Martin, por tu ayuda y por haber compartido experiencias.

John and Carmen, thank you for all the care and nice atmosphere. After long-days in the lab, it was the best place to be, you made me feel like home.

Amb tot això, després de voltar una mica pel món, he de dir que encara no he trobat un laboratori amb uns estudiants de doctorat com el nostre. Us trobaré a faltar. Per ser tant únics, esbojarrats, perquè de vegades hi ha tant bon rotllo que es fa difícil concentrar-se, i perquè amb vosaltres és més fàcil donar la volta als problemes i frustracions ocasionats per la tesi! Gràcies als companys que ara ja sou doctors i dels quals vaig aprendre als meus inicis, Pep Orellana, Aleix Ribas, Maria Margalef, Joan Serrano, Zara Pons, Ester Casanova, Anabel Fernandez i Neus Michaelo.

A les 5 fantàstiques, perquè el setembre 2017 es coneixerà com el tesis-boom, Aïda, Susu, Sarah i Maria José, això ja ho tenim! També m'agradaria donar molts ànims i sort a l'Àngela, que també està a les acaballes. Hem sigut veïnes, companyes d'habitació (i llit!) i de diverses aventures doctorals...com va dir

Humphrey Bogart, “sempre ens quedarà París”, amb les crêpes i la felicitat de quan vam anar de congrés. Tota l’energia positiva que transmets segur que l’aprofitaràs fins la recta final!!

També envio forces a tots els que esteu inmersos en la tesi en aquests moments, Kate, Andrea, Maria, Cristina, Albert, Lisard, Anna, Iris, Paula, Carlos, Miguel, Aleix, Roger, Andreu i Héctor. Tot i el gran esforç que suposa espero que gaudiu molt d’aquesta etapa per les recompenses que també té.

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Sònia, gràcies per ajudar-me amb els últims westerns, la recta final ha sigut més lleugera gràcies a la teva feina. Et desitjo molta sort en la teva carrera que tot just comença!!

Perquè quasi tot és possible comptant amb la Niurka i la Rosa, sou un exemple d’organització i eficiència i també un referent per a sobreviure al laboratori, espero sortir d’aquí i que se m’hagin encomanat algunes d’aquestes habilitats. No sé si seré capaç de combinar tot això amb la “chispa i sabrosura” que aporteu però ho tindré molt present!

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A continuació, no puc deixar de mencionar als amics que han viscut part d'aquest procés, m'he perdut moltes estones i celebracions, per la distància i la falta de temps, però sempre heu estat al meu costat, gràcies per fer que tot segueixi igual. Les més guapes, fem el ridícul, patrulla X, Anna Monzonís, Patri després d'això ho haurem de celebrar.

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Als meus pares, Josep i Emi, al Joan i al Feliu

&

To Jorrit,

So close despite the distance

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"The real discipline is not imposed.

It can only come from within ourselves."

Tenzin Gyatso

The 14th Dalai Lama

Nobel Peace Prize 1989

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CONTENT

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SUMMARY

Obesity is an increasing health problem and a major risk factor for a number of chronic diseases. Up to now, strategies to reduce and prevent obesity were unsuccessful. Therefore, novel approaches to treat obesity need to be developed. In this sense, several animal and human studies demonstrate that polyphenols protect against metabolic disorders including diabetes and cardiovascular disease. Thus, polyphenols emerge as bioactive compounds useful to reduce obesity and its associated metabolic diseases. Energy balance is regulated by leptin in the central nervous system, particularly in the hypothalamus where it activates POMC and inhibits AgRP neurons to produce satiety and promote energy expenditure. However, leptin action appears to be suppressed in obesity which is reflected by increased appetite and reduced energy expenditure. The aim of this thesis was to identify polyphenols that improve leptin sensitivity under obesogenic environments, which could ultimately result in a loss of body weight. We show that a chronic intake of a grape seed proanthocyanidin extract improves leptin signaling by increasing POMC gene expression and reduces food intake without decreasing body weight in obese animals. Furthermore, we investigated other polyphenols that could complement the effects of proanthocyanidins by enhancing body weight loss. Our results show that high doses of resveratrol effectively reduce body weight, fat mass and correct hyperleptinemia in obese animals acting as a leptin sensitizer compound. Additionally, we demonstrate the potential of seasonal fruits rich in polyphenols to modulate hypothalamic leptin signaling and downstream effectors in normal conditions and during obesity. Finally, the role of a novel target to modulate AgRP neurons activity is explained. The outcome of this research provides insights into the design of functional foods that combine bioactive compounds which could potentially be used as anti-obesity therapy.

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I. INTRODUCTION

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1. Obesity and body weight regulation

1.1. Obesity

Obesity and overweight are defined as excessive fat accumulation that increases health risks. Most recent reports indicate that 39% of adults are overweight and 13% obese [1]. Furthermore, childhood obesity rose by approximately 47% in last three decades. There is a high diversity depending on the world region and socioeconomic status. Developed countries had the highest increase rates but this trend is changing and obesity in the developing world is markedly rising [2]. The fact that obesity is associated with high all-cause mortality globally[3] and that up to now approaches to reverse obesity did not succeed [4], underscores the need to take action in preventing this disease.

Many studies focus on the causes of obesity. The genetics of obesity have been broadly investigated. Twin studies weather raised in similar environments or reared apart showed a high correlation for body mass index (BMI) and substantial genetic influence for obesity[5–7] Also,, the effect of genetic influence and family environment on the BMI of adopted children have been examined, reinforcing the ideathat genetics plays an important reole on the BMI [8]. Thes and other studies show that 40-70% of the variance corresponds to genetics and 30% to the environmental factors[7,9].Therefore, an obesogenic environment has also a critical impact on the high prevalence of obesity although it remains subject to genetic predispositions[4,10–13].

Most studies agree that the high prevalence of obesity is due to polygenic disorders rather than a single gene mutation[14]. However, the study of single gene mutations in rodents has been very useful to understand some of the mechanisms of the development of obesity[15].

The main genes involved in monogenic mutations that produce an obese phenotype are the ones encoding for leptin[16], leptin receptor (LEPR)[17], carboxipeptidase E[18], prohormone convertase I[19], proopiomelanocortin (POMC)[20] and the melanocortin receptor 4 (MC4R)[21,22]. The majority of these mutations share a common link. They are relevant to the efficacy of leptin signalling and melanocortin pathway which play a key role on body weight regulation, as demonstrated by many studies [23–26] (explained in next sections of this introduction).

However, the rapid increase in obesity in such a brief period of time suggests that genetic disorders are not the main cause[1427]. The high calorie intake, diet composition changes and decrease in physical activity are undoubtedly promoting the obesity epidemic[122]. Continuous research and development of therapeutic strategies as well as effective public health measures are needed. Thus, the mechanism of body weight regulation is essential to solve the obesity problem.

1.2. Body weight regulation

Energy homeostasis is the balance between energy intake, energy expenditure and energy storage. In most adults, although there is a large variation in daily food intake and energy expenditure, body weight is almost constant, which depends on the regulation of the balance between energy intake, in the form of food and drinks, and energy expenditure, in the forms of basal metabolism, physical activity and adaptive thermogenesis[28,29].

Adipose tissue serves as a crucial integrator of energy homeostasis, because a host of regulating hormones for energy balance can be secreted from the

adipose tissue (namely adipocytokines)[30]. Traditionally, adipocytokines are categorized as pro-storage of energy including resistin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and retinol-binding protein-4 (RBP-4), and anti-storage of energy such as leptin, adiponectin, visfatin and omentin[31] **(Figure 1).** However, the effect of some of these molecules is still controversial depending on the target tissue[32]

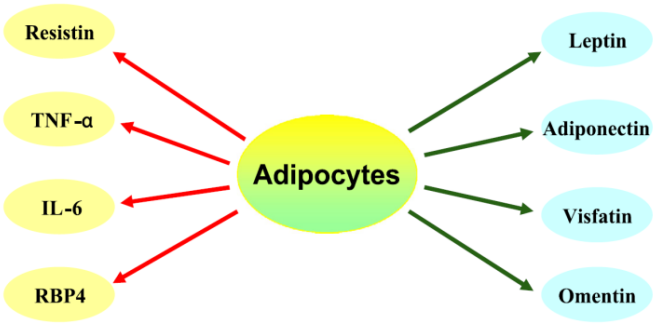


Figure 1. Adipocyte derived secreted proteins. [33]red arrows represent energy storage proteins and green arrows anti-storage proteins. Abbreviations: tumor necrosis factor-α, TNF-α; interleukin-6, IL-6; retinol-binding protein-4, RBP-4.

1.2.1 Brain regulation of food intake and energy expenditure

Most peripheral signals for energy homeostasis, mainly the adipocyte-derived regulating proteins, regulate various types of neuropeptides, which, in turn, modulate energy homeostasis and contribute to the stable state of body weight[34].

The hypothalamus and the brainstem are key areas for the homeostatic control of food intake. Additionally, the reward circuits mediated by neurons in the limbic system, responsible for eating behaviour are important for the acquisition of macronutrients and maintenance of energy homeostasis [35].

The energy regulation occurs through the central melanocortin system, which is a well-described neuronal pathway that receives hormonal and nutritional signals and produces neuronal responses to control food intake and energy expenditure[36,37].

The lesions of specific areas in the brain interfering with the signalling pathways will result in the energy metabolism disorders conducing to the development of obesity and related diseases such as diabetes[38,39].

The brain centres regulating energy homeostasis are the arcuate (ARC), ventromedial (VMH), lateral hypothalamus (LHA), dorsomedial (DMH) and paraventricular (PVN). However, the arcuate nucleus is especially important on the regulation of food intake since it integrates peripheral and brainstem signals[40].

1.2.2 Hypothalamic arcuate nucleus

The ARC is positioned at the base of the 3rd ventricle, above the median eminence (ME) where the blood brain barrier (BBB) is semi-permeable. The ME has fenestrated capillaries that selectively allow the flow of blood-borne compounds. This feature makes the neurons of the ARC highly susceptible to peripheral changes[39,40]. Importantly, the ARC contains neurons co-expressing neuropeptide Y and agouti-related protein (NPY/AgRP) and proopiomelanocortin and cocaine amphetamine-related transcript (POMC/CART) which are part of the melanocortin system[36]. These are called first-order neurons that will respond to the circulating signals and in turn project to second-order neurons located downstream[41].

POMC neurons express and synthetize POMC protein that is further cleaved producing biologically active peptides including adrenocorticotropine (ACTH), and α, β, γ-melanocyte-stimulating hormone (MSH)[37]. Among the melanocortins, the α-melanocyte stimulating hormone (α-MSH) is a peptide with an anorexigenic effect. This peptide acts as an agonist of melanocortin receptor type 3 and 4 (MC3/4R) in various hypothalamic nuclei to reduce food intake and energy expenditure[42].

Studies on the MC3 and MC4 receptors demonstrate different implications on the regulation of energy stores. Only MC4 deficiency produces severe obesity and hyperphagia, in contrast MC3 deficient mice are not obese, but present a higher feed efficiency and almost twice the amount of fat mass with decreased lean mass[43]. Human studies also show that mutations in MC4R produce a rapid development of obesity in early life[44]. Therefore, MC4R has a central role in food intake regulation whereas MC3 is involved in fat stores regulation but its mechanisms of action need further investigation[43,45].

On the other hand, AgRP neurons co-express NPY together with AgRP. Both neuropeptides contribute to increase food intake and promote weight gain, it's been reported that fasting increases mRNA levels of both molecules whereas POMC mRNA levels would be decreased in that condition. Importantly, these neurons are only found in the ARC[46]. AgRP acts as endogenous antagonist of MC4R and inverse agonist of the constitutive activity of MC4R[47]. AgRP shows long-term effects on food intake beyond 24h period to several days, not seen in other orexigenic neuropeptides and agrees with the hypothesis that melanocortins exhibit a tonic inhibition on food intake[48]. Powerful effects on food intake are also produced by NPY although in a different manner. NPY has higher effects on the first 24h compared to AgRP, inducing a short-term feeding response[49]. The signaling of NPY acts through Y (Y1, Y2, Y4, Y5, y6)

receptors and produces inhibitory responses. The Y1 receptors are involved in feeding-induced hyperphagia and together with the Y5 in the PVN they behave synergistically to control energy homeostasis[50,51].

POMC and AgRP neurons have opposite effects on energy balance and both neurons are regulated by leptin, which is the hormone that plays a central role on the regulation of energy homeostasis[52]. Olofsson et al. [53] demonstrated that 70% of AgRP compared to a 10% of POMC neurons are outside the blood brain barrier and are more sensitive to changes in the periphery such as circulating leptin levels.

2. Leptin

The word “leptin” originates from the Greek root “leptos”, which means “thin”, and it was discovered in 1994[54]. Leptin, a 16 kDa polypeptide and the product of the OB gene, is a cytokine-like circulating hormone produced and secreted predominantly by the adipocytes in the white adipose tissue (WAT), acting through their receptors (LEPRs) on specific populations of neurons in the brain, including the hypothalamic, midbrain, and brainstem neurons, and whose main function is to regulate energy stores[55]. Mirroring the body's fat stores, leptin plays a crucial role in the regulation of numerous neuroendocrine functions, from energy homeostasis to a variety of additional processes such as reproduction[56], bone function[57], cardiovascular regulation[58] and immune function [59]. Leptin decreases body weight by both suppressing appetite and promoting energy expenditure by directly targeting the hypothalamic neurons, including the increased expression of the anorexigenic peptide α-MSH, which is derived from POMC cells, and the decreased expression of the orexigenic

peptide neuropeptide Y (NPY) and agouti-related peptide (AgRP) [60–62] **(Figure 2).** Interestingly, besides the central regulation of energy balance exerted by leptin secreted from the adipose tissue, leptin produced in the stomach is gaining interest. Gastric leptin increases in response to a meal and several studies indicate that it might play a role on short-term regulation of food intake[63].

Figure 2. Neuronal mechanisms in energy homeostasis triggered by leptin. Abbreviations: arcuate nucleus, ARC; Agouti-related protein, AgRP; cocaine amphetamine-related transcript, CART; neuropeptide Y, NPY; melanocortin

receptor 4, MC4R; leptin receptor isoform B, OBRB; proopiomelanocortin, POMC; paraventricular nucleus (PVN); neuropeptide Y receptor Y1, Y1; neuropeptide Y receptor Y5, Y5.

2.1 Factors influencing leptin secretion

Various factors influence leptin secretion and expression. The most important factors are the distribution of fat and the status of its energy stores because leptin is mainly expressed in the adipose tissue, and circulating leptin concentrations in the fed state are highly correlated with the degree of adiposity[64,65]. Leptin expression also correlates with feeding and insulin level, as evidenced by the fact that insulin triggers leptin expression directly in isolated adipocytes[66] and enhances leptin levels when injected into rodents[67], as well as the discovery that decreased leptin levels accompanied with low insulin resistance states and circulating leptin concentrations increased after insulin treatment[68]. Glucocorticoids directly induce hyperleptinemia and stimulate leptin synthesis *in vitro* and *in vivo*[69]: For example, leptin expression increases in response to the chronic elevation of cortisol in humans[70]. Glucose and/or its metabolites play permissive roles in the secretion and expression of leptin[71] and leptin signalling[72], and glucose dose-dependently enhances leptin signalling and leptin sensitivity, at least in part, by attenuating the ability of AMP-activated protein kinase (AMPK) to inhibit leptin signalling. Circulating free fatty acids (FFAs) serve as suppressors of leptin secretion that may be associated with the hyposensitivity to food caused by FFAs in circulation[73]. The administration of thyroid hormone decreases leptin levels in rodents, but that is not a major determinant of plasma leptin levels[74]. Meanwhile, there is an interaction effect between leptin, sex hormones and growth hormones, which is likely to result in metabolic disorders involving sex hormones and growth retardation in obese adolescents[75]. Additionally, infections, endotoxins and some cytokines

stimulate leptin synthesis and secretion reported by several studies[76]. In summary, there are many influencing factors contributing to the level of expression and secretion of leptin that provides a wide field for further studies on leptin **(Figure 3).**

Figure 3. Diagram for leptin synthesis, secretion, biological actions, leptin resistance mechanisms and therapies[33]. Notes: +++: high correlation; ++: positive correlation; +: permissive role; − −: inhibition; −: Negative regulation; ●: on study; ▲: require further trials; FFAs: free fatty acids; ER: endoplasmic reticulum; ObRb: leptin receptor b.

2.2 Leptin signalling pathway

Plasma leptin has a central effect on the regulation of feeding behaviour and energy expenditure by activating the hypothalamic leptin receptors (LEPRs or

OBRs). The LEPR, the product of the *Lepr* gene, is a member of the class I cytokine receptor family, and has at least six splice variants, *Obra–Obrf*[77]. Notably, the long receptor OBRB mediates essentially all known physiological effects of leptin in energy homeostasis[78], because genetic deficiency of *Obrb* results in pronounced hyperphagia and morbid obesity in animals[79].

As depicted in **Figure 4**, leptin binding to the domain of extracellular of OBRB triggers the domain of activated box-1 and amino acid at 31–36 chain on OBRB, and the recruitment of the tyrosine kinase Janus kinase-2 (JAK2), resulting in the phosphorylation and activation of JAK2[80]. Activated JAK2 phosphorylates three tyrosine residues in the cytoplasmic domain of OBRB, which includes Tyr985, Tyr1077 and Tyr113[81]. Of these residues, phosphorylated Tyr1138 (pY1138) recruits signal transducer and activator of transcription 3 (a transcription factor, STAT3), which becomes phosphorylated by JAK2[82]. Phosphorylated STAT3 (pSTAT3) then homodimerizes and translocates to the arcuate nucleus in the hypothalamus, where it increases the expression and neuronal excitability of POMC and inhibits that of AGRP and NPY[83]. cause the inhibition of appetite and increasing energy expenditure[84]. This complicated physiological process is regulated by both positive and negative regulators. The negative regulators act as feedback inhibitors of leptin signalling by binding to pY985 and preventing the activation of the JAK2/STAT3 pathway, including suppressor of cytokine signalling protein-3 (*S*OCS3) [85] and protein tyrosine phosphatase 1B (PTP1B)[86].

In addition to the JAK2/STAT3 pathway, leptin mediates its effects through other pathways in the brain and the periphery. Noteworthy the phosphatidylinositol 3-OH kinasa (PI3K) pathway mediates acute leptin effects by targeting forkhead box protein O1 (FoxO1) and the mammalian target of rapamycin/S6 kinase (mTOR/S6K), factors with a relevant role on the

regulation of energy balance[87]. After the activation of the leptin receptor, JAK2 will recruit insulin receptor substrate (IRS) that will activate PI3K and produce anorexigenic effects through the phosphorylation and inactivation of FoxO1, a transcription factor that induces *Agrp/Npy* and inhibits *Pomc* expression. Similarly, PI3K will activate mTOR, a nutrient sensing molecule that contributes to leptin's anorexigenic effect via a reduction of AMPK activity[88,89]. Furthermore, another pathway that is activated by leptin involves the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinaseS (ERK). It is achieved by the SH2-containing protein tyrosine phosphatase 2 (SHP2) that binds pY985 and will recruit growth factor receptor-bound protein 2 (GRB2). All this will contribute to the anorectic effects of leptin[90].

There is limited information about the role of leptin in the periphery. OB-Ra receptors have been detected in several peripheral organs mainly in lungs, kidneys, and lymph nodes and to a lower extent in WAT, liver and skeletal muscle. Similarly OB-Rb can be found in these peripheral tissues but in lower levels compared to OB-Ra. This suggests that leptin has a peripheral action[91]. Additionally, *in vitro* studies with different cell lines and *in vivo* studies in rats demonstrated the existence of leptin signalling in peripheral tissues and, consequently, the actions that mediates at the periphery in different organs. Leptin is able to activate STAT1 and STAT3 and in a lower degree MAPK and PI3K in skeletal muscle, WAT and liver. The role of leptin at the periphery seems to be related with the regulation of adiposity and glucose homeostasis. Referring to glucose metabolism, there are evidences of a cross-talk between leptin and insulin. For instance, in skeletal muscle leptin increases glucose sensitivity and in WAT has opposite effects compared to insulin promoting lipolysis[92].

Figure 4. Leptin signalling pathway and downstream effectors[89](adapted). Abbreviations: AKT, protein kinase B; AMPK, AMP-activated protein kinase; AgRP, agouti-related protein; extracellular signal-regulated kinase, ERK; extracellular signal-regulated kinase, ERK; growth factor receptor-bound protein 2, GRB2; IRS insulin receptor substrate, JAK Janus kinase, Leptin receptor isoform b, LepRb; mammalian target of rapamycin, mTOR; PI3K phos- phatidylinositol 3-OH kinase; POMC, proopiomelanocortin; PTP1B, protein-tyrosine phosphatase 1B; S6K, S6 kinase; SH2-containing protein tyrosine phosphatase 2, SHP-2; SOCS3 suppressor of cytokine signalling 3; STAT signal transducer and activator of transcription

2.3 Leptin and photoperiod

Seasonal changes in light cycles exert an influence on animal's physiology in order to adapt to new conditions, providing an evolutionary benefit to guarantee the survival of the species. Photoperiod effects are mediated by the hormone melatonin which acts in the Hypothalamic-Pituitary-Thyroid axis and affects neuroendocrine systems that control growth, reproduction and energy balance[93].

Leptin secretion and signalling is affected by photoperiod as supported by several studies. In this sense, seasonal rodents such as Siberian hamster (*Phodopus sungorus*) and field vole (*Microtus agrestis*) develop leptin resistance as an adaptive response to overcome the challenges of different seasons. In addition, rat strains sensitive to photoperiod, such as Fisher 344 (F344) rats, increase their body weight in long-day (LD), like summer and spring, whereas reduce it during short-day (SD). Like winter and autumn[93–95]. Thus, .leptin resitance, may occur under physiological conditions such as in seasonal animals or during pregnancy.

Importantly, seasonal leptin resistance in Siberian hamster is characterized by an increase on inhibitors of the leptin signalling cascade, such as SOCS3 and PTP1B. Particularly SOCS3 is thought to be the molecular switch to adapt the physiology to the new season demands[94]. Interestingly, other species like field vole and sheep also increase Socs3 gene expression in LD, when food is abundant and easily available. This is accompanied by an increase in body adiposity that, in turns, increases plasma leptin concentration. This increase of serum leptin levels is not associated with the anorectic effect of this hormone, rather than a mechanism to induce energy storage to achieve future needs during SD season[94,95].

Photoperiod also affects neuropeptides that are regulated by leptin. Mercer *et al.* reported that Siberian hamster exposed to SD displayed a repression of *Pomc* and an overexpression of *Agrp* in the ARC, whereas *Npy* was not affected[96]. Besides, leptin gene expression in adipose tissue and OBRB in the ARC were downregulated. The possible biological meaning is the convenience of this modification to counteract the negative energy balance state that Siberian hamster is undergoing in SD periods[97]. In contrast, studies from Ross *et al*. using F344/N rats a sub-strain more sensitive to photoperiod, described an

overexpression of *Npy* and repression of *Agrp* without *Pomc* modification in the ARC in response to SD[98]. Remarkably, NPY has been shown to act as a negative regulator of growth axis. Thus, the overexprresion of NPYagrees with the growth reduction and the low mRNA levels of growth hormone releasing hormone (GHRH) displayed in this sub.stari of rats during SD. Furthermore, F344/N rats reduce the food intake in SD,which is consistent the repression of AgRP [93].

Other studies have focused on the effect of photoperiods on F344 rats fed a high-fat diet. In this situation, the gene expression of neuropeptides in the ARC nucleus and blood leptin levels are unaltered by photoperiod and. Therefore, it is remarkable that the HFD that developed obesity do not allow any changes in orexigenic and anorexigenic neuropeptides in the ARC under each of two photoperiods[93].

In conclusion the function of hypothalamic neuropeptides in different photoperiods cannot always be anticipated by its known role in energy balance. This introduces new questions of how leptin works in a physiological context involving different photoperiods. The study of these models could give valuable insights on how to reverse leptin resistance in obesity.

2.4 Leptin sensitivity

Noticeably, the reduced abilities of peripheral leptin and central OBRB to collaborate in the suppression of appetite and the promotion of energy expenditure can be taken as crucial risk factor for the development of obesity. Therefore, elevated peripheral leptin level is likely to control obesity. However, the hope that leptin might be a “miracle cure” in the treatment of obesity was

remote. Rather than being leptin deficient, most obese humans and animals have high levels of circulating leptin[99,100],and increased leptin fails to prevent the development of obesity[101–103]. Meanwhile, treatment with leptin alone in obese individuals fails to counteract common obesity[104]. This apparent leptin ineffectiveness and hyposensitivity has been identified as leptin resistance. A large number of studies demonstrated that most individuals with diet-induced obesity manifest leptin resistance characterized by increased leptin levels in the blood and decreased leptin sensitivity[105–110]**.**

In view of the great influence of leptin resistance on the development of obesity, it is an important subject for treating obesity to elucidate the mechanisms of leptin resistance. Currently, several non-genetic mechanisms have been proposed to explain leptin resistance, including defective leptin transport across the blood–brain barrier (BBB) and attenuation of leptin signalling, ER stress, inflammation, loss of sirtuin 1 activity and others[89,111]**(Figure 5).**

Figure 5. Cellular mechanisms of leptin resistance[89](adapted). Abbreviations: IL-6R, interleukin 6 receptor; IRS insulin receptor substrate, JAK Janus kinase, Leptin receptor isoform b, LepRb; myeloid differentiation primary response gene 88, MyD88; mammalian target of rapamycin, mTOR; NF-kB, nuclear factor kappa-light-chain enhancer of activated B cells; IKK-b IKK-e, inhibitors of nuclear factor kB; PI3K phos- phatidylinositol 3-OH kinase; PTEN, phosphatase and tensin homolog; PTP1B, protein-tyrosine phosphatase 1B; SFA, saturated fatty acid; SOCS3 suppressor of cytokine signalling 3; STAT signal transducer and activator of transcription; TCPTP, tyrosine-protein phosphatase non-receptor type 2; Toll-like receptor 4, TLR4; TNF-R, tumor necrosis factor receptor 1.

2.4.1 Defective transport of leptin across the BBB

The results of a large number of studies displayed that obese individuals exhibit high peripheral leptin levels but relatively lower cerebrospinal fluid (CSF) concentrations, suggesting that defective leptin transport into the central nervous system (CNS) across the BBB is part of the mechanism of leptin

resistance[112–117]. Indeed, leptin mediates the inhibition of appetite and increased energy expenditure only when circulating leptin is transported across the BBB and ultimately binds to its receptors initiating the signals for energy homeostasis in some hypothalamus neuron populations. Triglycerides act as the regulators for the process because decreasing triglycerides can strengthen the anorectic effect of leptin by enhancing leptin transport across the BBB[118]. Acute phase C-reactive protein (CRP) is also likely to contribute to leptin resistance by preventing leptin from crossing the BBB[119].

The process of leptin transport balance is regulated by two LEPRs, OBRA and OBRE. OBRA mediates leptin transport across the BBB [117,120] OBRE inhibits leptin transport by counteracting the function of OBRA[121]. Under normal conditions, the actions and number of these two receptors are in balance. Thus, subsequent studies are needed to clarify the equilibrium correlation between OBRA and OBRE under normal and obese states.

2.4.2 Attenuation of OBRB signalling

The attenuation of OBRB signalling is mainly due to two parallel molecular mechanisms including the up-regulation of suppressor of cytokine signalling (SOCS3) in the cytoplasm and that of protein tyrosine phosphatase-1b (PTP1B) in the endoplasmic reticulum. Both of them are all involved in the regulation of OBRB signalling pathway, particularly JAK2/STAT3.

SOCS3 is a critical protein that inhibits the signal transduction process of various cytokines in the body, including leptin. The most crucial event for the inhibition of appetite and increased energy expenditure is the leptin-mediated STAT3 phosphorylation. This factor increases POMC expression as well as

inhibits the activities of NPY and AGRP. By binding to Tyr985 of OBRB and JAK2, SOCS3 inhibits the leptin-induced phosphorylation of STAT3 signalling through a feedback negative mechanism. Mice with deletions of *Socs3* in the whole brain or in POMC neurons are resistant to diet-induced obesity[122,123]. In addition, the incidence of diet-induced obesity and leptin resistance is decreased in rats with hypothalamic *Socs3* silencing by RNAi[124].

When obesity is accompanied by hyperleptinemia, or decreased response to leptin administration, we use the term "leptin resistance" [125]. However, studies from Ottaway *et al.* [126]reveal that responsiveness to endogenous leptin is preserved in hyperleptinemic DIO mice therefore, maintaining leptin-mediated suppression of food intake. Their findings suggest that the reduced OBRB signalling is due to the increase in SOCS3 levels instead of decreased leptin action or a defect on OBRB functionality[126]. Referring to this, Myers et al. stated that leptin resistance could be defined as the failure of pharmacologic (exogenous) leptin to increase OBRB signalling and physiologic responses in obesity[127]. Agreeing with this hypothesis, others propose that pSTAT3 activated neurons in DIO mice would produce a constant response to endogenous leptin.. Nevertheless, the role of the STAT3 phosporylation in leptin resistance is contradictory. Whereas some groups show that DIO mice present high pSTAT3 levels, compared to lean controls, others found decreased pSTAT3 levels in obesity[87]. Why obesity develops leptin resistance in animals that fully respond to leptin is a question that remains to be answered. Therefore, new approaches are needed to study the mechanisms to increase leptin sensitivity.

PTP1B is a class 1 non-receptor protein tyrosine phosphatase , which is attached to the cytoplasmic face of the endoplasmic reticulum [128]. PTP1B binds to and dephosphorylates JAK2, thereby inhibiting leptin signalling[129]. A high-fat diet is accompanied by elevated *Ptp1b* expression, suggesting that PTP1B

may play a crucial role in the aetiology of leptin resistance[130]. Both systemic, neuron-specific and POMC neuron-specific deletions of *Ptp1b* improve leptin sensitivity and protect individuals from diet-induced obesity[131,132]. The exact correlation between the expression and activity of PTP1B and leptin resistance remains unclear; however, this particular correlation is the focus of gene therapy for leptin resistance in the future.

3. Polyphenols

Polyphenols are plant secondary metabolites that despite not being essential for growth and development they play important roles protecting against UV radiation, herbivore aggressions, providing defence against pathogens, or acting as pollinators attractants among other functions[133].

Polyphenol consumption has been linked to beneficial effects on health[134,135]. Interestingly, a 5 year follow up of PREDIMED study shows that a polyphenol rich-diet is negatively associated to body weight and obesity in elderly subjects at high-cardiovascular risk[136]. Furthermore, there are evidences that polyphenols are able to cross the BBB and may have neuroregulatory actions on energy expenditure and food intake which are negatively associated to diet-induce obesity in animal and human studies[137]. There is limited information about the mechanisms of action of these bioactive compounds on leptin signalling pathway. Therefore, it is a promising research field, since it could bring interesting insights on how to prevent and reduce obesity.

In this section the structure and classification of the main classes of polyphenols are presented. Besides, polyphenols used on the studies contained in this thesis

will be discussed in more detail from a health perspective, focusing on their effects in energy balance and leptin signalling pathway.

3.1 Structure and classification

Phenolic compounds and polyphenols have one or more aromatic ring with a hydroxyl group respectively. Flavonoids and non-flavonoids are usually referred to as polyphenols. Nonetheless, a few compounds mentioned, including phenolic acids, have only one phenolic ring. In this section the term polyphenols will be used indistinctively. They range from low to large molecular weight and are usually conjugated with sugars and organic acids. They are classified in two main groups: **flavonoids** and **non-flavonoids**. According to the number of phenol rings and the structural elements binding the rings different subgroups can be designated **(Fig. 6).** Polyphenols occur in plant-derived foods and beverages[138].

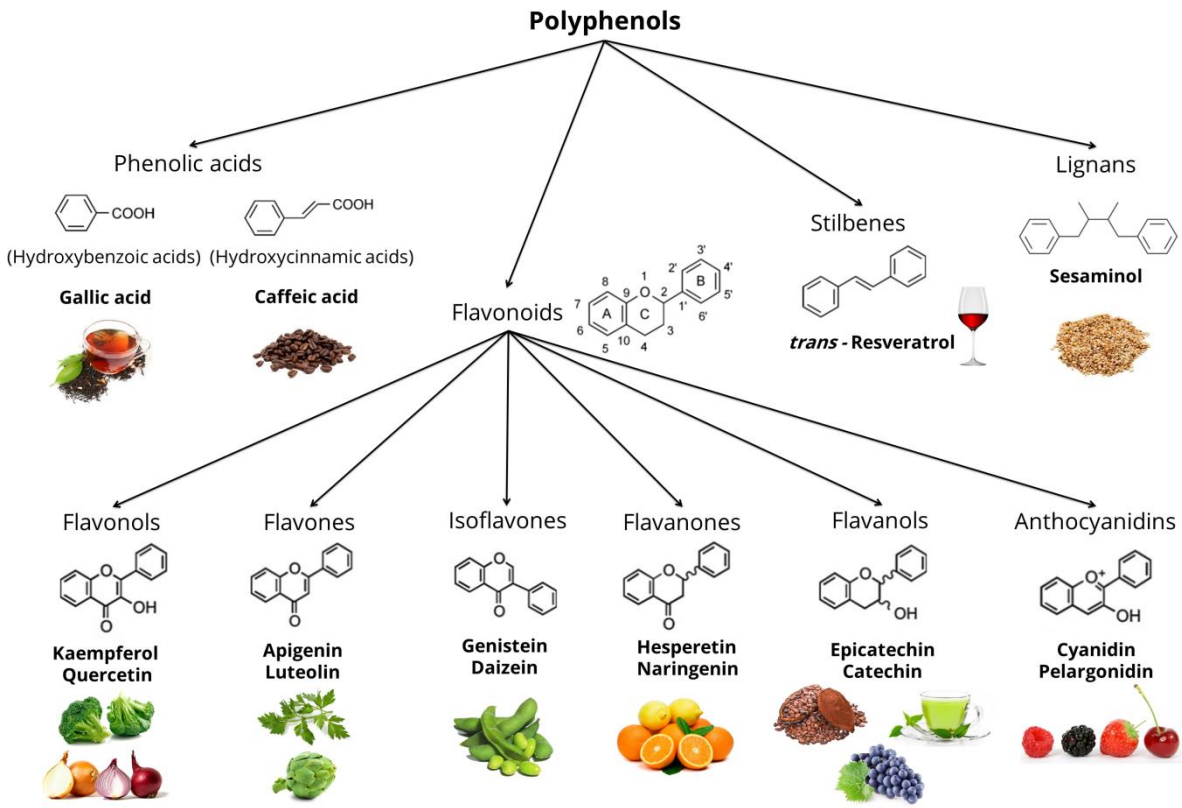


Figure 6. Basic structural skeletons of phenolic compounds. Representative molecules of each subgroup and dietary sources.

3.1.1 Phenolic acids

Phenolic acids are the main non-flavonoids that are considerably abundant in diet. They can be divided into hydroxybenzoic acids (protocatechuic and gallic acid) and hydroxycinnamic acids (coumaric, caffeic and ferulic acid). Among them, gallic acid is the most common form and is mainly found in black tea, green tea, grapes and wine, as non-sugar galloyl ester. Ellagic acid and ellagitannins are also present in a variety of fruits including raspberries, strawberries and pomegranate and are usually glycosylated. Hydroxycinnamic

acids are also named chlorogenic acids when esterified with tartaric or quinic acid. Coffee and grapes represent important sources[133,139].

3.1.2 Stilbenes

Stilbenes are substances produced by plants as a response to injury, pathogens or stress. They are found in very low amounts in human diet. Resveratrol (3,5,4'-trihdroxystilbene) is the main compound and appears as *cis* and *trans* isomers as well as conjugated with glycosides. *Trans*-resveratrol **(Fig. 7)** is the predominant form and it is mainly found in red wine and peanuts. Minor quantities have been identified in spinach, red cabbage, berries and pistachio. Although its presence in diet is scarce resveratrol is important since numerous health effects have been reported in animal studies using high doses compared to those achieved by a dietary intake[133,138].

Figure 7. Molecular structure of *trans*-resveratrol.

Resveratrol has gained interest in the obesity research field since the evidences suggesting that this molecule could have anti-obesity properties. Resveratrol is a potent activator of sirtuin 1 (Sirt1), a deacetylase involved in energy homeostasis and that is induced by caloric restriction and exercise. Therefore, resveratrol is able to mimic this conditions, that are linked to improved health and negative energy balance[140].

Several mechanisms of action have been described to explain the beneficial effects of resveratrol, involving the regulation of adipogenesis, *de novo* lipogenesis, lypolisis, thermogenesis and fatty acid oxidation in peripheral tissues[141]. However, little is known about the modulation of central energy homeostasis and leptin signalling pathway by resveratrol. Nonetheless,it has been reported that the treatment with resveratrol (30 mg/kg per day) rescue hyperleptinemia and pSTAT3 levels in the ARC of the offspring of high-fat diet, without affecting food intake [142].,Furthermore, in vitro(142) and other vivo(143) studies show reduced leptin levels with resveratrol treatment. In contrast, a mice study using intraperitoneal doses of 100 mg/kg of resveratrol reported supressed food intake and downregulation of *Npy* and *AgRP* genes[143]. Intriguingly, a meta-analysis of randomized controlled trials do not find changes on plasma leptin of obese and non-obese subjects supplemented with resveratrol [144]. Furthermore, leptin concentration in plasma of these subjects was independent of the length of treatment and the dose.[144].

The mechanisms by which resveratrol affects energy homeostasis still need to be clarified; especially, in what refers to leptin signalling pathway in the hypothalamus. For this reason, in this thesis different doses of resveratrol were used to investigate the anti-obesity effects and the modulation of leptin signalling in the hypothalamus in dose-response experiments. This topic will be addressed in **Chapter 2.**

3.1.3 Lignans

Lignans are phytoestrogens, together with isoflavones. Due to structural similarities with estrogens, they can act as agonists or antagonists of estrogen receptors. The parent forms of lignans, such as secoisolariciresinol and

matairesinol, are all converted to enterodiol and enterolactone by human gut microbiota. *In vitro* and *in vivo* studies support a beneficial role of these metabolites. Flaxseed and sesame seeds are rich sources of lignans whereas only traces can be found in grains, cereals, some fruits and vegetables[133,139].

3.1.4 Flavonoids

Flavonoids are the broadest group of polyphenols and are found all through the plant kingdom. They contain a total of 15 carbons with two aromatic rings linked through a three-carbon bridge **(Fig. 6).** The most abundant and principal subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. They are usually conjugated with sugars as glycosides[138].

Flavonols are the most extensive group of flavonoids. The main compounds are kaempferol and quercetin and are usually found forming glycosides with glucose or rhamnose. They are found in different amounts in foods depending on variety or seasonal changes and accumulate in leaves or the skin since their production is enhanced by light. Yellow and red onion are an important source of flavonols[133,138,139].

Flavones include apigenin and luteolin as principal compounds. Structurally flavones are similar to flavonols, but they lack the oxygen at C-3 **(Fig. 6).** They can undergo several modifications such as hydroxylation, methylation, gylcosylation and alkylation. Their distribution is limited to celery, parsley, some herbs and the skin of citrus fruits. Small quantities are found in rooibos tea.

Isoflavones are found in leguminous plants, mainly in soybeans, which contain considerable amounts of daidzein and genistein. Due to their structural

similarity to estrogens, they are considered phytoestrogens as the non-flavonoid lignans. Isoflavones may have pseudohormonal effects since they have the ability to bind estrogen receptors. Glycosidated forms are more common, except in fermented soy products which can be rich in aglycones[133,139].

Flavanones are present in high amount only in citrus fruit. Small amounts are found in tomatoes and some aromatic herbs. The main aglycones are hesperetin in oranges, naringenin in grapefruit, and eriodictyol in lemons. However, they are usually glycosylated, hydroxylated or methylated. The most common form is hesperetin-7-O-rutinoside (hesperidin) that is tasteless. In contrast, neohesperidoside conjugates confer the characteristic taste of bitter oranges. Interestingly, the majority of these compounds are found in the solid parts of citrus fruits, mainly the peel[133,139].

Flavanols, are structurally complex. They occur as monomers such as catechin and epicatechin that can undergo hydroxylation and form gallocatechins and be esterified with gallic acid **(Fig. 8).** Green tea and chocolate are the main sources of flavan-3-ol monomers although they are also present in red wine. Contrarily to other flavonoids they are not glycosylated in foods.

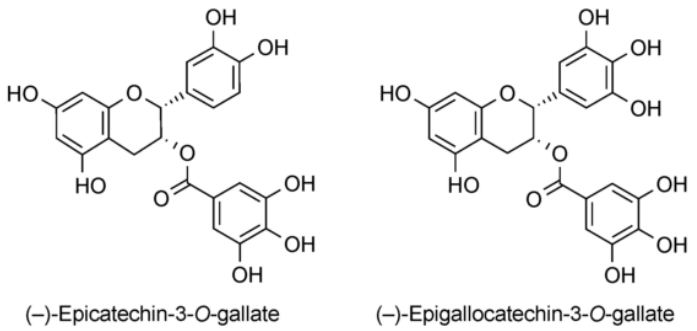


Figure 8. Basic structural skeletons of flavanol monomers[138].

Additionally, they can form oligomers and polymers known as condensed tannins or proanthocyanidins (PACs) **(Fig. 9).** The ones that consist only in (epi)catechin units are named procyanidins and are the most abundant class in plants. Remarkably, proanthocyanidins are the main source of polyphenols in human diet, being present in grapes, apples, pears, kakis, peaches and beverages such as red wine, cider, tea and beer[138,139]. Since proanthocyanidins are abundant in foods, our research group have focused years on the study of these compounds for their potential to improve metabolism and health.

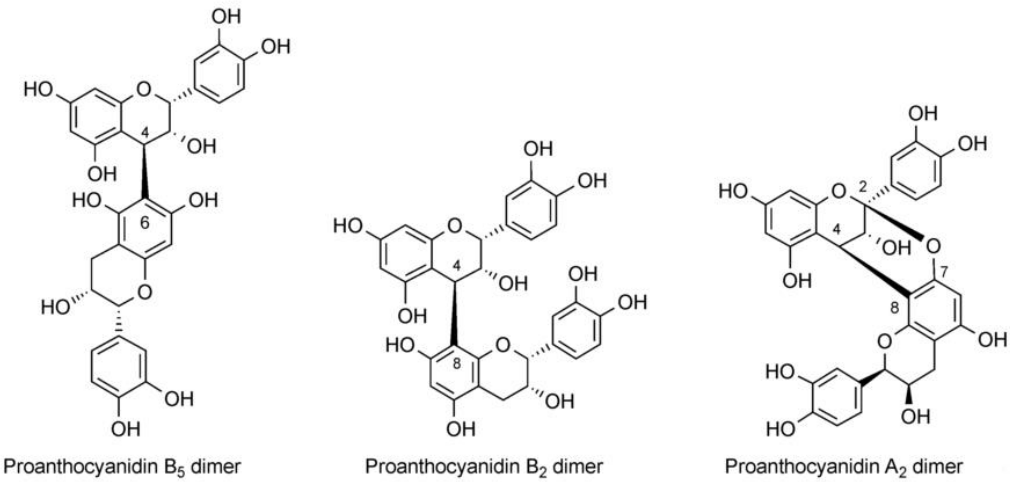


Figure 9. Basic structural skeletons of proanthocyanidins[138].

The Nutrigenomics Research Group has extensively studied the effects of PACs in metabolism, mainly in peripheral tissues. PACs have been studied for its antioxidant, anti-inflammatory and hypolipidemic effects [145]. Specially, it has been reported that a grape seed proanthocyanidin extrat (GSPE) rescues dyslipidemia in HFD fed rats by supressing lipogenesis and increasing cholesterol efflux in liver, reducing fatty acid accumulation[146]. Many of these effects are mediated by the modulation of gene expression through the regulation of the nuclear receptors farnesoid-X receptor (FXR) and Small Heterodimer Partner (SHP), the transcription factor SREBP1 and some microRNAs (mirRNA), such as mirR-33a and miR122 in liver [147,148]. Moreover, GSPE is able to induce lipolysis in 3T3-L1 adipocytes, modulate insulin-signalling pathway in diabetic rats, exerting an anti-hyperglycemic effect, improve muscle oxidative capacity in obese rats and decrease blood pressure in animals with metabolic syndrome [149–153].

According to this data, GSPE is able to improve many of the features of metabolic syndrome such diabetes, dyslipidemia and hypertension. Nevertheless, none of the previous studies have shown that GSPE could a reduce body weight at physiological doses. However, some studies have reported that a high and acute dose of GSPE is able to reduce food intake[154]. . Accordingly, GSPE could exert some of these effects partly via the modulation of leptin signalling in the hypothalamus. Thus, the study of specific mechanisms involving the main factors controlling food intake was required. This topic will be addressed in **Chapter 1.**

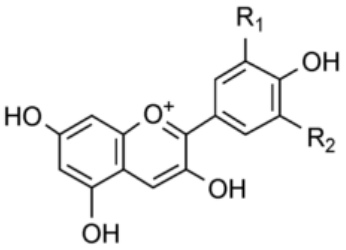
Anthocyanidins are widely distributed in plants and give the red, blue and purple colors characteristic of some fruits and flowers. Cyanidin is the most present in foods, followed by pelargonidin, delphydin, peonidin, petunidin and malvidin. To prevent their degradation, they are forming sugar conjugates in

plants, which are known as anthocyanins, and they can also be esterified with phenolic and organic acids **(Fig. 10).** Dietary intake of anthocyanins comes mainly from red wine and berries, although they are also present in leafy and rood vegetables like aubergines, onion, and cabbage and radish. Usually anthocyanins predominate in skin with the exception of cherries and strawberries where they also occur in the flesh.

Anthocyanins are particularly interesting since some studies show their obesity reducing properties. Prior et al. showed that the supplementation with an anthocyanins-rich extract from blueberry decrease body weight in mice fed a high-fat diet and fat mass mainly due to a decrease in retroperitoneal and epididymal fat pads[155]. Furthermore, serum leptin levels were also reduced. Interestingly, mice that consumed anthocyanins showed a reduction on the ratio of leptin to adipose tissue which suggests a direct effect of this extract on the production of leptin in the adipose tissue[155]. In contrast, another study from the same group, using black raspberry anthocyanins, do not reveal any of the previous effects[156].

Recently, Wu et al reported that high doses of blueberry anthocyanins decrease the body weight by a 19.4% and serum leptin levels of obese mice after 8 week treatment[157] . Another study from the same group, using a high dose of anthocyanins extracted from sweet cherry also showed a 11.2% reduction on body weight decreased serum leptin levels of high-fat diet fed mice [158]. Thus, the source of anthocyanins could be relevant on the capacity of these polyphenols to reduce body weight. Despite to that, the study of anthocyanidins could bring promising insights to target obesity. To date, the anthocyanin effects on hypothalamic regulation of leptin has yet to be investigated since the majority of studies have focused on the effects of anthocyanins on leptin secretion from adipose tissue[159]. Therefore, in this thesis an anthocyanin rich

extract, or fruits rich in these compounds, have been used to investigate their effects to modulate the hypothalamic leptin system. This topic will be addressed in **Chapter 2 and 3.**



Pelargonidin (**35**) $R_1 = H, R_2 = H$
Cyanidin (**36**) $R_1 = OH, R_2 = H$
Delphinidin (**37**) $R_1 = OH, R_2 = OH$
Peonidin (**38**) $R_1 = OCH_3, R_2 = H$
Petunidin (**39**) $R_1 = OCH_3, R_2 = OH$
Malvidin (**40**) $R_1 = OCH_3, R_2 = OCH_3$

Figure 10. Basic structural skeletons of main anthocyanidins and Cyanidin conjugated with rutin[133].

3.2 Intake, bioavailability and metabolism

Bioavailability refers to the proportion of the nutrient that is digested, absorbed and metabolized through physiological pathways. Depending on the structure of the polyphenols the bioavailability will be different. These compounds have to be hydrolyzed by intestinal enzymes or colonic microflora before being absorbed[160].

The absorption can vary among phenolic compounds. F**igure 11** displays the basic mechanism of absorption of flavonoid glycosides in the small intestine. Firstly, the sugar is cleaved by the action of lactase phloridzin hydrolase (LPH) and the aglycone may enter by passive diffusion inside the enterocyte. Alternatively, flavonoids can be hydrolyzed by the cytosolic b-glucosidase

(CBG) once they have been transported inside the cell via sodium-dependent glucose trans- porter 1 (SGLT1)[133]. The body recognizes flavonoids as xenobiotic, so they are subject to phase-II in the small intestine or later in the liver. In the small intestine, specialized enzymes such us sulfotransferases (SULTs), uridinedi- phosphate glucuronosyltransferases (UGT), and catechol-O- methyltransferases (COMTs) will produce sulfate, glucuronide and/or methylated forms[133,161]. Efflux of polyphenols back into the lumen can also occur and involves multidrug resistance proteins (MRP) and the glucose transporter GLUT2[133]. Therefore, polyphenol metabolites reaching systemic blood and tissues are different from dose present in food[160]. The majority of polyphenols reach the maximum concentration in plasma after 1-2h of their ingestion[162,163].

Figure 11. Proposed mechanisms for the absorption and metabolism of polyphenols in the small intestine. CBG, cytosolic b-glucosidase; COMT,

catechol-O-methyl transferase; GLUT2, glucose transporter; LPH, lactase phloridzin hydrolase; MRP1-2–3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenol sulfate/glucuronide/methyl metabolites; SGLT1, sodium-dependent glucose transporter; SULT, sulfotransferase; UGT, uridinediphosphate glucuronosyltransferase[133].

Polyphenols that are not metabolized by small intestine are metabolized by colonic microflora into aglycones and phenolic acids. The rate and site of absorption depends on the chemical structure, degree of glycosylation/acylation, conjugation with other phenolics, size and degree of polymerization[163]. Polyphenol determination in the ileal fluid of ileostomists after ingestion of food rich in phenolic compounds shows that important amounts arrive to the colon were they might play a physiological role[164]. These studies are critical to understand the absorption and metabolism of polyphenols and to elucidate which are the bioactive metabolites that exert beneficial effects.

After their absorption, circulating flavonoids bind to albumin and are transported to the liver where will be metabolized [165]. There are evidences that flavonols and their metabolites may also accumulate in several organs such as heart, lungs, liver adipose tissue and muscle[166]. Finally, the excretion is through xenobiotics detoxification pathway. This restricts the potential toxic effects and helping their biliary and urinary elimination[165].

4. Effects of Polyphenols on leptin signalling

Several studies investigated the effect of polyphenols on leptin signalling pathway in the hypothalamus and the peripheral tissues. Interestingly, some of them demonstrated that certain polyphenols are able to modulate leptin signalling and could potentially increase leptin sensitivity in obesity.

Next, we present a review written by members of the Nutrigenomics Research Group that focuses on bioactive food compounds that interact with leptin system and may improve leptin resistance. Importantly, among the cited compounds are polyphenols and phenolic compounds.

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Modulation of leptin resistance by food compounds

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Abstract

Leptin is mainly secreted by white adipose tissue and regulates energy homeostasis by inhibiting food intake and stimulating energy expenditure through its action in neuronal circuits in the brain, particularly in the hypothalamus. However, hyperleptinemia coexists with the loss of responsiveness to leptin in common obese conditions. This phenomenon has been defined as leptin resistance and the restoration of leptin sensitivity is considered to be a useful strategy to treat obesity. This review summarizes the existing literature on potentially valuable nutrients and food components to reverse leptin resistance. Notably, several food compounds, such as teasaponins, resveratrol, celastrol, caffeine and taurine among others, are able to restore the leptin signaling in neurons by overexpressing anorexigenic peptides (POMC) and/or repressing orexigenic peptides (NPY/AgRP), thus decreasing food intake. Additionally, some nutrients, such as vitamins A and D, can improve leptin transport through the blood brain barrier. Therefore, food components can improve leptin resistance by acting at different levels of the leptin pathway; moreover, some compounds are able to target more than one feature of leptin resistance. However, systematic studies are necessary to define the actual effectiveness of each compound.

1. Introduction

Overweight and obesity, the epidemic of the 21st century according to World Health Organization (WHO) information, are defined by chronic disease with an elevated accumulation of adipose tissue due to an imbalance between energy intake and energy expenditure, which affects both physical and psychosocial health. Currently, obesity is considered a health problem in both developed and developing countries, significantly increasing in prevalence in many nations worldwide with the expectation that more than 300 million people will be obese in 2035. However, presently, there is no successful long-term treatment for obesity, with the exception of bariatric surgery, which is expensive and risky. Thus, society needs innovative anti-obesity strategies that cause a significant reduction in food intake and/or an increase in energy expenditure with higher efficacy, safety, and selectivity. In this sense, the discovery of leptin in 1994 [1] opened a new field within the therapeutic strategies driven to combat obesity, and fortunately, leptin therapy has been found to be relevant for patients with very low leptin or leptin deficiency [2]. However, the administration of leptin is absolutely inefficient in decreasing the body weight of obese humans who are not leptin-deficient but instead have high levels of circulating leptin associated with loss of responsiveness to leptin [3]. This hyposensitivity to leptin is, currently, identified as leptin resistance, and its prevention and treatment could represent a major challenge in obesity research for the next decade.

Understanding the biological function of leptin and its receptors is an important step to identify potential dietary food compounds that could provide new strategies for restoring leptin sensitivity. Accordingly, dietary food compounds, such as amino acids, terpenoids, and flavonoids, act through a variety of mechanisms to improve leptin sensitivity. Herein, we review the roles of dietary

food compounds that have demonstrated a clear improvement in leptin signaling, as well as briefly summarize the latest advances in the molecular mechanisms involved in leptin resistance, to supply new ideas for the management of obesity.

2. Biology of leptin

Leptin, which is a 16-KDa circulating protein with 167 amino acids synthesized from the LEP gene, is mainly secreted by white adipose tissue (WAT) and acts in the brain to regulate energy homeostasis [4]. The quantity of leptin released into circulation is directly proportional to the amount of body fat in the organism, reflecting the status of long-term energy stores [5]. Apart from WAT, there are other tissues with the capacity to secrete leptin, such as the placenta, mammary glands, ovaries, skeletal muscle, stomach, pituitary gland, lymphoid tissue and brown adipose tissue. Notably, leptin is secreted in a pulsatile fashion and displays a circadian rhythm. Its levels fluctuate according to changes in calorie intake, decreasing during starvation and increasing in overfed and obese states [6]. Additionally, women have higher levels of circulating leptin than men because of their higher estrogens levels, which increase the leptin serum concentration; meanwhile, male androgens suppress the leptin serum levels [7]. In addition to sex steroids, circulating leptin levels are also modulated by other hormones, including catecholamine, insulin, glucocorticoids and cytokines[8–10].

To date, six isoforms of the leptin receptor are identified, including five short isoforms (namely LEPRa, LEPRc, LEPRd, LPERe and LEPRf) and one long isoform (LEPRb) [11]. All of the isoforms have an extracellular domain to link leptin, but only LEPRb has the complete intracellular domain required to

activate the cellular signaling cascade of leptin. LEPRb belongs to the gp130 class I cytokine receptor family and is the main receptor implicated in leptin signaling in neurons. The highest expression of LEPRb in the brain is located in the hypothalamus, particularly in the arcuate nucleus and ventromedial hypothalamus. However, LEPRb is also expressed in many extra-hypothalamic brain regions, such as the ventral tegmental area, hippocampus and brainstem [12], as well as in peripheral tissues[13].

After crossing the blood-brain barrier (BBB) through a receptor-mediated process, leptin directly targets two different neuronal populations in the arcuate nucleus, one co-expressing the proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) and the other co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) [14]. Leptin stimulates POMC/CART expression and inhibits AgRP/NPY expression, reducing food intake and increasing energy expenditure, which consequently decrease body weight. In addition, leptin also inhibits feeding by reducing the expression of the melanin-concentrating hormone (MCH) and orexins in the lateral hypothalamic area, as well as by enhancing the expression of brain-derived neurotrophic factor and steroidogenic factor-1 (SF-1) in the ventromedial hypothalamus [15].

Leptin is also implicated in the regulation of other physiological functions, such as glucose and lipid metabolism, reproduction and sexual maturation, thermogenesis, heart rate and blood pressure, the hypothalamic-pituitary-adrenal system, neuroendocrine and neuroprotection functions, thyroid and growth hormones, angiogenesis and platelet-aggregation, hematopoiesis, immune and pro-inflammatory responses and bone remodeling.

3. Molecular leptin signaling

When leptin interacts with LEPRb, the conformational change and dimerization induced in the receptor promote the activation of JAK2 and its auto-phosphorylation. Moreover, JAK2 phosphorylates three tyrosine (Tyr) residues, which include Tyr985, Tyr1077 and Tyr1138, in the cytoplasmic domain of the receptor, activating it and initiating different intracellular signaling pathways.

Phosphorylated Tyr1138 (pY1138) recruits the signal transducer and activator of transcription 3 (a transcription factor, STAT3), which also becomes phosphorylated [16].Subsequently, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to POMC and AgRP promoters, stimulating POMC expression and inhibiting AgRP [17].This is what is known as the JAK2/STAT3 signaling pathway, which is regulated by both positive and negative regulators. The suppressor of cytokine signaling protein-3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) act as feedback inhibitors of leptin signaling by binding to pY985 and preventing the activation of the JAK2/STAT3 pathway [18,19]. However, the Src-homology 2 domain 1 (SH2B1) markedly enhances JAK2 activity, which is conducive to the activation of this signaling pathway [20].

In addition to the JAK2/STAT3 signaling pathway, the activation of LEPRb also activates the extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K) pathways (Figure 1). The ERK pathway is activated through the recruitment of the protein tyrosine phosphatase non-receptor type 11 (PTP11 or also called SHP2) to pTyr985 of LEPRb [21], whereas the activation of the PI3K pathway is mediated by the phosphorylation of insulin receptor substrate 2 (IRS2) [22]. The PI3K pathway also affects the neuronal activity and neuropeptide release of AgRP and POMC neurons [22].

Notably, there is evidence that this pathway inhibits PTP1B and forkhead box protein O1 (FoxO1). FoxO1 stimulates the expression of NPY and AgRP, inhibits the expression of POMC, and blocks STAT3 action in these neurons; therefore, the inactivation of FoxO1 via PI3K allows STAT3 to bind to POMC and AgRP promoters [23].

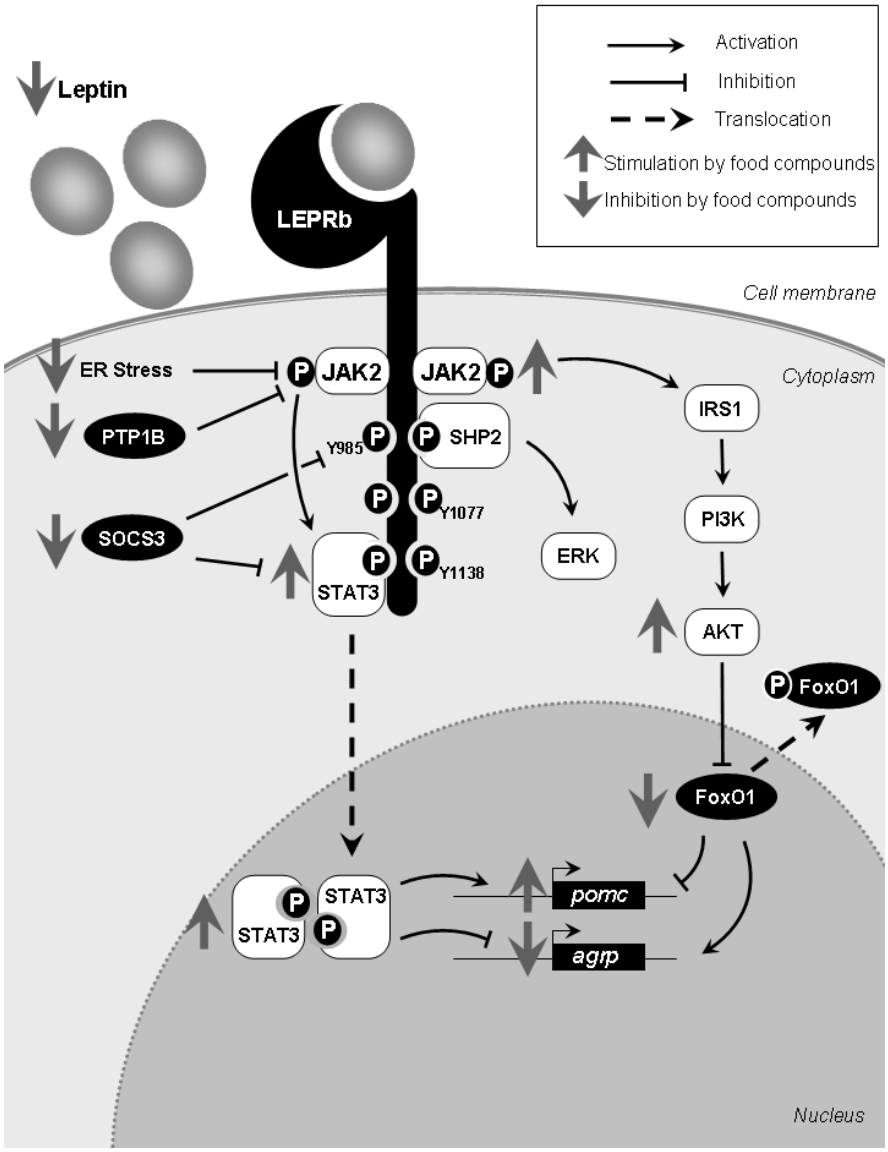

Figure 1: Schematic representation of the main components and regulators of leptin pathway targeted by food compounds. Leptin signalling pathway is

modulated by the effect of several food compounds on targets from different upstream and downstream levels.

Finally, the AMPK and mTOR pathways are also involved in the regulation of leptin signals. Specifically, leptin inhibits AMPK in several hypothalamic regions stimulating hypothalamic acetyl-CoA carboxylase (ACC) action, consequently decreasing food intake and body weight [24].

4. Mechanisms of leptin resistance in obesity

As previously explained, the term leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Although the exact mechanisms that lead to leptin resistance are still unclear, some have been proposed, including impaired leptin transport across the BBB and the disruption of the leptin signaling cascade within neurons from specific brain areas. Besides, hypothalamic inflammation, endoplasmic reticulum (ER) stress and loss of Sirtuin1 activity also promote leptin resistance.

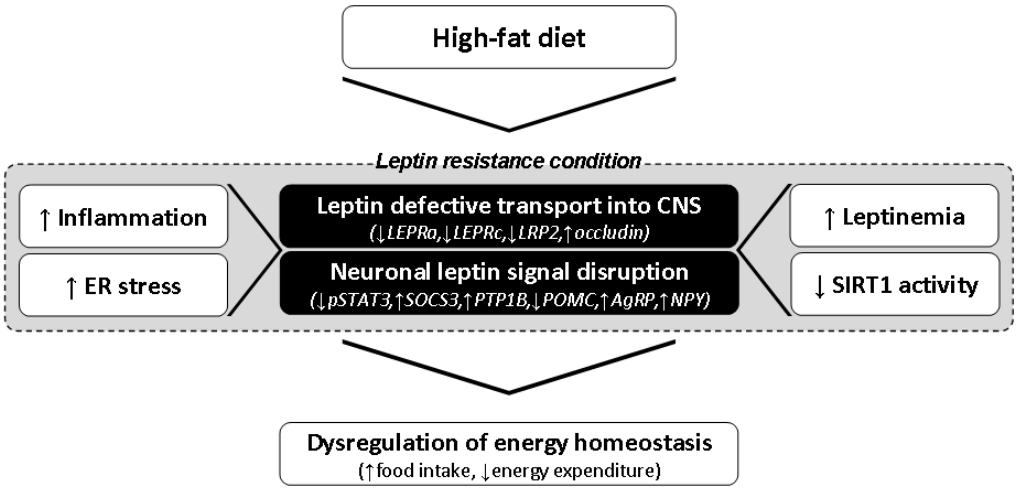

Figure 2: Schematic diagram for molecular mechanisms of leptin resistance. The molecular basis for leptin resistance is not yet completely understood but it have been mainly attributed to several mechanisms. These include reduced transport across the blood-brain barrier and the enhancement of intracellular processes that attenuate cellular signalling such as inflammation, endoplasmic reticulum (ER) stress, hyperlepinemia and sirtuin 1 dysfunctionality. CNS: central nervous system; ER: endoplasmic reticulum; SIRT1: sirtuin 1.

4.1 Defective leptin transport across the blood-brain barrier (BBB)

Most known functions of leptin within the central nervous system (CNS) are presumably mediated by leptin produced in the periphery. Therefore, leptin must be transported across the BBB. Several studies have shown that this is most likely conducted using a specific and saturable transport system that is located at both the endothelium of the cerebral microvessels and the epithelium of the choroid plexus [25]. LEPRa and LEPRc short form leptin receptor isoforms have both been shown to be highly expressed in the microvessels of the brain and are suggested to facilitate the BBB transport of leptin [26]. Furthermore, low density lipoprotein-related protein 2 (LRP2), also known as megalin, has been identified as potential novel leptin transporter protein at the choroid plexus epithelium. It functions by binding circulating leptin and transporting the hormone into the CNS [27].

Several reports in obese humans and rodents, in which the leptin levels in the cerebrospinal fluid are significantly decreased compared with control group, have suggested that leptin resistance is associated with a defect in the transport of leptin through the BBB [28].From these studies some hypotheses have been proposed as causes of this defective transport of leptin. For example, polyunsaturated fatty acids have been reported to induce peripheral leptin

resistance via an increase in the expression of hypothalamic occludin, one of the main proteins of the tight junctions, reducing paracellular transport of leptin into the brain [29]. Alternatively, an impaired expression of the transporters LEPRa and LEPRc at the BBB has also been suggested, but this aspect is quite controversial because some studies have suggested that a decreased capacity of the transporter to bind and transport leptin into the brain is the major cause of the defective leptin transport [30,31]. Additionally, some studies indicate that high levels of both triglycerides and protein C reactive (PCR) also reduce leptin transport across the BBB [29]; therefore, in states of hypertriglyceridemia and/or inflammation, such as obesity or starvation, decreased leptin transport into the CNS is expected.

4.2 Attenuation of the leptin signaling cascade in the hypothalamus

Another mechanism involved in the development of leptin resistance is the disruption of LEPRb signaling in the hypothalamus. In fact, this mechanism has been considered to be one of the leading factors and the primary defect that induces central leptin resistance. This loss of leptin signaling is mainly due to two parallel molecular mechanisms, including the up-regulation of SOCS3 and PTP1B.

As explained before, SOCS3 is a key protein that inhibits the signal transduction process of various cytokines in the body, including leptin. By binding to Tyr985 of LEPRb and JAK2, SOCS3 inhibits the leptin-induced phosphorylation of STAT3 through a negative feedback mechanism [18]. In this sense, the incidence of diet-induced obesity and leptin resistance is significantly

decreased in rats with brain-specific deletion of SOCS3 [32]. Moreover, PTP1B is a non-receptor protein tyrosine phosphatase located in the cytoplasmic face of the endoplasmic reticulum (ER) in the hypothalamic regions enriched with leptin-responsive neurons [19]. PTP1B dephosphorylates JAK2, thereby inhibiting leptin signaling. The expression of PTP1B is increased by high-fat feeding and inflammation, suggesting that PTP1B may play a crucial role in the etiology of leptin resistance[33]. Additionally, brain-specific deletions of PTP1B improve leptin sensitivity and offer protection from obesity [34]. Thus, as important targets to increase leptin sensitivity and improve obesity, SOCS3 and PTP1B have emerged as their inhibition may facilitate this process.

In addition to the JAK2/STAT3 signaling cascade, other signaling pathways such as the P13K/Akt and AMPK pathways, as mentioned above, jointly participate in the LEPRb signal transduction. Consequently, the study of regulators in these secondary pathways, including the phosphatase tensin homolog deleted on chromosome 10 (PTEN) and ACC, among others, can also potentially provide new targets for the management of leptin resistance.

4.3 Hypothalamic inflammation

Most evidence indicates that inflammation, both in peripheral tissues and the hypothalamus, is a cause of the development of leptin resistance in obesity. Specifically, the inflammatory pathway IκB kinase-β/nuclear factor-κB (IKKβ/NF-κB) is activated in the hypothalamus of rodents fed a high-fat diet (HFD) [35]. Notably, genetic inhibition of this pathway in the neurons of the arcuate nucleus [36] or AgRP neuron-specific deletion of IKKβ [37]protects from HFD-induced obesity, enhances leptin signaling and reduces SOCS-3

expression. Remarkably, the promoter of SOCS-3 has two putative motifs for binding NF-κB [37], thus connecting hypothalamic inflammation with leptin resistance.

Toll-like receptor 4 (TLR4), a membrane receptor that functions in the innate immune system, is activated by saturated fatty acids in the hypothalamus, triggering the IKKβ/NF-κB pathway [38]. Moreover, genetic and pharmacological inhibition of TLR4 restored leptin signaling in rodents fed a HFD [38]. Therefore, TLR4 is proposed to act upstream from IKKβ/NF-κB in the inflammatory process induced by a HFD in the hypothalamus.

Besides IKKβ/NF-κB pathway, c-Jun N-terminal kinase (JNK) is another pro-inflammatory signalling component up-regulated in the arcuate nucleus of murine fed a HFD [35]. However, the actual implication of JNK on inducing leptin resistance and obesity is controversial.

Recently, 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), which regulates key aspects of immunity, has been also involved in the development of leptin resistance[39]. Specifically, it has been described that 15d-PGJ2 inhibits the leptin-induced phosphorylation of STAT3 *in vitro* and the leptin-induced anorexia *in vivo* [39].

In addition to leptin signaling dysfunction, inflammatory processes are also responsible for structural changes in the hypothalamus, which alter the hypothalamic circuits. Interestingly, neuronal injury has been observed in specific brain areas that regulate food intake in obese humans and rodents [40].

4.4 Endoplasmic reticulum stress

The transmembrane proteins are synthesized and folded by ER in order to form active proteins in the ER lumen. Miss-folded and un-folded proteins are eliminated via proteasome complex. An imbalance in this process causes an accumulation of defective proteins in RE lumen, forming ER-stress and, consequently, the induction of unfolded protein response (UPR) [41]. If there is a short-term ER-stress, the UPR restores the ER homeostasis reducing the protein synthesis, increasing their folding capacity and degrading the miss- and un-folded proteins [41].

Notably, ER stress has also been suggested to be an inducer of leptin resistance. In this sense, long-term ER stress inhibits the leptin signaling pathway, leading to hyperleptinemia and obesity [37], and several studies have shown that obese animals display significant ER stress in multiple tissues (i.e., liver, adipose, and brain tissues) [42,43]. Therefore, the inhibition of ER stress increases leptin sensitivity and reduces food intake and body weight [44]. At molecular level, hypothalamic ER stress results in decreased post-translational conversion of POMC to α-MSH in HFD fed rats [45], thus connecting ER stress and leptin resistance.

The pro-inflammatory IKKβ/NF-κB pathway may act both upstream and downstream from ER stress and experimental evidences show that hypothalamic IKKβ/NF-κB pathway and ER stress positively feedback each other under conditions of overnutrition [35] further worsening leptin resistance.

4.5 Sirtuin1 activity

Recently, a reduced activity of sirtuin 1 (SIRT1), which is a NAD+dependent protein deacetylase, has been implicated in the appearance of leptin resistance [46,47]. On the contrary, the activation of hypothalamic SIRT1 increases energy expenditure and reduces food intake [46]. SIRT1 can improve leptin sensitivity by decreasing the levels of PTP1B [46], SOCS3 [46] and FOXO1 [48]. Moreover, SIRT1 activation reduces inflammation [49] and ER stress [50], two dysfunctions that also induce leptin resistance. Therefore, SIRT1 appears as a new promising target to improve leptin resistance.

5. Food compounds useful for counteracting leptin resistance

Several nutrients and food components with the ability to reverse leptin resistance have been described. This review compiles food compounds that are able to reduce hyperleptinemia, promote leptin transport across the BBB or modulate leptin cascade in the hypothalamus. However, food compounds able to reduce inflammation and ER stress or to increase SIRT1 activity but without experimental evidence of promoting hypothalamic leptin sensitivity, are not included in this review.

5.1 Food compounds controlling circulating leptin levels

Hyperleptinemia is one of the characteristics of leptin resistance, and numerous studies have been conducted to identify compounds with anti-hyperleptinemic activity. It should be noted that most of these studies are performed in animal

models, using basically rats and mice. Table 1 summarizes a list of food compounds and extracts that have shown the ability to decrease the levels of circulating leptin in *in vivo* studies.

Among the molecules listed in Table 1 numerous phenolic compounds can be found. This group of compounds, which is widely distributed in fruits and vegetables, has been shown to reduce the level of circulating plasmatic leptin in a large range of *in vivo* studies, using different types of models and treatments. This capacity has been observed by both using pure phenolic compounds, such as resveratrol [51], oleuropein [52] and myricetin [53], as well as some precursors or derivatives of phenols, such as polydatin [54] and KMU-3 [55] (a synthetic derivative obtained from gallic acid). In addition, some polyphenolic rich-extracts, obtained from natural sources such pecans [56], brown algae [57] and peach and plum juices [58], have shown the same behavior. Among all of these studies carried out with phenolic compounds, it is worthwhile to highlight that some of them have been conducted in humans. For example, fraxin, a glucoside of an *o*-methylated coumarin, and curcumin have been confirmed to reduce hyperleptinemia in overweight and obese humans [59], thus emphasizing their suitability for use in the formulation of functional foods directed towards weight reduction. Taking into consideration all of these studies, it seems clear that phenolic compounds are a very interesting family of molecules for finding new molecules from natural sources that could be used to improve hyperleptinemia and leptin resistance.

Table 1. Food compounds that reduce hyperleptinemia

Class	Compound/s	Dietary source	Experimental model	Reference
Phenolic compounds	Resveratrol	Grapes, red wine	Wistar rats	51
	O-coumaric acid	Vinegar	Male Wistar rats	107
	7-O-galloyl-D-sedoheptulose	*Cornifructus*	*db/db* mice	108
	Neohesperidin	Citrus	Male KKAyand C57BL/6 mice	109
	Polydatin	*Polygonum cuspidatum*	Male Sprague Dawley rats	54
	Myricetin	Vegetables, fruits, nuts, berries, tea, red wine	Male C57BL/6J mice	53
	Fraxin	*Fraxinus sp*	Elderly overweight/obese human	59
	Gingerol	Ginger	Male Wistar rats	110
	Oleuropein	Olives	C57BL/6JOlaHsd mice fed HFD	52
			C57BL/6 mice fed HFD	111
	Polyphenol-rich extracts	Pecan nut	Male Wistar rats	56
		Brown algae	High-fat diet induced obese mice	57
		Peach and plum juice	Zucker rats	58
		Zygophyllum album	Female Wistar rats	112

Terpenes	Thymol	Thyme	High-fat diet-induced obese mice	61
	Lycopene	Tomatoes, watermelon, papaya, orange	Male Wistar rats	63
	Teasaponin	Tea	Male C57Bl/6J mice	62
	Ginsenoside Rb1	Ginseng	Male C57Bl/6 mice	88
PUFAs	DHA, EPA	Fish oils, golden algae oil	Male C57BL/6J mice	55
	CLA	Beef, lamb, dairy foods	Human and mice	67
Soluble fiber	Pectin	Fruits	Male Wistar rats	96
Other	Protein lysates	Rice bran	A high carbohydrate diet-induced obese rats	65
	Isothiocyanates	*Moringa oleifera*	Male C57BL/6J mice	60
	Mate aqueous solution	*Ilex paraguariensis*	Male Wistar rats weaned prematurely	102

CLA, conjugated linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFAs:polyunsaturated fatty acids

In addition to phenolic compounds, other plant secondary metabolites, such isothiocyanates [60] and some terpenoids including thymol [61], saponins [62] and lycopenes [63] among others, have also been proven to be effective in reducing circulating leptin levels when administered to rodents. Furthermore, other compounds different from plant secondary metabolites have shown effects reversing leptin resistance. For example, some peptides [64] and protein hydrolysates [65], as well as polyunsaturated fatty acids (PUFAs), such as docosahexanoic acid (DHA) [66], eicosapentaenoic acid (EPA) [66] and conjugated linoleic acid (CLA) [67], also exhibit anti-hyperleptinemic action. Interestingly, some of them have been proven in humans. For example, this is the case for the CLA isomer t10c12-CLA, thus highlighting its suitability and potential to be used in treatments directed towards the reversal of leptin resistance [67].

Although there are several studies describing a reduction in the leptin level due to the effect of natural compounds, only some of them describe the mechanisms by which this reduction is produced. One of these mechanisms is through the repression of the leptin gene. Notably, cranberries [68] and KMU-3 (a synthetic gallic acid derivative) [55] have the ability to repress leptin gene expression in the adipocyte cell line 3T3 L1 (Table 1). However, to confirm the contribution of leptin gene repression on the anti-hyperleptinemic effect of a food component, it would be necessary to determine whether the expression of the leptin gene in WAT correlates with the reduction in the leptin level induced by a specific food compound in *in vivo* models. In this sense, one promising phenolic compound that can be highlighted is oleuropein, found in olives. In the study performed by van der Stelt *et al.* [52] a reduction in the levels of serum leptin is correlated with a reduction of the expression of the leptin gene in epididymal WAT of mice fed HFD supplemented with oleuropein compared to HFD mice.

Another target to take into account to reverse leptin resistance involves the CB1 cannabinoid receptor (CB1R). CB1R is the main cannabinoid receptor found in the brain, and it is present in endocrine cells and other peripheral tissues [69], such as pancreas, fat, liver and skeletal muscle tissues [70]. It is known that the use of brain-penetrating CB1R antagonists can cause neuropsychiatric side effects, but a selective targeting of peripheral CB1R results in an improved hormonal-metabolic profile without the observation of the brain secondary consequences [70]. The antagonistic action of CB1R causes an increase in appetite, insulin resistance and an increase in the hepatic lipogenesis, suggesting its implication in obesity [70]. Therefore, there are synthetic CB1R antagonists that increase the leptin sensitivity [70] or CB1R inverse agonists that reverse the leptin resistance, decreasing leptin expression and secretion by adipocytes and increasing leptin clearance via the kidney [71]. Although, to date, no natural compounds have been described as CB1R antagonists, the search and use of molecules from natural sources that antagonize this target could be one mechanism to reverse leptin resistance.

As indicated above, many food compounds have been described as anti-hyperleptinemic. However, food compounds could improve leptin sensitivity by a direct action in the brain, targeting hypothalamic leptin signalling and leptin transport across the BBB, or by a secondary effect resulting from body weight reduction, as a result of targeting peripheral tissues such as liver and adipose tissue. In this sense, serum leptin levels strongly correlate with the reduction of body weight induced by several polyphenols in rats fed a HFD [72]. Therefore this review focuses on food compounds that target hypothalamic leptin signalling and leptin transport across the BBB.

5.2 Food compounds that modulate leptin transport across the brain blood barrier

As stated in previous sections, leptin resistance can be the consequence of the impairment of its transport across the BBB, reducing leptin accessible to the CNS. Thus, increasing leptin transport across the BBB could be a good strategy to increase central leptin sensitivity. Despite that several transporters have been described; most studies have focused on the capacity of food compounds to increase the expression of the transporter megalin. Some of these compounds are listed in Table 2. Notably, increased bile acid production due to a lithogenic diet [73] produce an overexpression of megalin in mice, whereas vitamin A and vitamin D [74] are effective in producing the overexpression of megalin in several cell lines. Interestingly, synthetic PPARα and PPARγ agonists induce the expression of megalin [75]. Therefore, it can be hypothesized that food components that could act as PPAR agonists, such as PUFAs [76], coumarins [77], flavonoids [78] or even polyphenol rich extracts from fruit juices [58], could be good candidates to improve leptin transport across the BBB.

Table 2. Food compounds that modulate leptin transport across the brain blood barrier

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Fraxin	*Fraxinus sp*	Upregulates clusterin gene expression	HUVEC cells	[80]
	Quercetin	Onion, broccoli	Increases LEPRa protein levels	HUVEC cells	[82]
Vitamins	Retinoic acid	Sweet potatoes, carrot	Upregulates LPR2 gene expression	Male C57BL/6J mice	[74]
	Cholecalciferol	Fish liver oils, fatty fish species, beef liver			
Bile acids	Cholic acid	Lithogenic diets	Upregulates LPR2 gene expression	RPT, JEG-3 and EC-F9 cells	[73]
	Chenocholic acid				

LEPRa, leptin receptor isoform a; LPR2, low density lipoprotein-related protein 2 also known as megalin

In addition to megalin, other proteins can modulate the transport of leptin across the BBB. In this sense, clusterin (also called ApoJ) is a plasma leptin-binding protein for which megalin has been identified as an endocytic receptor [79]. Additionally, clusterin modulates leptin signaling in cell lines expressing LepRb [79]. Therefore, increasing the expression and/or the activity of clusterin could be a good way to increase leptin interactions with LepRb and megalin. Notably, the coumarin fraxin upregulates clusterin gene expression in cell lines [80]. As an overexpression of clusterin induces the overexpression of megalin [81], fraxin is considered a potential stimulator of leptin transport across the BBB.

It is also important to highlight the findings obtained by Indra *et al*. [82], in which the administration of quercetin, a flavonol found in many fruits, produced an overexpression of LepRa in HUVEC cells. This finding is very important due to the involvement of this receptor in the transport of leptin through the BBB. However, as quercetin is actively sulphated and glucoronidated upon intestinal uptake, further experiments should be carried out using these quercetin metabolites in this cell line to obtain stronger conclusions.

5.3 Food compounds enhancing leptin signalling in the hypothalamus

Several food compounds, most of them from vegetal sources, have the capacity of reaching the hypothalamus, where they regulate leptin signaling pathways. These food compounds can either cross the BBB or pass through fenestrated capillaries of circumventricular organs (CVO) and medial eminence (ME) and target arcuate nucleus (ARH)neurons, which express LEPR[83], i.e., POMC/CART and AgRP/NPY neurons, where they can act at different levels.

Some compounds improve central leptin sensitivity by increasing the expression of LEPRb, the phosphorylation of STAT3 and the expression of downstream components, mainly neuropeptides [84]. Additionally, some food compounds target the negative regulators of the leptin cascade, such as SOCS3 and PTP1B, reduce ER stress and/or modulate other leptin cascades, such as PI3K/Akt [85]. Food components that modulate leptin signaling activity are summarized in Table 3.

Table 3. Food compounds involved in the modulation of the leptin signaling cascade

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Tissue	Reference
Phenolic compounds	Curcumin	Turmeric	Reduces LEPRb phosphorylation Reduces STAT3 phosphorylation	Male Sprague Dawley rats	Hepatic stellate cells	[113]
	Resveratrol	Grapes, red wine	Increases STAT3 phosphorylation	Male Wistar rats	Hypothalamus	[84]
			Increases AMPK phosphorylation	Male Wistar rats	Liver, muscle	[95]
	Fucoxanthin	Brown algae	Increases AMPK phosphorylation	Male C57BL/6J mice	eWAT	[97]
Terpenes	Teasaponin	Tea	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male C57BL/6J mice	Hypothalamus	[62]
	Ginsenoside Rb1	Ginseng	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Males C57Bl/6 mice	Hypothalamus	[88]
			Increases AKT phosphorylation	Male Long-Evans rats	Hypothalamus	[94]
	Celastrol	*Tripterygium wilfordii* *Celastrus regelii*	Increases STAT3 phosphorylation Upregulates SOCS3 mRNA levels	Male C57BL/6J mice	Hypothalamus	[89]

			Reduces ER stress			
Soluble fibre	Pectin	Fruits	Increases STAT3 phosphorylation Increases AMPK phosphorylation	Male Wistar rats	Liver, eWAT	[96]
Plant extracts	Triterpenoids	*Schisandra chinensis*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[114]
	Phenolic compounds	*Cyclocarya paliurus*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[115]
	Xanthones and fllavonoids	*Cudrania tricuspidata*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[116]
Others	Caffeine	Coffee beans, tea bush, kola nuts	Increases STAT3 phosphorylation Reduces ER stress	SH-SY5Y-Ob-Rb cells	Liver, eWAT	[90]
	Leucine	Soybeans, beef	Increases LEPRb phosphorylation Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male Sprague-Dawley rats	Hypothalamus prWAT	[117]
	Taurine	Shellfish, turkey dark meat	Increases STAT3 phosphorylation Reduces ER stress	Male C57Bl/6 mice	Hypothalamus	[91]
	Safranal	Saffron	Inhibits PTPB1 activity	Male C57BL/6J mice	C2C12 myoblast	[118]

Abbreviations: AKT, protein kinase B; AMPK, 5' adenosine monophosphate-activated protein kinase; ER, endoplasmic reticulum; LEPRb, leptin receptor; PTP1B, protein-tyrosine phosphatase 1B, SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; prWAT, perirenal white adipose tissue; eWAT, epididymal white adipose tissue.

To the best of our knowledge, only leucine has been described as a food component that increases the expression of LEPRb [86]. Currently, the best marker of leptin signaling activity is the level of pSTAT3 which is the transcription factor that mediates leptin anorexigenic actions [87]. Notably, several food compounds increase the level of pSTAT3 in the hypothalamus of rodents fed a high-fat diet, such as teasaponin [62] and ginsenoside Rb1 (the main bioactive compound of ginseng) [88], which also inhibit SOCS3, thus increasing leptin sensitivity. Interestingly, resveratrol increases pSTAT3 levels in the hypothalamus concomitantly with a decreased adiposity [51]. Other compounds, such as celastrol (a pentacyclic triterpene extracted from the roots of thunder god vine) [89], caffeine [90] and taurine [91] are also STAT3 activators, and their actions seem to be mediated through the decline of ER stress in the hypothalamus.

Other studies have focused instead on negative factors that attenuate leptin receptor signaling, such as PTP1B. However, only *in vitro* studies had found potential natural compounds that would inhibit this target [92]. Therefore, the capacities of these compounds to increase leptin sensitivity in the hypothalamus are speculative and more research is needed to confirm these findings.

Current studies have shown that the activation of PIK3/Akt and AMPK pathways is essential to maintain energy homeostasis, as they are involved in the anorexigenic effect of leptin [93]. Notably, ginsenoside Rb1 activates AKT in both the hypothalamus of obese rats and in hypothalamic cell lines [94]. Moreover, it has been hypothesized that SIRT1 activation would inhibit proteins involved in leptin resistance, thus reducing ER stress [47]. Therefore, compounds that either directly or indirectly activate SIRT1 , such as resveratrol, could increase central leptin sensitivity [95].

Other studies have focused on the capacity of several food compounds, which lower body weight and visceral fat mass, to modulate leptin signaling in peripheral tissue. For example, it has been confirmed that pectin [96] and fuxocanthin [97] increase pSTAT3 levels and AMPK activity in adipocytes, thus relating it with the reversal of leptin resistance.

First order neurons POMC and AgRP, will project to second order neurons in the paraventricular nucleus (PVH), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and lateral hypothalamic area (LHA), where they produce the anorexigenic and orexigenic effects, respectively [98]. Leptin anorectic effects are mediated by CART and POMC neurons. The later produces α-MSH peptide, which binds to the MC3/4 receptor in second order neurons and will inhibit food intake [99,100]. Many natural compounds are able to increase either POMC or CART levels. In this sense, apigenin [101], ginsenoside Rb1 [88], teasaponin [62], taurine [91], leucine [86] and yerba mate extracts [102] have been shown to increase the expression of POMC in the hypothalamus and to reduce food intake and body weight (Table 4). Moreover, some of these compounds, including resveratrol [103], ginsenoside Rb1 [88] and taurine [91], are also able to inhibit the orexigenic neuropeptides AgRP and NPY, suggesting an increased effectiveness to modulate food intake and energy expenditure. However, celastrol, which reduces body weight in DIO mice, increases AgRP mRNA levels [89]. Therefore, more studies are necessary to thoroughly understand this mechanism.

Table 4. Food compounds targeting neuropeptides that regulate energy homeostasis

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Apigenin	Fruits, vegetables	Upregulates POMC mRNA levels Upregulates CART mRNA levels	N29-2 and SH-SY-5Y neuronal cells	101
	Resveratrol	Grapes, red wine	Downregulates AgRP mRNA levels Increases NPY protein expression	N29-4hypothalamic cells	51
Terpenes	Teasaponin	Tea	Upregulates POMC mRNA levels	Male C57Bl/6 mice	62
	Ginsenoside Rb1	Ginseng	Downregulates AgRP mRNA levels Upregulates POMC mRNA levels	Male C57Bl/6 mice	88
			Increases NPY protein expression Upregulates POMC mRNA levels	Male Long-Evans male rats	94
	Celastrol	*Tripterygium wilfordii* *Celastrus regelii*	Upregulates AgRP mRNA levels	Male C57Bl/6 mice	89
Others	Retinoic acid	Sweet potatoes, carrot	Increases methylation levels in the POMC gene	Leukocytes of obese men	119
	Taurine	Shellfish, turkey dark meat	Increases NPY protein expression Upregulates POMC mRNA levels Upregulates CART mRNA levels	Male C57Bl/6 mice	91

Abbreviations: AgRP, Agouti-related protein; CART, cocaine-and amphetamine-regulate transcript; NPY, neuropeptide Y, POMC, proopiomelanocortin;

5.4 Food compounds against leptin resistance: a holistic overview

In the previous sections, food compounds have been listed according to the level where they improve leptin resistance. However, it is important to take into account that some of these compounds act at several levels of the leptin pathway. Thus, some food compounds stand out from the others. For example, resveratrol, a phenolic compound obtained from the skin of grapes, is able to reduce the circulating level of leptin not only by reducing its secretion but also by promoting the activation of STAT3 in the hypothalamus, thereby increasing leptin sensitivity and to down-regulating AgRP and NYP. Others compounds that can be highlighted are teasaponin and ginsensoside, which reduce hyperleptinemia, activate hypothalamic STAT3, increase POMC mRNA levels, and inhibit SOCS3. In addition, ginsensoside increases p-FOXO1 and inhibits PTP1B.

It is also worthwile to take into account that some food compounds also modulate proinflammatory cytokines. These cytokines contribute to the development of leptin resistance, and some food compounds reduce proinflammatory cytokines, such as tesaponin and ginsensoside.

Therefore, the selection of a compound taking into consideration its influence within the entire set of mechanisms involved in the onset of leptin resistance is the best way to assure its functionality. Figure 1 shows the leptin pathway components and regulators targeted by food compounds.

6. Methods for the identification of new natural compounds with anti-leptin resistance activities.

To deeply study the mechanisms involved in the development of leptin resistance and its reversal using natural compounds, several strategies have been followed by researchers in the last two decades. These strategies involve a broad variety of techniques, ranging from the more theoretical ones, which are based in the use of bioinformatics-aided tools, to biological studies that evaluate the *in vivo* response in different models.

Traditionally, both *in vitro* cellular models and *in vivo* studies have been used to study leptin resistance. Focusing on cell models, different points of view can be followed. For example, it is very important to evaluate if the compounds being studied are able to cross the BBB and reach the selected targets. To do this, a specific cellular model using endothelial cells should be used. Until now, several endothelial cell lines are described in the bibliography for the simulation of the BBB, such as RBEC1, GP8/3.9, GPNT and RBE4 [104]. Other studies are focused on the evaluation of the signaling cascade pathway. These studies are carried out using astrocyte cultures and neuronal cell lines.

Regarding the *in vivo* studies aimed to evaluate leptin resistance, several models of metabolic syndrome using animals with a non-functional leptin pathway have been used. In general, these animals can be classified into two groups: genetically altered or diet-induced altered animals. In the first group, all of the animals (in general mice and rats) that present a modification in any of the genes involved in the onset of leptin resistance can be included. These modifications can be either from natural origin or generated in the laboratory, including knock-out animals that had a deficiency in the leptin receptor or

animals that do not produce leptin (ob/ob mice, db/db mice, etc.). In the other group, animals in which leptin resistance originates by means of the nutritional composition of the diet are included. For example, a high fat diet produces the onset of leptin resistance in animals. This last group of animal models is very interesting to simulate the most typical situation that originates leptin resistance in humans.

The most novel approach to study leptin resistance is the so-called *in silico* strategy, which is based in the use of bioinformatics. This set of techniques is focused on the search through the virtual screening of new potent molecules from natural products with the capacity to directly act in one of the mechanisms that is implicated in leptin resistance, including molecules that have the capacity to directly bind to therapeutic targets and consequently either inhibit or activate them. To carry out these *in silico* studies and successfully do the virtual screening, some prerequisites have to be accomplished. For example, the existence of crystallized structures within databases is necessary to know their 3-D conformations, which is essential in this process. The existence of ligands described for the selected targets is also required to complete the design of the pharmacophore, which contains the information of the biological conformation and electrostatic features that the ligands should have to interact with the binding site of the target proteins. To date, some bioinformatics results focusing on the reversal of leptin resistance have been carried out. For example some papers have reported molecules with the ability to inhibit PTP1B [105,106], whereas no results against SOCS3 and SH2B1 have been found. Following this virtual screening, the selected set of molecules that fulfil the prerequisites should be used in an in vitro assay to confirm the theoretical activity described.

Taking into consideration this wide spectrum of strategies, the most logical sequence of action in the search for compounds that could revert leptin

resistance is as follows: start with *in silico* studies to select potentially active food compounds, test the molecules selected in the virtual screening *in vitro* by using several cellular models, and finally test the most actives ones in *in vivo* conditions, first in animals models and then in humans, to determine their efficacies.

Concluding remarks

Leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Notably, numerous food components, mainly polyphenols, are able to reduce hyperleptinemia, suggesting that these compounds could improve leptin resistance. However, only a few studies have focused on the mechanism by which these food components could primarily restore leptin sensitivity. The results of these studies indicate that food components can reverse leptin resistance by increasing leptin access to the brain and/or activating the intracellular signaling cascade of leptin in the hypothalamus. Nevertheless, the conclusions that could be extracted from these studies are limited because they focus only on one of the levels of leptin resistance. Thus, new studies considering the activity of a particular food compound at all levels of leptin resistance and using validated markers of leptin sensitivity, such as pSTAT3, are indispensable to clearly ascribe the property of leptin sensitizer.

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II. HYPOTHESIS AND OBJECTIVES

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This PhD thesis has been performed in the Nutrigenomics Group of the Universitat Rovira i Virgili, within the frame of two research project carried out in this group: “Development of an integrated food to maintain body weight and to prevent the risk of obesity related pathologies (AGL2013-40707-R)” and “Illegitimate signaling of fruit consumption and obesity pathogenesis (AGL2013-49500-EXP).

Dietary polyphenols are recognized for their health promoting effects. Several studies report their anti-inflammatory, antioxidant, cardio- and neuroprotective properties. Moreover, great attention is being paid to these compounds since they are present in multitude of plant-derived foods. The Nutrigenomics Group focus on the potential health benefits of proanthocyanidins and other polyphenols. In particular, our group has an extensive experience studying the metabolic effects of proanthocyanidins, using a grape seed proanthoycianidin rich extract (GSPE) which was proved to ameliorate the main components of metabolic syndrome in diet-induced obesity models. Furthermore, the next goal after the identification of bioactive compounds will be to the design functional foods that prevent or treat metabolic risk factors.

To date, strategies to counteract obesity epidemic have been unsuccessful. Leptin has been a research focus since it is the key hormone that regulates central energy homeostasis and, although controverted, it is suggested that obesity coexists with ‘leptin resistance’ .In recent years, potent leptin sensitizers, such as betulinic acid, celastrol and withaferin A, have been identified. They increase leptin sensitivity either suppressing PTP1B, a negative regulator of leptin signaling, or decreasing endoplasmic reticulum stress.

Data about the ability of polyphenols to modulate leptin signaling pathway is scarce. However, the wide protective effects exerted by polyphenols on obesity

models, targeting several of the multifactorial disorders associated to obesity, allows us to **hypothesize that polyphenols or polyphenol-rich fruits might have the potential to modulate central leptin signaling in the hypothalamus and, thereby, restore leptin sensitivity.**

The **aim** of this thesis was **to assess whether polyphenols can improve central leptin sensitivity in obesity and determine the mechanisms by which these compounds affect leptin signaling pathway**. For this aim to be achieved, the following **goals and objectives** were proposed:

1. **To evaluate the ability of different polyphenols to modulate leptin signaling pathway in the hypothalamus and its downstream neuropeptides.**

   In previous studies our group demonstrated that proanthocyanidins are able to prevent metabolic syndrome except for body weight and hypertension. Proanthocyanidins only showed a tendency to reduce body weight and the reduction of blood pressure was only achieved with supraphysiologic doses. Thus, the main goal of the AGL2013-40707-R project is to elaborate what we designate 2.0 functional food, that will contain more than one ingredient, combining proanthocyanidins with other compounds, that complement their effects, targeting metabolic syndrome from a multifactorial point of view.

   To prevent hypertension, researchers in our group already found a peptide hydrolysate that could be effective at low doses. Then, two approaches were taken to find bioactive compounds with the ability of reducing body weight: compounds able to induce the browning process and compounds effective modulating the **leptin signaling pathway,** which appears to be altered in obesity. In this situation, leptin is not exerting its anorectic effects as in

normal conditions. The last approach was investigated in this thesis. Therefore, the objectives were:

a. **To assess the effect of GSPE on central and peripheral leptin signaling pathway on a diet-induced obesity model.** The effect of GSPE on the hypothalamic leptin system had not been investigated yet, but moderate doses of proanthocyanidins tend to reduce body weight. This suggests that GSPE could potentially modulate leptin signaling or downstream factors **[Chapter 1].**
b. **To evaluate the ability of anthocyanins and resveratrol to increase leptin sensitivity.** In order to find other ingredients that could complement GSPE reducing obesity, we opt for the use of an anthocyanin rich extract and resveratrol. The selection was based on the reported anti-obesity effects of both classes of polyphenols in different animal models. The capacity of these polyphenols to modulate the central leptin system was evaluated first by studying the effects of these compounds on the gene expression of leptin signaling pathway in the hypothalamus in healthy mice, as a preliminary study. The most effective compound, resveratrol, was selected and tested on a diet-induced obesity model **[Chapter 2].**

2. **To determine whether seasonal fruits rich in polyphenols are able to modulate the hypothalamic leptin system in rats placed to long-day or short-day photoperiod [Chapter 3].**

The xenohormesis theory states that polyphenols synthesized by plants, as a response to environmental stress, provide a chemical signature of the surrounding conditions. This mark may be recognized by heterotrophs

consuming these plants, to detect changes when conditions are still favorable, such as the need of food, which allows animal survival. Polyphenol structures and content on plants depend on genetic aspects but also on photoperiod, temperature, harvest season and crops conditions. Numerous studies correlate photoperiod alterations with obesity. The hypothesis of the project AGL2013-49500-EXP is that the composition and concentration of polyphenols in fruits inform animals about the season, and the consumption of fruits out-of season may stimulate metabolic diseases, such as obesity. Indeed, circulating leptin levels experience seasonal rhythms. For this reason, the out-of season fruit consumption could be involved in the attenuation of leptin signaling present in obesity. Thus, the objectives of this project were:

a. **To assess the effect of seasonal fruits (grape or cherry) on leptin signaling pathway when they are consumed in or out-of season**
b. **To evaluate whether the effect of seasonal fruits on the leptin system is influenced by an obesogenic diet**

3. **To study hypothalamic neurons that could be potential targets of polyphenols [Chapter 4].**

Leptin action in the hypothalamic arcuate nucleus produces the activation of Pomc neurons and suppresses AgRP/Npy neurons, generating satiety signals and increasing energy expenditure thereby maintaining body weight. Interestingly, Olofsson and colleagues showed that AgRP neurons are mainly located outside the blood brain barrier, being more sensitive to metabolic peripheral signals. Although, the polyphenols used in this thesis have been detected in brain samples, we think that AgRP neurons would be more susceptible to polyphenol since they can directly be affected by blood borne substances.

This part of the PhD thesis has been performed in Professor Allison W. Xu laboratory in the Diabetes Center at University of California, San Francisco. This group discovered that AgRP neurons are outside the blood brain barrier and has extensive knowledge and expertise on the study of hypothalamic neurons and how AgRP neurons sense and integrate peripheral signals, such as leptin, This allowed me to get fully involved in one of their projects that aimed to investigate new mechanisms by which AgRP neurons are activated in both physiological conditions (chapter 4) and after ethanol consumption (data not shown).

Recent data gives evidences that AgRP neurons are activated by a class of G protein-coupled receptors (GPCRs). According to this and other studies, the group of Professor Xu hypothesizes that adenosine receptor 2B could play a role on the activation of AgRP neurons and that this receptor has metabolic implications in normal or pathological conditions.

The research work performed in this Ph.D. has been supported by the grants AGL2013-40707-R and AGL2013-49500-EXP from the Spanish government. This thesis was carried out in the Nutrigenomics Research Group laboratories of the Universitat Rovira i Virgili under a FPI predoctoral fellowship from the Spanish Government. An international phase has been completed in the Diabetes Center at University of California San Francisco under the supervision of Prof. Allison W. Xu to obtain the International Doctorate. Mention. This phase was supported by a personal grant from the Spanish Government.

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III. RESULTS

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CHAPTER 1

Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and *Pomc* gene expression in rats with diet-induced obesity

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Abstract

Dietary obesity is usually linked with hypothalamic leptin resistance, in which the primary impact is interference in the homeostatic control of body weight and appetite. Notably, proanthocyanidins (PACs), which are the most abundant phenolic compounds present in human diet, modulate adiposity and food intake. The aim of this study was to assess whether PACs could re-establish appropriate leptin signalling in both the hypothalamus and peripheral tissues. Male Wistar rats were fed either a standard chow diet (STD group, n=7) or a cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either grape-seed PAC extract (GSPE) at 25 mg per kg of body weight per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) for the last 21 days of the study period. Specific markers for intracellular leptin signalling, inflammation and endoplasmic reticulum stress in the hypothalamus, liver, mesenteric white adipose tissue and skeletal muscle were analyzed using immunoblotting and quantitative PCR. GSPE treatment significantly reduced the food intake but did not reverse the hyperleptinemia and body weight gain assessed. However, the animals treated with GSPE exhibited greater hypothalamic activation of STAT3, which was associated with a rise in the *Pomc* mRNA levels compared to the CD group. In addition, this restoration of leptin responsiveness was accompanied by lower local inflammation and increased *Sirt1* gene expression. The effects of the GSPE treatment in the peripheral tissues were not as evident as those in the hypothalamus, although the GSPE treatment significantly restored the mRNA levels of *Socs3* and *Ptp1b* in the skeletal muscle. The use of GSPE reduces hyperphagia and improves the central and peripheral leptin resistance associated with diet-induced obesity. Our results suggest that GSPE could exert these effects partially by increasing Sirt1 expression and preventing hypothalamic inflammation.

1. Introduction

Obesity has reached truly epidemic proportions worldwide and has become one of the most prevalent health problems that our world currently faces.[1] In mammals, energy balance is regulated by controlling food intake and energy expenditure by means of the interactions of peripheral nutrients and hormones with different neuronal subpopulations. The critical cell populations include the anorexigenic proopiomelanocortin (POMC)- and orexigenic Agouti-related protein (AgRP)-expressing neurons, both located in the arcuate nucleus of the hypothalamus.[2]

Leptin, a hormone secreted mainly from white adipose tissue, is the main molecule that transmits information regarding the energy stores of the periphery to the hypothalamus.[3] The interaction of leptin with its longest receptor isoform (Obrb) in the POMC- and AgRP-expressing neurons promotes the phosphorylation of the signal transducer and activator of transcription-3 (STAT3). Then, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to the POMC and AgRP promoters. This stimulates the expression of POMC and inhibits that of AgRP reducing food intake and increasing energy expenditure.[4,5]

However, leptin is completely ineffective in decreasing food intake and suppressing body weight gain in subjects with diet-induced obesity. In this condition, instead of a leptin deficiency, high circulating levels of the hormone are observed, but the high levels are associated with a loss of responsiveness.[6–8] Although the basis of leptin resistance is not completely understood, it has been generally related to several mechanisms[9–11]including reduced leptin transport across the blood-brain barrier and the enhancement of intracellular processes

that attenuate OBRB signalling. These process include hypothalamic inflammation and endoplasmic reticulum (ER) stress, which, in turn, up-regulate the expression of negative regulatory molecules, including suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B).[12] Moreover, the hypothalamic NAD^+-dependent deacetylase sirtuin 1 (SIRT1) has been confirmed to be a mediator of leptin action in POMC- and AgRP-expressing neurons in which it suppresses nuclear factor-κB (NF-κB) signalling and/or regulates ER stress reactions through the deacetylation of the active spliced form of X-box binding protein 1 (XBP1s).[13] Therefore, hypothalamic SIRT1 activity may be an additional mechanism that is involved in the regulation of leptin signal transduction.[14]

Within this context, because pharmacologic methods to restore the leptin levels and sensitivity have not yet been found, the use of bioactive food compounds may be a useful approach that could complement the existing therapeutic strategies.[15] In this sense, natural dietary polyphenols, specifically proanthocyanidins (PACs), which are a class of flavonoids structurally complex, are bioactive food compounds present in fruits and vegetables and are significantly implicated in health promotion.[16] In particular, our group and others have reported many beneficial effects of grape-seed PACs on various obesity-associated diseases including insulin resistance, dyslipidemia, hypertension and local and systemic inflammation.[17–21] Moreover, although there have been some questionable results regarding the potential effects of these compounds on the control of body weight in diet-induced obesity,[22] latest studies have shown that grape-seed PACs have the potential to significantly modulate food intake and adiposity.[19,23,24] Thus, we hypothesized that the chronic consumption of dietary PACs could rescue the anorexigenic actions of leptin by interfering with those metabolic abnormalities that attenuate leptin

signalling in diet-induced obesity. Accordingly, the aim of the present study was to evaluate the effects of a grape-seed PAC extract (GSPE), administered for 21 days to rats previously fed a cafeteria diet (CD), on the central and peripheral leptin resistance induced by the CD.

2. Materials and methods

2.1 Grape-seed PAC extract

The grape-seed PAC extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract is mainly composed of phenolic compounds (total content higher that 96 %) including procyanidin monomers or flavan-3-ols (21.3%), and dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of procyanidins. The phenolic composition of this extract was further analyzed by Quiñones M *et al*.[25]

2.2 Animals and diet

The investigation was carried out in accordance with the ethical standards and according to the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249 by Generalitat de Catalunya).

Male Wistar rats of 200±50 g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were singly housed in a 12 h light-dark cycle at 22ºC, fed a standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum* and were provided access to tap water during the adaptation week. After the adaptation week, animals were distributed into three equivalent groups of 7 animals and housed individually in cages to permit control of their food intake. One group was fed with standard chow diet (STD) with a calorie breakdown of 14% protein, 8% fat and 73%

carbohydrates, and the other groups were fed with STD plus cafeteria diet (CD) composed by 14% protein, 35% fat and 51% carbohydrates. The animals had access during the dark phase to STD and water and to CD *ad libitum.* CD consisted of bacon, carrots, cookies, foie-gras, cupcakes, cheese and sugary milk. Ten weeks later, an oral treatment was administered in combination with the CD diet for 21 days. The treated group (CD+GSPE) received 25 mg of GSPE/kg body wt dissolved in 5% gum arabic (G9752, Sigma-Aldrich, Madrid, Spain), and the CD group received 1 mL of gum arabic. The rats received the treatment in the afternoon and then were allowed *ad libitum* access to STD and CD at night. On day 21 of treatment, the rats were fasted for 3 hours before anesthesia with 50 mg/kg body wt of sodium pentobarbital *(*Fagron Iberica, Barcelona, Spain) and euthanized by abdominal aorta exsanguination. Blood was collected using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. The plasma was obtained by centrifugation (1 500 x *g*, 4ºC, 15 min) and stored at -80ºC. The hypothalamus, liver, muscle and mesenteric white adipose tissue (mWAT) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80ºC until analysis.

2.3 Adiposity index

The adiposity index was determined by the sum of the mesenteric white adipose tissue (mWAT), perirenal white adipose tissue (pWAT) and epididymal white adipose tissue (eWAT) depot weights. Results were expressed as percentage of the total body wt.

2.4 Plasma leptin levels

The plasma leptin levels were determined using an Enzyme Immunoassay Kit according to the manufacturer's instructions (Biosource International, Inc., San Diego, CA, USA).

2.5 Indirect calorimetry

Indirect calorimetry analyses were performed on day 20 of the treatment using a Oxylet Pro System (PANLAB, Barcelona, Spain). Food was removed at 09:00 a.m., and the animals were fasted for 7 hours (from 09:00 am to 04:00 pm). After an initial acclimatization period of 1 hour, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 6 h. The respiratory quotient (RQ), energy expenditure (EE) and substrate oxidation were calculated as previously described.[26] Briefly, the RQ was calculated as the VCO2/VO2 ratio and the EE in kcal/day/kg0.75 as VO2×1.44×[3.815+(1.232×RQ)]. The rate of carbohydrate and fat oxidation were calculated in g/min as 4.55 × VCO2 – 3.21 × VO2 – 2.87 n and as 1.67 × VO2 – 1.67 × VCO2 – 1.92 n, respectively. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed. Finally, to obtain the values of fat and carbohydrate oxidation in kJ/min, the fat and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor.

2.6 Total RNA isolation

Total RNA from the hypothalamus, liver, muscle and mWAT was extracted using the TRIzol LS Reagent (Life Technologies, Uppsala, Sweden) and RNeasy Mini Kit (Qiagen) according to the manufacturers' protocols. The quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA integrity was evaluated on denaturing electrophoretic gels stained with SYBR Green dye (Bio-Rad, Barcelona, Spain), and only samples with an adequate RNA concentration (A260/A280 ≥1.8) and purity (A230/A260 ≥2.0) were selected for reverse transcription.

2.7 Gene expression analysis

The cDNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Life Technologies and was subjected to quantitative PCR (qPCR) using the CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad)

with SYBR Green PCR Master Mix (Bio-Rad). The forward and reverse primers for the various genes used in this study are shown in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). A cycle threshold (Ct) value was defined by setting the threshold during the geometric phase of the cDNA sample amplification. The fold change in expression of each mRNA was calculated with respect to the STD group using the ΔΔCt method corrected for the primer efficiency and converted to relative expression ratio with *Ppia* as the reference gene.[27]

2.8 Western blot analysis

Activated STAT3 in the hypothalamus, liver, mWAT and skeletal muscle was visualized using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). Additionally, the protein levels of the ObRb leptin receptor isoform as well as total and phosphorylated eIF2α in the hypothalamus were also determined by western blot analysis. Tissues were homogenized at 4°C in 0.5 mL of Radio-Immunoprecipitation Assay lysis buffer containing protease and phosphatase inhibitor cocktails using a TissueLyser LT (Qiagen). The homogenate was incubated for 30 minutes at 4°C and then centrifuged at 20 000 × *g* for 15 min at 4°C. The supernatant was used for total protein and western blot analyses. The total protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific).

A total of 100 μg of protein was solubilized and boiled for 10 min in a loading buffer solution containing Tris HCl 0.5 M, pH 6.8; glycerol, SDS, β-mercaptoethanol and Bromophenol Blue. The total protein extracts were separated using SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and electrotransferred onto supported PVDF membranes (Trans-Blot Turbo Mini PVDF Transfer Packs from Bio-Rad). After blocking, the membranes were incubated with agitation overnight at 4ºC with antibodies specific for ObRb (Abcam, Cambridge, UK), p-STAT3 (Abcam) or total or phosphorylated eIF2α (Cell Signalling, Izasa SA, Barcelona, Spain), diluted 1:1 000 and then with the goat anti-rabbit secondary antibodies (Sigma-Aldrich), diluted 1:10 000. For β-actin analysis, the membranes were incubated with a rabbit anti-actin

primary antibody (Abcam) and then with a goat anti-rabbit secondary antibody (GE Healthcare, Barcelona, Spain), using the same dilutions specified above. The protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Finally, protein band quantification was performed using ImageJ (W.S Rasband, NIH, MD, USA).

2.9 Statistical analysis

The data are expressed as the means ± s.e.m. A two-tailed Student *t* test was used to evaluate the differences between two groups. Multiple independent groups were compared with a one-way ANOVA followed by Tukey or Dunnett's T3 *post hoc* test when necessary. Outliers were determined by Grubbs' test. The statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

GSPE treatment ameliorated food intake but did not reverse the obesity and hyperleptinemia induced by the CD

As shown in Table 1, CD for 13 weeks consistently resulted in obesity and loss of leptin sensitivity in our experimental model as indicated by significant increases in the body wt and circulating leptin levels. Moreover, CD increased the adiposity index and the wt of all of the WAT depots studied including the mWAT, pWAT and eWAT depots, which confirmed the robust metabolic correlation between leptin and fat mass (rho=0.901, P <0.001). However, the administration of GSPE to the CD-fed rats for 21 days did not significantly exert anti-obesity effects, indicating that GSPE consumption during 21 days did

not prevent the total body wt gain measured at the end of the experimental period and did not significantly reduce the adiposity index and the weights of all the WAT depots studied (Table 1). A very similar pattern was also observed for the leptin levels, which exhibited a slight decrease (9.4% lower) in CD+GSPE group, but the difference from that of the CD group was not statistically significant (P=0.41).

Table 1. Body and adipose tissue weights, adiposity index and plasma concentrations of leptin in rats fed the STD or CD and treated with GSPE or the vehicle

	STD	CD	CD+GSPE
Initial body wt (g)	288±9	289±14	292±17
Final body wt (g)	443±11*	531±15	511±17
Body wt gain (g)	155±23*	242±31	219±43
Mesenteric WAT (g)	6.26±0.6*	14.00±1.5	13.33±0.9
Perirenal WAT (g)	8.96±0.4*	20.17±2.0	19.51±0.6
Epididymal WAT (g)	10.02±0.6*	21.61±2.5	18.10±0.8
Adiposity index (%)	5.80±0.3*	10.42±0.8	9.89±0.2
Leptin (ng/mL)	10.09±1.4*	27.85±2.5	25.24±2.4

Abbreviations: GSPE, grape-seed proanthocyanidin extract; WAT, white adipose tissue; wt, weight. The rats were fed a standard chow diet (STD group) or a cafeteria diet for 10 weeks. After 10 weeks, the rats fed the cafeteria diet were treated orally with GSPE (25 mg/kg of body wt) (CD-GSPE group) or the vehicle (CD group) for 21 days. The adiposity index was computed for each animal as the sum of the different WAT weights, expressed as a percentage of body weight. The values are the means ± s.e.m. of seven samples from each group. * denotes significant differences ($P < 0.05$) with respect to both CD and CD+GSPE groups assessed using one-way ANOVA and Tukey's *post hoc* test.

Notably, the energy intake in the GSPE-treated group was significantly lower than that of the CD rats (Table 2). In addition, because leptin has been reported to maintain high-energy expenditure even though it reduces energy intake, we reasoned that the GSPE treatment, despite its effects on energy intake, could also enhance energy expenditure and lead to the utilization of fat as the main energy source. Thus, we measured the energy expenditure and the respiratory quotient (RQ) in both CD- and GSPE-treated rats. However, no significant differences were observed between the groups in the RQ values, energy expenditure or substrate oxidation (Table 2).

Table 2. Food intake, substrate oxidation and energy expenditure in in rats fed with a CD and supplemented with GSPE or vehicle

	CD	CD+GSPE
Energy intake (KJ/day/animal)	1197.32±31.1	1105.83±45.4*
Respiratory quotient	0.89±0.1	0.88±0.1
Energy expenditure (Kcal/day/kg$^{0.75}$)	68.28±2.2	66.73±5.3
Carbohydrate oxidation (kJ/min/kg$^{0.75}$)	127.39±17.9	152.59±34.9
Fat oxidation (kJ/min/kg$^{0.75}$)	51.76±11.4	61.33±9.5

Abbreviations: GSPE, grape-seed proanthocyanidins extract. Rats were fed with a cafeteria diet for 10 weeks. After 10 weeks rats fed a cafeteria diet (CD) received oral treatment of GSPE (25 mg/kg of body wt) (CD-GSPE group) or vehicle (CD group) for 21 days. Values are mean ±s.e.m. of seven samples from each group. * denotes significant differences *(P* < 0.05) with respect to STD group assessed by Student's *t* test.

GSPE treatment activated the hypothalamic leptin receptor-STAT3 pathway in the CD-fed rats.

To determine whether the observed decrease in energy intake indicated that the GSPE treatment affected the functions of the POMC- and AgRP-expressing neurons, we initially assessed the leptin signalling pathway by measuring the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). Indeed, CD did lead to a significant decrease in the basal levels of p-STAT3 in the hypothalamus, and when GSPE was administered to the CD group, the levels of p-STAT3 increased significantly to basal levels. This showed that the GSPE treatment reversed the phenomenon observed in CD rats (Figure 1A) and indicated that treatment of the CD rats with GSPE for 21 days was sufficient to rescue the leptin signalling in this tissue.

Next, we determined whether the modulation of hypothalamic p-STAT3 was directly mediated by enhanced cell surface content of the long leptin receptor isoform Obrb. However, the CD group showed only a slight decrease in the mRNA levels of O*brb* compared to the STD group, and no statistically significant difference between these groups were detected. In addition, the mRNA levels of *Obrb* were not enhanced after the administration of GSPE to the CD rats (Figure 1B). This result indicated that if GSPE is, indeed, a true leptin sensitizer, its effects are not mediated by a rise in the *Obrb* content of the hypothalamic cells. Accordingly, the Obrb protein levels as assessed by western blotting were also not affected by the GSPE treatment. Finally, the gene expression of both the short leptin receptor isoform Obra and low density lipoprotein-related protein 2 (Lrp2), also called megalin, was assessed to determine whether the modulation of hypothalamic p-STAT3 was a result of an enhanced transcellular transport of leptin into hypothalamus. However, the

mRNA levels of both *Obra* and *Lrp2* were similar in all three groups of animals, and the differences among the groups did not reach statistical significance (Figure 1C).

To further investigate the effects of GSPE treatment on the regulation of the leptin signalling pathway, the gene expression of negative feedback regulatory molecules, namely suppressor of cytokine signalling 3 (*Socs3*) and protein-tyrosine phosphatase 1B (*Ptp1b*), was also assessed by qPCR. Notably, the *Socs3* mRNA levels were decreased in the CD-fed rats compared with the STD group, and the GSPE treatment significantly increased its transcript levels relative to the CD-fed rats. In addition, the mRNA expression of *Ptp1b* was reduced in the CD-fed rats compared with STD group, but the GSPE treatment did not induce any significant change after the treatment period compared to the CD-fed rats (Figure 1D).

Figure 1. Effect of GSPE treatment on the hypothalamic leptin signalling. The leptin signalling pathway was primarily assessed by evaluating the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3) (A) and by the determination of the cell surface content of the long leptin receptor isoform b (Obrb) (B). Additionally, qPCR was used to investigate the gene expression of both the short leptin receptor isoform a (Obra) and low density lipoprotein-related protein 2 (Lrp2) (C). Finally, the gene expression levels of the negative feedback regulatory molecules suppressor of cytokine signalling 3 (Socs3) and protein-tyrosine phosphatase 1B (Ptp1b) were also determined in this tissue (D). The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment selectively regulated the expression of hypothalamic peptides involved in appetite regulation

Then we evaluated the hypothalamic mRNA levels of proopiomelanocortin (*Pomc*), agouti-related peptide (*Agrp*), and neuropeptide Y (*Npy*). Interestingly, the *Pomc* mRNA levels were significantly increased in the GSPE-treated rats compared with both the STD group and CD group (Figure 2A). Furthermore, and in contrast to our expectations, we found that the *Agrp* gene expression levels were also significantly increased although to a much lower degree than the *Pomc* levels (Figure 2B). The *Npy* mRNA levels were not statistically altered by either the CD or GSPE treatment (Figure 2C).

Figure 2. Effect of GSPE treatment on the regulation of hypothalamic neuropeptide gene expression. The mRNA levels of hypothalamic proopiomelanocortin (Pomc) (A), agouti-related peptide (Agrp) (B) and neuropeptide Y (Npy) (C) were assessed using qPCR. The mRNA levels of the

selected neuropeptides were normalized to those of Ppia. The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment significantly potentiated the gene expression of hypothalamic *Sirt1* in a manner consistent with an attenuation of local inflammation

To elucidate the intracellular effects by which GSPE treatment potentially rescues leptin signalling in the hypothalamus, we assessed the impact of these compounds on the molecular processes associated with leptin signalling disruption, including local inflammation, ER stress and loss of SIRT1 activity in this tissue.

The contributions of these processes to leptin resistance were initially investigated using the gene expression of inducible nitric oxide synthase (*inos*), which is an important marker of neuroinflammation. Indeed, although the CD did not induce local inflammation in this tissue, as indicated by the similar mRNA levels in the STD and CD groups, the GSPE treatment significantly down-regulated the *inos* gene expression (Figure 3A), which confirmed the ability of these compounds to prevent local inflammation. Notably, GSPE treatment resulted in a significant up-regulation (3-fold higher) of the *Sirt1* mRNA levels in a manner consistent with reduced hypothalamic inflammation (Figure 3B).

Finally, to address the molecular implications of ER stress in leptin resistance, hypothalamic ER stress markers, including the spliced form of X-box binding

protein-1 (XBP1s) and the levels of ATF4 and CHOP mRNA, were also determined by qPCR. However, our results indicated that GSPE treatment did not modify the gene expression levels of any of these indicators (Figure 3C). Additionally, other ER stress markers were assessed by western blotting. However, the total eIF2α protein levels did not display significant differences among the groups (Figure 3D), and the phosphorylated form of eIF2α was not detected in any group of animals. These results indicate not only that the CD did not induce ER stress in this tissue but that these markers of ER stress were also not significantly affected by the GSPE treatment.

Figure 3. Effect of GSPE treatment on hypothalamic inflammation, sirtuin expression and ER stress. To evaluate the possible mechanisms responsible for the GSPE effects on hypothalamic leptin signalling, the gene expression of inducible nitric oxide synthase (inos) (A) and sirtuin 1 (Sirt1) (B) was investigated in this tissue. Additionally, the hypothalamic mRNA levels of the ER stress markers X-box binding protein-1 (XBP1) spliced form, and ATF4 and CHOP were determined using qPCR (C). The mRNA levels of these selected genes were normalized to those of Ppia. In addition, the protein levels of total and phosphorylated eIF2α were also assessed by immunoblotting (D). The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

GSPE treatment distinctively modulated leptin signalling in the liver, mWAT and skeletal muscle of the CD-fed rats

Alternatively, to assess the contribution of the metabolic signals derived from peripheral tissues to the regulation of energy intake and energy homeostasis, we next investigated the leptin signal transduction in the liver, mesenteric WAT (mWAT) and skeletal muscle. Indeed, a decrease in the level of p-STAT3 was observed in the livers of the CD and GSPE groups compared to STD group but the differences did not reach statistical significance (Figure 4A). Furthermore, no significant differences were observed in the mRNA levels of *Obrb*, *Socs3* and *Ptp1b* among the three groups of animals indicating that GSPE had no effect on restoring leptin sensitivity in this tissue.

In the mWAT (Figure 4B), the CD-fed rats showed a significant increase in p-STAT3 compared to the STD-fed rats, whereas the GSPE treatment significantly restored the p-STAT3 values to those of the STD-fed rats. Furthermore, the mRNA levels of *Obrb*, *Socs3* and *Ptp1b* in mWAT were also similar in all three groups of animals.

Finally, a slight increase of p-STAT3 was observed in the skeletal muscles of the GSPE-treated rats compared to STD and CD groups (Figure 4C). Importantly, in contrast to the liver and mWAT, the *Socs3* and *Ptp1b* gene expression levels were significantly lower in the CD-fed rats than in the STD group; furthermore, the GSPE treatment induced a robust increase of their expression levels compared to the CD-fed rats. These results indicated that the skeletal muscle is the tissue most sensitive to the GSPE treatment with respect to leptin signalling. In addition, as observed in the hypothalamus, the *Obrb* mRNA levels were not altered by the GSPE treatment.

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Figure 4. Effect of GSPE treatment on the peripheral leptin signalling. The leptin signalling pathway was investigated in the liver (A), mesenteric WAT (mWAT) (B) and skeletal muscle (C). STAT3 phosphorylation (p-STAT3) was assessed using western blotting, and the mRNA levels of the long leptin receptor isoform b (Obrb), suppressor of cytokine signalling 3 (Socs3) and protein-tyrosine phosphatase 1B (Ptp1b) were determined using qPCR. The mRNA levels of these selected genes were normalized to those of Ppia. The rats were fed either the standard chow diet (STD group, n=7) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, n=7) or with the vehicle (CD group, n=7) during the last 21 days of the study. The values shown are the means ± s.e.m. * indicates significant differences between the groups at $P \leq 0.05$, as assessed using one-way ANOVA.

4. Discussion

Previous results from our group have indicated that chronic consumption of grape-seed PACs are unable to counteract the body weight gain and the hyperleptinemia induced by a CD in rats, but consumption of these compounds significantly reduces the food intake.[23] The hyperphagia observed in animals fed a highly palatable diet has been related to a dysfunctional melanocortin system[28]. We therefore determined whether chronic ingestion of GSPE was able to reverse this dysfunction and normalize the leptin signalling in the hypothalamus of rats fed a CD.

Importantly, our CD model exhibited central leptin resistance as indicated by the decrease in the hypothalamic p-STAT3 levels in rats fed the CD for 13 weeks. Notably, the impairment of leptin-induced STAT3 phosphorylation in the hypothalamus has been considered to be one of the leading markers for cellular leptin signal attenuation in hyperleptinemic rats with diet-induced obesity.[29] Conversely, the CD-fed rats in this study did not display altered gene expression of *Obrb*, *Pomc*, *Agrp* or *Npy* in the hypothalamus. Contradictory

results have been published regarding the effect of a CD on *Pomc* expression in the hypothalamus, with both repression[30] and overexpression[31] having been reported. Thus, the duration of the CD and the grade of obesity achieved can affect the severity of the melanocortin system dysfunction in rats.

Remarkably, 21 days of GSPE treatment normalized the level of p-STAT3 and the gene expression of *Socs3* and *Ptp1b* in the hypothalamus, all of which had been repressed by the CD. Interestingly, this normalization was associated with a high overexpression of *Pomc*, suggesting that the up-regulation of *Pomc* could be mediated by the increase in p-STAT3 induced by the GSPE treatment. Together, these results indicate that chronic consumption of GSPE clearly improved the central leptin signalling in the CD-fed rats. Notably, POMC is an anorexigenic neuropeptide.[2] Thus, the overexpression of POMC induced by GSPE treatment could mediate the significant reduction of food intake and adiposity observed in the CD+GSPE group. Other polyphenols and polyphenol extracts modulate the neuropeptides involved in food intake and energy expenditure (reviewed in [32]). For instance, resveratrol reduces *Npy* and *Agrp* expression[33], and apigenin increases *Pomc* expression[34] in neuronal cell lines. These results reinforce the idea that specific polyphenols could improve central leptin signalling.

The induction of central leptin resistance in diet-obesity models has been mainly attributed to hypothalamic inflammation[35,36] as a result of the induction of the pro-inflammatory signalling molecules JNK and NF-κB and ER stress resulting from over-nutrition. Remarkably, our results showed that GSPE treatment reduced the hypothalamic inflammation, as indicated by the *inos* gene expression levels, which suggested that the local anti-inflammatory activity of proanthocyanidins in this tissue could be one of the mechanisms by which GSPE treatment re-established normal central leptin sensitivity. In addition,

SIRT1activity has been highlighted as a mediator of central leptin action.[13,14] Thus, the hypothalamic overexpression of *Sirt1* induced by GSPE treatment could be another part of the mechanism by which GSPE treatment reduced the central leptin resistance.

The improvement of central leptin signalling could be secondary to the peripheral actions of GSPE on metabolism and hormones or the communication by the afferent nervous system to the brain. However, the capacity of GSPE to modulate *Sirt1* gene expression levels and inflammation in the hypothalamus itself, together with the facts that GSPE compounds can cross the brain-blood barrier [37] and that their metabolites have been found in the rat brain,[38] suggest a direct action of the PACs at hypothalamic level.

In addition to the hypothalamus, peripheral tissues such as the liver, skeletal muscle and adipose tissue are targets of leptin, and peripheral leptin resistance has been associated with obesity.[39] Notably, the GSPE treatment normalized the leptin cascade disruptions caused by the CD in the mWAT and skeletal muscle. Remarkably, leptin resistance in WAT has been associated with the excessive fat mass accumulation that characterizes obesity.[39] Accordingly, the normalization of leptin signalling observed in the mWAT of rats supplemented with GSPE was associated with a decline in the body adiposity. Moreover, we have demonstrated in a previous study that CD-fed rats supplemented with GSPE at the same dose and time period used in this study show an activation of muscle β-oxidation and an improvement in mitochondrial function.[24] These effects of GSPE in muscle are also consistent with the up-regulation of fatty acid oxidation, which is an insulin-sensitizing effect of leptin in this tissue.[40]

Therefore, these results clearly indicate that GSPE treatment was also effective in improving peripheral leptin sensitivity in CD-fed rats.

Despite this improvement of leptin sensitivity, rats treated with GSPE did not displayed a significant body weight reduction indicating that GSPE, at the dose and time used in this experiment, was not sufficient to totally reverse leptin dysfunction induced by a high-fat diet. However, body weight gain and epididymal fat mass of rats treated with GSPE were 10% and 16% lesser that those of rats not treated, respectively. Therefore, the improvement of leptin sensitivity induced by GSPE could behind a body mass rearrangement that, in turn, can improve obesity outcomes.

Intriguingly, GSPE did not reduce significantly the body weight, despite GSPE decreasing energy intake and not affecting energy expenditure nor substrate utilization. However, measurements of energy expenditure and substrate utilization were performed in the fasted state rather than the fed state. Thus, the lack of effects of GSPE on body weight combined with a decrease in energy intake suggest that energy expenditure in the fed state may be reduced by GSPE.

Importantly, translation of the daily dose of PACs (25 mg per kg of body wt per day) used in this study to the human doses[41] estimated for a 70 kg human indicates that the equivalent intake would be between 250 and 280 mg of GSPE/day. Humans consuming a polyphenol-rich diet can easily achieve or even exceed this PAC intake.[42] Therefore, the inclusion of PAC-rich foods in diets of obese people could be a good strategy to reduce the appetite and improve central and peripheral leptin sensitivity, thus complementing dietary therapies intended to promote weight loss in obese subjects. However, the effect of an obesogenic diet on leptin resistance have been described to be sex-

dependent.[43] Therefore, as this study has been performed in males, further studies are warranted in order to determine the potential sex differences of PACs in relation to energy intake and body weight.

In conclusion, 21 days of GSPE treatment normalized the CD-induced leptin signalling disorders observed mainly in the hypothalamus and in the skeletal muscle of rats with diet-induced obesity. This improvement in leptin signalling resulted in part from neuroprotection against diet-induced inflammation and from the increase in hypothalamic sirtuin expression. Together, these results strongly suggest that PACs could reduce energy intake and adiposity by re-establishing central and peripheral leptin sensitivity.

Conflict of interest

The authors declare no conflicts of interest.

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Supplementary information is available at International Journal of Obesity's website

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Supplementary material

Table S1. A summary of the rat-specific primer sequences used for qRT-PCR analysis.

Primer Name (Rat)	Primer sequences (5'-3')	Product size (bp)	Gen Bank accession no/reference
Agrp	GAG AAC TCT GGG AAC AGG GC CAA GCA AAG GCC ATG CTG AC	140	NM_033650.1
Atf4	TAT GGA TGG GTT GGT CAG TG CTC ATC TGG CAT GGT TTC C	145	NM_024403.2
Chop	AAG ATG AGC GGG TGG CAG CG CCG GTT TCT GCT TTC AGG TGT GGT	112	NM_001109986.1
Lpr2	GGA GCC AGT CAG TAG CCA AG CCT GGG AGG ACA GCC AAT TT	136	NM_030827.1
iNos	GGA TCT TCC CAG GCA ACC A AAT CCA CAA CTC GCT CCA AGA TT	60	Martínez-Micaelo N *et al.* [1]
Npy	CTA TCC CTG CTC GTG TGT TTG G TGG TGA TGA GAT TGA TGT AGT GTC G	136	Sun B *et al.* [2]
ObRa	CAC TGT TAA TTT CAC ACC AGA G GTC ATT CAA ACC ATA GTT TAG	235	AF304191.1
ObRb	CCA GTA CCC AGA GCC AAA GT GGA TCG GGC TTC ACA ACA AGC	122	NM_012596.1
Pomc	CAT AGA CGT GTG GAG CTG GT TCA AGG GCT GTT CAT CTC CG	149	NM_139326.2
Ppia	CTT CGA GC TGT TTG CAG ACA A AAG TCA CCA CCC TGG CAC ATG	138	NM_017101.1
Ptp1b	CCC TTT TGA CCA CAG TCG GA TTG GTA AAG GGC CCT GGG TG	119	NM_012637.2
Sirt1	TTG GCA CCG ATC CTC GAA ACA GAA ACC CCA GCT CCA	217	XM_006223877.1
Socs3	CTG GAC CCA TTC GGG AGT TC CTG GGA GCT ACC GAC CAT TG	148	NM_053565.1
Xbp1	GCT GAA GAG GAG GCG GAA G GTC CAG AAT GCC CAA CAG G	172	Mulero M *et al.* [3]

Abbreviations: Agrp, Agouti related protein; Atf4, activating transcriptor factor 4; Chop, C/EBP homologous protein; Lpr2, Low density lipoprotein-related protein 2; iNos, inducible nitric oxide synthase; Npy, neuropeptide Y; Obra, leptin receptor isoform a; Obrb, leptin receptor isoform b; Pomc, proopiomelanocortin; Ppia, peptidylprolyl isomerise A; Ptp1b, protein tyrosine phosphate 1B; Sirt1, sirtuin 1; Socs3, suppressor of cytokine signaling 3; Xbp1, X-box binding protein 1.

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POLYPHENOL EFFECTS ON CENTRAL LEPTIN SENSITIVITY IN OBESITY
Maria Ibars Serra

CHAPTER 2

Resveratrol, but not anthocyanins, improves hypothalamic leptin sensitivity and potentially contributes to body weight loss in obesity

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Abstract

Changes in diet composition and increased calorie intake have a big impact in most common forms of obesity. Concomitant to the excess body fat, obesity also causes hyperleptinemia. In this condition leptin sensitivity is decreased in the hypothalamus and the ability to regulate energy balance is deeply blunted. Phenolic compounds may have a role in body weight control and metabolic regulation. Therefore, the aim of this study was to determinate whether resveratrol or anthocyanins could individually increase leptin sensitivity in the hypothalamus and reduce body weight and total body fat mass under obesogenic conditions.

We initially performed a preliminary study in healthy mice fed standard chow diet and supplemented with 100 mg/kg/day of either anthocyanin-rich extract (ARE) or resveratrol (RSV) for 15 days. RSV consumption, but not ARE, enhanced leptin signalling and 24h-energy expenditure by increasing both hypothalamic phosphorylation of STAT3 (pSTAT3) and *Obrb* gene expression. Then, we designed a second study in order to assess whether RSV could re-establish appropriate leptin sensitivity in hyperleptinemic obese animals. Accordingly, male Wistar rats were fed a cafeteria diet (CD) during 8 weeks, afterwards animals were supplemented with a daily dose of 50, 100 or 200 mg/kg of RSV for 22 days together with de CD. We observed that the consumption of 200 mg/kg/day of RSV was significantly associated with both reduced leptin levels and increased hypothalamic leptin sensitivity. Notably, this restoration of leptin action at this dose was accompanied by lower body weight and higher 24h-energy expenditure and fat oxidation compared to untreated obese rats, but not by reduced food intake. Although, these metabolic effects on body weight and energy balance were not so evident at lower doses of RSV, 100 mg/kg/day significantly showed higher levels of hypothalamic

protein content of pSTAT3, ObRb and SOCS3, indicating that RSV at this intermediate dose could be also effective in improving leptin sensitivity in this tissue. In contrast, no significant changes on leptin system were observed at dose of 50 mg/kg/day.

Therefore, these results suggest that the anti-obesity effects of RSV could be at least partly through the modulation of leptin sensitivity in the hypothalamus. Future studies should focus on analyzing more extensively the molecular mechanism by which RSV directly modulates leptin signalling and affects body weight.

1. Introduction

Obesity and overweight are associated with higher all-cause mortality and it is increasing nowadays. Strategies to counteract obesity are a main focus for global health[1]. The hypothalamus is a brain area involved in the regulation of energy homeostasis. Specifically, the arcuate nucleus (ARC) receives signals from peripheral organs that inform about energy status in the body[2]. One of this chemical signals is leptin, the key hormone in energy regulation. It is mainly produced in the adipocytes and is proportional to body fat stores[3]. Circulating leptin reaches the ARC in the hypothalamus and binds to the long form of leptin receptor (ObRb). This interaction activates the transduction pathway through the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) in different neurons to supress food intake and allow energy expenditure[4]. Specifically, leptin activates anorexigenic proopiomelanocortin (POMC) neurons that produce α-MSH peptide responsible to bind melanocortin receptor 4 (MC4R) and produce satiety signals. Concomitantly, leptin also

inhibits orexigenic neurons producing agouti-related protein (AgRP) and neuropeptide Y (NPY)[5].

However, in obesity, leptin is not able to promote energy expenditure and satiety despite plasmatic levels of this hormone appears to be highly increased. This lost in leptin sensitivity is known as 'leptin resistance', although recent data postulate the term should be redefined[6]. Several mechanisms could reduce leptin sensitivity. These include defective transport across the blood brain barrier (BBB) and attenuation of ObRb signalling through increased endoplasmic reticulum stress and/or inflammation, impaired NAD^+-dependent deacetylase sirtuin 1 (SIRT1) function and the overexpression of inhibitory factors such as suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase (PTP1B)[7,8].

As current pharmacological treatments to improve leptinemia and leptin sensitivity in diet-induced obesity did not succeed, several authors, among whom we find ourselves, support that several phenolic compounds present in plants may target hypothalamic leptin system, which makes them a promising strategy to complement the existing therapies against obesity[9]. In particular, we have recently reported that a grape-seed PACs extract is able to reduce circulating plasmatic leptin levels and improve hypothalamic leptin signalling by increasing *Sirt1* gene expression and preventing inflammation[31]. This capacity has been also observed in other polyphenolic-rich extracts obtained from natural sources as well as in several pure phenolic compounds such as oleuropein, quercetin, curcumin and apigenin[9].

In this context, both resveratrol and anthocyanins have consistently demonstrated their anti-obesity effects by lowering total body fat mass and leptin serum levels[10–17]. However, only a few studies have focused on the mechanism by which these phenolic compounds could primarily alleviate

obesity and there is not enough evidence about their effects on leptin signalling. Therefore, as these compounds or its derived-metabolites can cross the BBB and interact with different neuronal subpopulations in the ARC[10], the aim of the present study was to examine whether either anthocyanins or resveratrol could exert part of their anti-obesity effects by modulating leptin sensitivity in these tissue. Initially, we evaluated hypothalamic leptin signalling only in non-obese animals in order to investigate the effects of these compounds under normal physiological conditions. We reasoned that if the consumption of resveratrol or anthocyanins is able to exert a minimal effect in healthy animals, it could give the indication that this effect could potentially be higher under pathological conditions. Thus, after testing them under physiological conditions, we also investigated the impact of these compounds in diet-induced obese animals with hyperleptinemia and impaired central leptin signalling in order to confirm if they could restore leptin sensitivity appropriately in these unfavorable metabolic conditions.

2. Materials and methods

2.1 Natural compounds

Resveratrol (RSV) of >98% purity degree was purchased from Fagron (Fagron Iberica, S.A.U, Barcelona, Spain). MEDOX®, an anthocyanin-rich extract (ARE) was provided by (MedPalett AS, Sandnes, Norway) which contained purified anthocyanins isolated from bilberries (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) (a mixture of 3-*O*-rutinosides of cyanidin and delphinidin, and 3-*O*-*β*-galactosides, 3-*O*-*β*-glucosides, and 3-*O*-*β*-arabinosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin). The 3-*O*-*β*-

glucosides of cyanidin and delphinidin constituted at least 40–50% of the total anthocyanins.

2.2 Animals and diet

The investigation was conducted in accordance with the ethical standards and according to the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the University Rovira i Virgili.

2.2.1 Study 1: Healthy mice

Male NMRI mice aged 8 weeks and of 40.14±0.47g body weight were purchased from Charles River Laboratoires (Bercelona, Spain). Animals were housed in a 12h light-dark cycle at 22ºC and fed with a standard rodent diet (Panlab 04, Barcelona, Spain) with a calorie breakdown of 20% protein, 8% fat and 72% carbohydrate and water *ad libitum*. After one week of adaptation, mice were trained for another week to lick low-fat condensed milk diluted in water 1:1 which was used as vehicle. Then, the animals were divided in 3 groups (n=8). The ARE group received 100 mg/kg of the anthocyanin rich-extract and the RSV group received 100 mg/kg of resveratrol every day. The control animals (STD group) received the same volume of the vehicle. The treatment lasted for 15 days. Weight was monitored every two days and indirect calorimetry was performed at the beginning and end of the experiment. Food intake was recorded 48h before sacrifice. On day 15th of treatment animals were fasted after treatment administration and sacrificed three hours later. Animals were anesthetized with Pentobarbital (60 mg/kg), cardiac puncture for plasma collection was performed and finally cervical dislocation. Blood was collected

using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. The plasma was obtained by centrifugation (1,500 x *g*, 4°C, 15 min) and stored at -80°C. The hypothalamus was excised and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Visceral fat depots were weighed and adiposity was calculated with the absolute weight of epididymal, mesenteric and retroperitoneal adipose tissues. Data was expressed as percentage of total body weight.

2.2.2 Study 2: Diet-induced obese rats

Male Wistar rats aged 5 weeks and of 122.56±3.11g body weight were purchased from Charles River Laboratories (Barcelona, Spain). Animals were housed in a 12h light-dark cycle at 22ºC, fed with a standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum* and provided access to tap water during 10 days of adaptation. After the adaptation period animals were divided into five equal groups composed by 6 rats. One group was fed with standard chow diet (STD group) with a calorie breakdown of 20% protein, 8% fat and 72% carbohydrate and the other groups were fed with STD plus cafeteria diet (CD groups) composed by 15% protein, 25% fat and 60% carbohydrate *ad libitium*. Animals had free access to fresh STD and cafeteria diet every day at 11:00 am. CD diet consisted of cookies, cheese, bacon, foie-gras, sugary milk, ensaïmada (a typical Majorcan pastry) and carrots. Weight gain and food consumption were monitored every week for 9 weeks until animals gained more than 15% body weight compared to STD group. Then, oral treatment was administered together with CD diet for 22 days. Treatment groups were daily supplemented with 50, 100 or 200 mg/kg body weight of resveratrol dissolved in low-fat condensed milk diluted in water 1:1. STD and CD diet group were supplemented with the same quantity of vehicle. Body weight and food intake

was weekly monitored until the end of the experiment. The day before sacrifice body composition was analysed from rats using magnetic resonance imaging system EchoMRI-700 (Echo Medical Systems, LLC., TX, USA). On day 22 of treatment rats were sacrificed by decapitation. Blood was collected and plasma was obtained as previously mentioned. Hypothalamus was excised and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.3 Circulating leptin levels

Leptin concentrations were measured in plasma using a specific Enzyme Immunoassay kit according to the manufacturer's instructions (Millipore, Madrid, Spain).

2.4 Respiratory exchange ratio and energy expenditure

Animals were acclimated in the respirometry system cages for a period of 2h. After this time they were subject to 16h of indirect calorimetry analyses using Oxylet Pro System (Panlab, Cornellà, Spain). Measurements were taken for a 8:8 hours light:dark cycle .The oxygen consumption (VO2) and carbon dioxide production (VCO2) measures were used by the software Metabolism 2.1.02 (Panlab) to calculate Respiratory Quotient (RQ) as VCO2/VO2 and energy expenditure as VO2x1.44x[3.815+(1.232xRQ] (Kcal/day/Kg^0.75) according to Weir equation[15]. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed[16]. Total activity was measured by recording infrared beam breaks (Oxylet Pro System, Panlab).

2.5 Gene expression analyses

Mice hypothalamus (n=4) was homogenized to extract total RNA using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) followed by RNeasy Mini Kit (Qiagen, Barcelona, Spain) according to manufacturer's instructions. Quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only samples with A260/A280 ratio ≥1.8 and A230/A260 ratio ≥2 were chosen to perform reverse transcription. RNA was converted to cDNA using High-Capacity complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain). Gene expression was determined by Real-Time PCR using the iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain) in the CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad) using primers obtained from Biomers.net (Ulm, Germany) (**Supplementary Table 1**). Relative expression of each gene was calculated referring to *Ppia* and normalized to the STD group. ΔΔCt method was used and corrected for primer efficiency [17].

2.6 Immunoblot analysis

Leptin signalling in the hypothalamus was assessed by calculating the activation of STAT3 using a phospho-specific antibody that identifies Tyr705phosphorylated STAT3 (pSTAT3). The assessment of pSTAT3 levels is the gold standard experimental marker for cellular leptin signalling[18]. Moreover, the protein levels of the ObRb leptin receptor isoform, SOCS3 and FOXO1 were also determined by Western blot analysis. Both mouse and rat hypothalamus (n=4 and n=6, respectively) were homogenized at 4°C using a Tissue Lyser LT (Qiagen, Barcelona, Spain) in 0.5 mL of Radio-Immunoprecipitation Assay lysis (RIPA) buffer (50mM Tris-HCl, 150mM

NaCl, pH 7.4, 1% Tween, 0.25% Na-deoxycholate, 0.1M phenylmethylsulfonyl fluoride) containing protease and phosphatase inhibitor cocktails (Sigma Aldrich, Madrid, Spain). The homogenates were incubated for 30 min at 4ºC and centrifuged at 20,000 x *g* for 15 min at 4ºC. The supernatant was placed in fresh tubes and was used to determine total protein and for immunoblotting analyses. The total protein content was quantified using the Pierce BCA protein assay kit (Thermo Scientific, Barcelona, Spain). Samples were denatured by mixing with loading buffer solution (Tris HCl 0.5M pH 6.8, glycerol, SDS, β-mercaptoethanol and Bromophenol Blue) and then heated at 99ºC during 5 min using a thermocycler (Multigen Labnet, Barcelona, Spain). Acrylamide gels were prepared using TGX Fast Cast Acrylamide Kit, 10% (Bio-Rad, Barcelona, Spain) and 30µg of protein were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using electrophoresis buffer (glycine 192mM, Tris base 25mM and 1% SDS). Proteins were electrotransfered onto supported polyvinylidene difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad). After blocking with 5% of non-fat dried milk, membranes were incubated with gentle agitation overnight at 4°C with specific antibodies for ObRb (Abcam, Cambridge, UK), diluted 1:1000, pSTAT3 (Abcam), diluted 1:2500, SOCS3 (Cell Signalling, Izasa S.A., Barcelona, Spain), diluted 1:1000 or FoxO1 (Cell Signalling), diluted 1:1000. For β-actin analysis as a loading control, membranes were incubated with a rabbit anti-actin primary antibody (Sigma, Madrid, Spain), diluted 1:1000. Finally, membranes were incubated with anti-rabbit horseradish peroxidase secondary antibody (GE Healthcare, Barcelona, Spain), diluted 1:10000. Protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Lastly, protein bands were quantitated by densitometry using ImageJ software (NIH, Bethesda, MD, USA) and each band was normalized by the

corresponding β-actin band and finally the treatment groups were normalized by the control group (STD).

2.7 Leptin sensitivity assessment

Consistent with hypothalamic pSTAT3 levels are typically attributable to leptin action, leptin sensitivity was estimated as the ratio of hypothalamic pSTAT3 levels to leptin concentration in plasma.

2.8 Statistical analyses

The results are expressed as mean ± standard error of the mean (S.E.M). Two tailed Student *t* -test was used to find differences between two groups. Multiple independent groups were compared with one-way ANOVA followed by LSD or Tukey post-hoc test. Repeated-measures ANOVA was used in indirect calorimetry analyses. GraphPad Prism 6 was used for statistical analyses and graphs (GraphPad Software, La Jolla, CA, USA). A P value ≤ 0.05 was considered statistically significant.

3. Results

Resveratrol, but not anthocyanins, increased hypothalamic leptin signalling and 24h-energy expenditure in healthy mice by modulating *ObRb* expression

Given the multiple line of evidence supporting leptin as a potential target for the energy balance regulation, we specifically tested the impact of resveratrol (RSV) or anthocyanin-rich extract (ARE) in hypothalamic leptin system. Thus, both plasmatic leptin concentrations and hypothalamic leptin signalling were initially evaluated in healthy mice treated for 15 days at dose of 100 mg/kg of either ARE or RSV in order to investigate them under physiological conditions.

At the end of the trial, any of the treated groups showed significant differences in plasma leptin levels compared to the STD group **(Fig. 1A)**. In order to assess the levels of leptin signalling in the hypothalamus, the activation of STAT3 was determined by immunoblotting in this tissue. The consumption of RSV demonstrated a trend to enhance pSTAT3 protein content compared to STD group (P=0.1) **(Fig. 1B)** while ARE consumption did not exert significant changes in pSTAT3 levels. We also determined whether the gene expression of the long leptin receptor isoform Obrb was up-regulated by any of the treatments. Interestingly, both ARE and RSV consumption produced higher mRNA levels of *Obrb* with respect to untreated animals (4- and 5-fold, respectively), but these changes were only statistically significant in RSV group **(Fig. 1C)**.

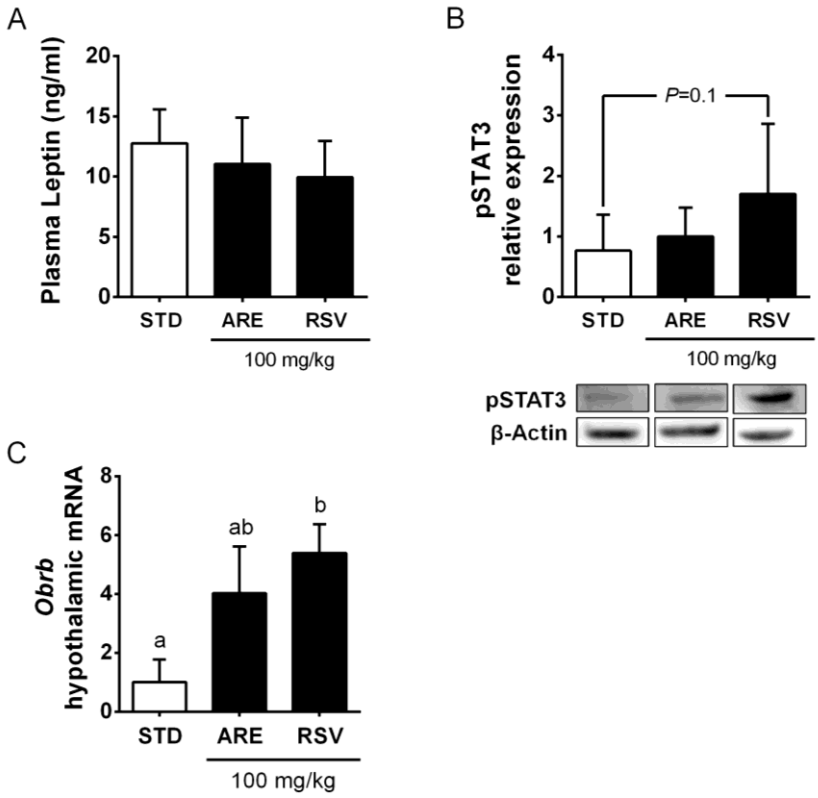

Figure 1. Plasma leptin levels and hypothalamic pSTAT3 and ObRb expression in mice treated with 100 mg/kg of either ARE or RSV. To study leptin signalling we initially determined the plasmatic levels of the hormone after 15 days of treatment with either vehicle (STD), anthocyanins (ARE) or resveratrol (RSV) (A). Additionally, both STAT3 phosphorylation (B) the mRNA levels of the long form of leptin receptor (Obrb) (C) were also assessed by immunoblotting and quantitative PCR, respectively. Values are mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

To further investigate the main regulatory factors involved in the leptin system, the mRNA levels of *Socs3*, *Ptp1b* and *Sirt1* were also analysed by quantitative PCR **(Fig. 2A).** Although, *Ptp1b* and *Sirt1* gene expression were not altered by any of the treatments, RSV consumption, but not ARE, exerted a notably trend to decrease the mRNA levels of *Socs3* with respect to untreated group (*P*=0.09)

Next, we also assessed the hypothalamic mRNA levels of *Pomc*, *Agrp* and *Npy* neuropeptides. However, none of them presented statistically significant changes in their hypothalamic expression after consumption of ARE or RSV **(Fig. 2B)**, although the levels of all of them seemed to be increased in RSV group when compared with both ARE and untreated groups.

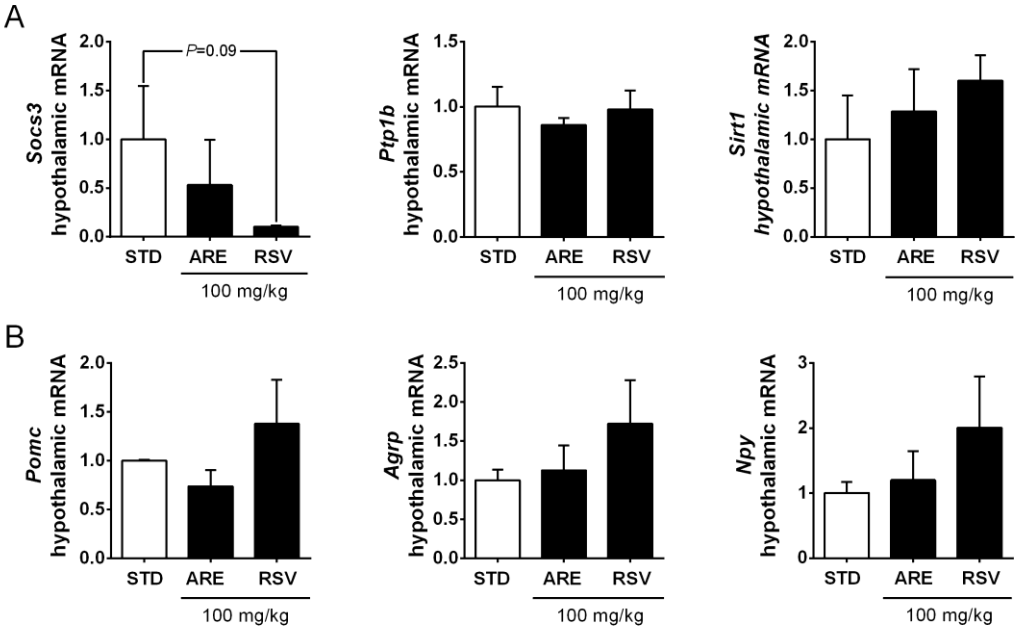

Figure 2. Hypothalamic gene expression in healthy mice. To further investigate leptin signalling, the expression of the genes involved in the signalling pathway was analysed in the hypothalamus of healthy mice. Quantitative PCR was used to determine the mRNA levels of the regulators of the leptin cascade *Socs3*, *Ptp1b* and *Sirt1* **(A)**, followed by the gene expression of anorexigenic (*Pomc*) and orexigenic neuropeptides (*AgRP* and *Npy*) **(B)**. mRNA levels of each gene were normalized to the constitutive gene *Ppia*. Values are mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

Finally, body weight, visceral adiposity, food intake and respiratory quotient (RQ) were not significantly modified after 15 days of treatment with either ARE or RSV (**Table 1**). However, the consumption of RSV significantly

increased 24-hour energy expenditure compared to both ARE-treated group and STD group.

Table 1. Body weight, visceral adiposity, food intake and indirect calorimetry measurements.

	STD	ARE	RSV
Body weight (g)	40.38±0.8	41.75±0.98	41.37±0.91
Visceral adiposity (%)	3.11±0.29	2.86±0.27	3.30±0.50
Food intake (g)	11.27±0.66	9.91±0.72	10.07±0.79
RQ	0.80±0.06	0.79±0.02	0.86±0.06
Energy expenditure (kcal/day/kg^0.75)	125.47±16.58^{ab}	119.94±6.32^{a}	155.13±6.82^{b}

Abbreviations: STD, standard chow diet; ARE, anthocyanin rich extract; RSV, resveratrol; RQ, respiratory quotient. Mice were fed a STD and were orally treated with vehicle (STD group), or either 100 mg/kg of ARE or RSV for 15 days. Visceral adiposity index was estimated for each animal as the sum of mesenteric, epididymal and perirenal fad pads, expressed as percentage of body weight. Values are the mean±SEM. Differences were assessed by one-way ANOVA followed by LSD post-hoc test.

High doses of resveratrol reduced circulating leptin levels and improved hypothalamic leptin sensitivity in obese rats

After performing the study in healthy mice, we decided to exclusively confirm the effect of RSV on hypothalamic leptin system using, at this time, diet-induced obese animals that presented both hyperleptinemia and impaired leptin sensitivity. For practical reasons and for similarity to humans, in this study we used Wistar rats fed cafeteria diet (CD) during 12 weeks instead of genetically obese mice[19]. Thus, the main goal was to observe if different doses of RSV could modulate hypothalamic leptin signalling in obesogenic conditions.

Accordingly, rats were divided in a low, intermediate and high dose groups (n=6) and were daily supplemented with 50 mg/kg (CD+50), 100 mg/kg (CD+100) or 200 mg/kg (CD+200) of RSV together with a cafeteria diet for 22 days.

At the end of the trial, circulating leptin levels in plasma showed a significant increase in CD group compared to the STD group **(Fig. 3A)**, indicating that our experimental model developed hyperleptinemia after 12 weeks of CD. Interestingly, at dose of 200 mg/kg/day, RSV consumption significantly reduced leptin levels compared to the CD animals, without any effect at lower doses.

Next, the activation of STAT3 was analysed by immunoblotting **(Supplementary Fig. 1)**. Contrary to our expectations, CD did not lead to a significant decrease in the basal levels of pSTAT3 in the hypothalamus, but when RSV was administered to obese rats, the levels of pSTAT3 significantly increased with respect to CD, although only the dose of 100 mg/kg/day of RSV reached statistical significance **(Fig. 3B).** Considering that hypothalamic pSTAT3 levels are mainly attributable to leptin action, next we performed a ratio between hypothalamic pSTAT3 and plasmatic leptin levels in order to estimate the degree of sensitivity to leptin of this tissue. In particular, the leptin sensitivity of CD animals was significantly reduced when compared to the STD group (**Fig. 3C**). However, we observed that, at dose of 200 mg/kg/day, RSV consumption significantly increased the hypothalamic leptin sensitivity, indicating that the consumption of high doses of this phenolic compound could be a valid tool to rescue leptin action in this tissue. In addition, the consumption of low and intermediate doses also increased the sensitivity to leptin, although no statistically significant changes were detected in comparison with the CD group.

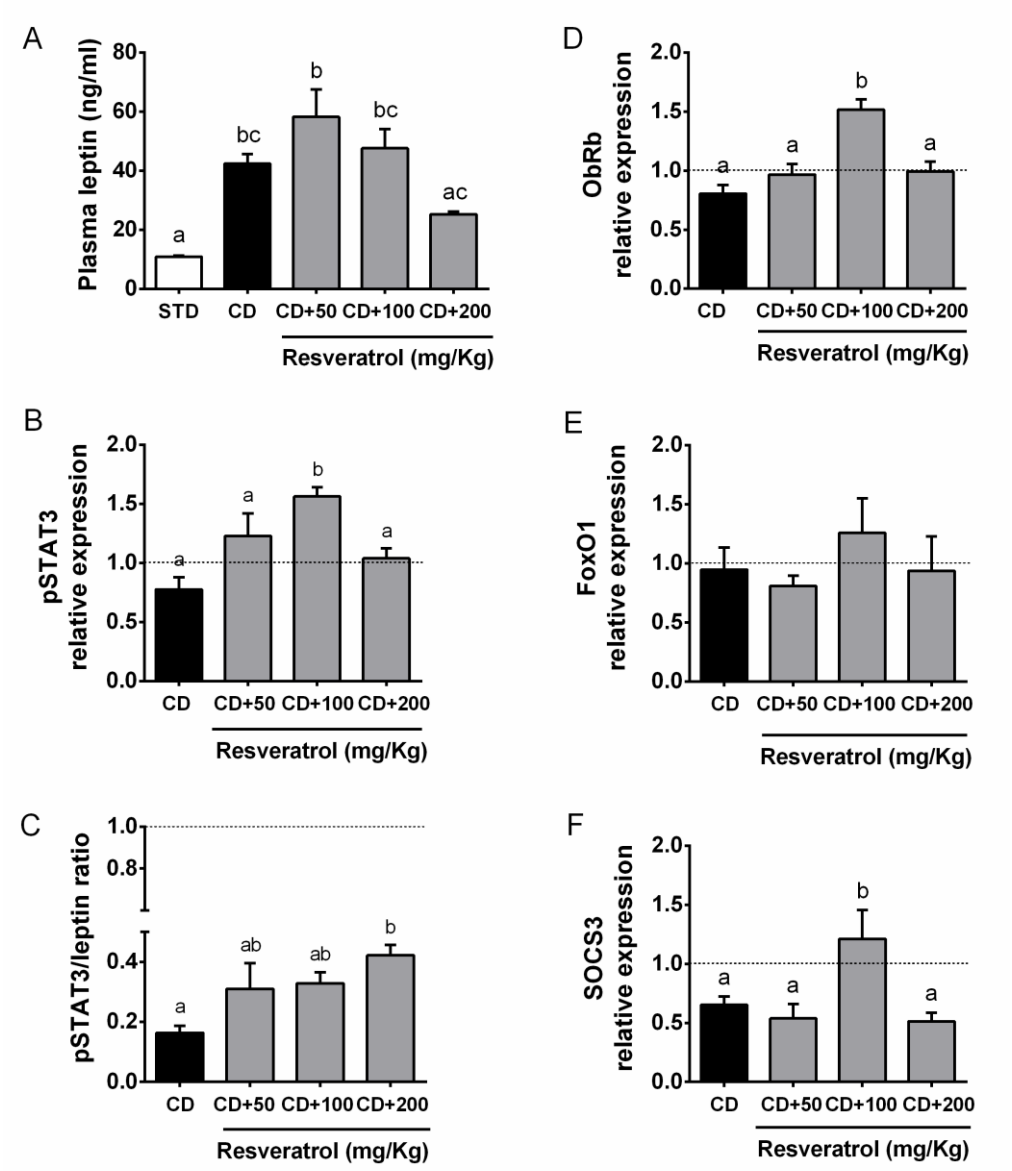

Figure 3. Plasma leptin levels and protein content of regulatory factors involved in leptin signalling in the hypothalamus. Plasma leptin levels of lean (STD) and obese animals treated with resveratrol (CD groups) were analyzed **(A).** Expression levels of pSTAT3 were evaluated by immunoblot in the hypothalamus, as a gold standard of leptin signalling **(B).** To determine the level of leptin sensitivity in this tissue a pSTAT3/leptin ratio was calculated **(C).** In addition the protein content of ObRb **(D),** FOXO1 **(E)** and SOCS3 **(F)** were determined by immunoblotting**.** Intensity signals of each protein were normalized by β-Actin

followed by normalization with the STD group, represented as a dotted line. Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CD+50), a intermediate dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

Next, we determined whether the modulation of hypothalamic leptin sensitivity was directly mediated by enhanced protein content of Obrb. Again, only the consumption of 100 mg/kg/day of RSV resulted in a significant increase in the protein levels of ObRb with respect to the CD group, and no statistically significant differences in the other doses were observed (**Fig. 3D**).

To further assess the effects of RSV on the regulation of the leptin system, the protein content of the negative regulatory factors FOXO1 and SOCS3 were also determined by immunoblotting (**Supplementary Fig. 1**). FOXO1 protein levels were not affected by RSV consumption at any dose (**Fig. 3D**) and only those animals consuming the dose of 100 mg/kg/day showed a significant increase in the protein levels of SOCS3 (**Fig. 3E**).

High doses of resveratrol reduced body weight and fat mass accumulation in obese rats by modulating 24h-energy expenditure but not food intake

RSV consumption increased hypothalamic leptin sensitivity in obese animals. Thus, we next evaluated whether these effects on leptin system could be related to changes in body weight, total body fat content and food intake.

At doses of 50 and 100 mg/kg/day, RSV did not significantly reduce body weight, indicating that the consumption of this phenolic compound for 22 days

did not exert any beneficial effects with respect to body weight at these doses **(Fig. 4A)**. In contrast, when animals were treated with 200 mg/kg/day, the body weight was significantly decreased with respect to CD group, and this reduction was associated with a significant decrease in total body fat mass **(Fig. 4B)**.

In contrast, our results showed that RSV consumption did not modify the levels of food intake in these animals **(Fig. 4C)**. In fact, we could not even detect a statistically significant reduction at dose of 200 mg/kg/day compared to the CD group, indicating that the reduction in body weight and fat mass observed in these animals were not due to a direct decrease in energy intake.

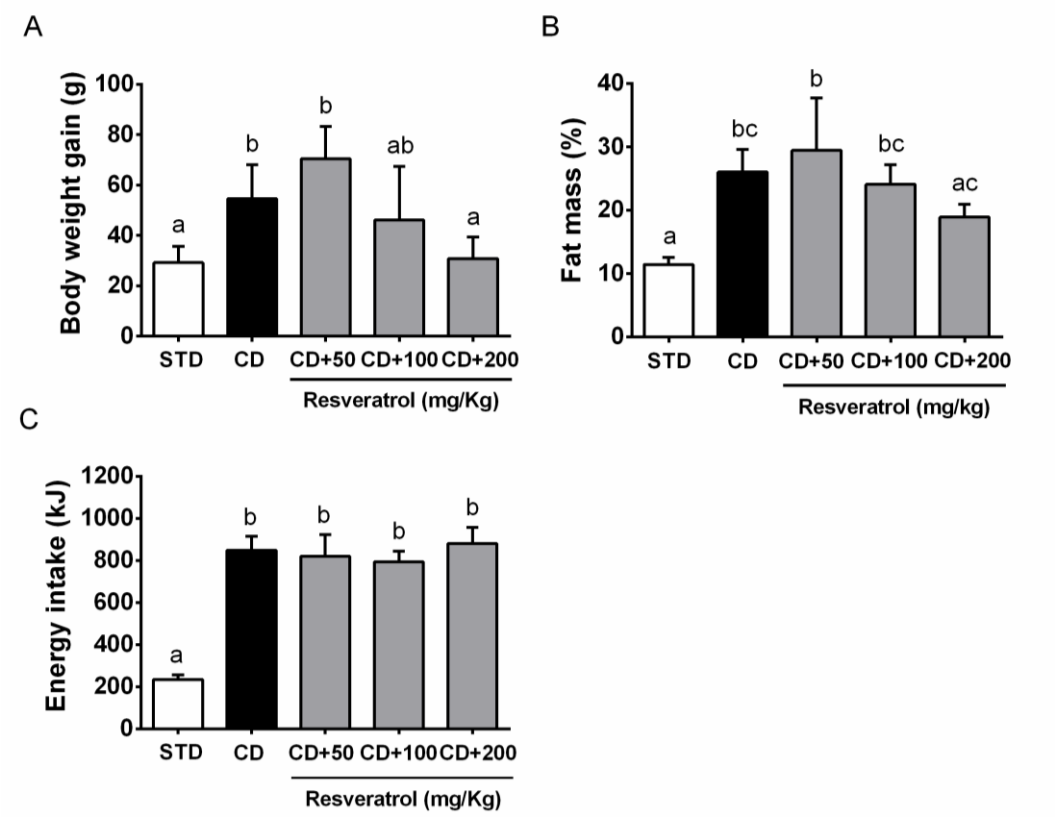

Figure 4. Effect of resveratrol treatment at different doses on body weight, fat mass and energy intake of obese rats. Body weight measurements were

taken the day of the sacrifice **(A),** fat mass was assessed one day before the sacrifice by magnetic resonance and is expressed as percentage of body weight **(B)** and energy intake refers to the last week of treatment **(C).** Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CD+50), a medium dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

Finally, we reasoned that if the consumption of RSV did not have significant effects on food intake, it could enhance 24 hours-energy expenditure and lead to the utilization of fat as the principal energy source. As expected, animals fed with standard chow diet used carbohydrates as a main energetic substrate and, therefore, presented RQ values very close to 1 (**Fig. 5A**). In contrast, the CD group presented lower RQ values compared to the STD group, indicating that they are not fully using carbohydrates since they have a diet rich in fat. Notably, RSV consumption significantly modulated RQ in a dose-dependent manner. Specifically, the consumption of RSV induced a significant decrease in RQ values in comparison to untreated animals, indicating that RSV consumption significantly potentiate the utilization of lipid as the principal energetic substrate. In fact, when the levels of both carbohydrates and lipid oxidation were analyzed by indirect calorimetry (**Fig. 5B and 5C**), RSV consumption at doses of 100 and 200 mg/kg/day significantly favoured 24 hours fat oxidation compared with untreated group. Surprisingly, at dose of 50 mg/kg/day, the consumption of RSV did not enhance lipid oxidation, confirming that, at this dose, RSV did not exert any beneficial effects in our experimental model.

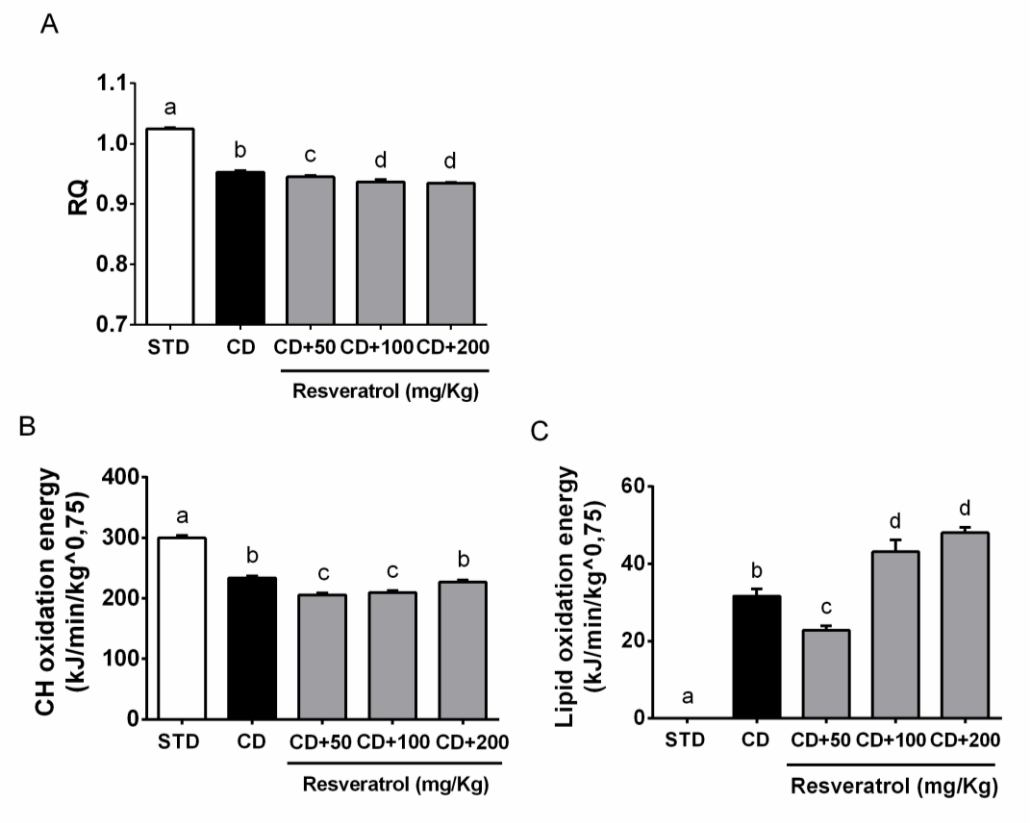

Figure 5. Effect of resveratrol treatment on RQ, carbohydrate oxidation and lipid oxidation of obese rats. In order to know how efficiently animals used the available energy, 24 hours-indirect calorimetry was performed as displayed with the respiratory quotient (RQ) **(A),** carbohydrate **(B)** and lipid **(C)** oxidation energy. Animals were fed a STD or a CD for 9 weeks. After this, STD group was supplemented with vehicle (n=6) and CD group (n=18) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CD+50), a medium dose of 100 mg/kg (CD+100) or a high dose of 200 mg/kg (CD+200) of resveratrol for a period of 22 days. Values are mean±SEM of 6 animals per group. Differences were assessed by one-way ANOVA followed by Tukey post-hoc test.

4. Discussion

Phenolic compounds have gained importance over the years for their beneficial effects in promoting health and preventing diseases[20]. Our group has special focus on these compounds and particularly has been investigating the effects of grape-seed proanthocyanidins (PACs) during last ten years. In this context, we have recently reported that a chronic treatment of 25mg/kg/day of PACs for three weeks in obese rats significantly decreased both food intake and circulating plasmatic leptin levels by presumably restoring the hypothalamic leptin signalling[10]. However, it is necessary to keep investigating other compounds with complementary or more powerful effects to fight against obesity and metabolic diseases. Thus, in the present study, we evaluated whether the consumption of other dietary phenolic compounds such as resveratrol (RSV) and anthocyanins are able to decrease body weight, food intake and leptinemia with significant changes in hypothalamic leptin signalling.

The hypothalamus is the metabolic tissue responsible for homeostatic regulation of body weight by modulating both energy expenditure and food (energy) intake. Under physiological conditions, leptin efficiently modulates the activity of hypothalamic POMC neurons to reduce meal size and to increase energy expenditure. However, in obesity, a decreased sensitivity to leptin occurs, resulting in an inability to control energy balance despite high energy stores[10]. For this reason, we investigated the effects of RSV and anthocyanins extract in both non-obese and obese animals in order to evaluate the effects of these compounds under physiological but also pathological conditions.

Despite anthocyanins consumption had demonstrated promising effects on hypothalamic energy balance regulation[12], our results in non-obese animals did

not give any indication that the consumption of 100 mg/kg/day of anthocyanins for 15 days could significantly affect hypothalamic leptin system. In fact, our data suggest that if anthocyanins have the ability to suppress body weight gain and body fat accumulation, their effects are not supported by an increase in hypothalamic leptin sensitivity. Remarkably, some authors have attributed the effects of anthocyanins to a direct interaction with adipose tissue inducing changes in both lipid synthesis and adipocytokines expression levels[10,11]. Moreover, as the bioavailability of anthocyanins is very low (about 0.1%)[14], further studies are needed to confirm the molecular mechanism of action of these compounds and to elucidate which phenolic metabolites derived from anthocyanins are responsible of their bioactivity[21].

Regardless of the current evidence that RSV has anti-obesity effects[22], majority studies have focused on the role of this compound in regulating adipogenesis, lipolysis, mitochondrial function and thermogenesis in peripheral organs such as liver, muscle and adipose tissues[23], but little is known about RSV effects on the modulation of hypothalamic energy homeostasis. In this context, our results show the ability of RSV to normalize plasmatic leptin levels and enhance hypothalamic leptin action in both obese and non-obese animals. Accordingly, it had been previously reported that the offspring of high-fat diet fed mice treated with 30 mg/kg/day of RSV rescued hyperleptinemia and increased pSTAT3 levels in ARC[24]. However, contradictory results have been widely published regarding the effect of this compound on circulating leptin levels. A recent meta-analysis of randomized controlled trials did not find changes on plasma leptin of obese and non-obese subjects supplemented with different doses of RSV. In contrast, other *in vitro*[25] and *in vivo*[26] studies showed a reduction of leptin levels. Possible issues explaining these controversial data could be the low bioavailability of this compound to reach the target tissues, the

different range of doses used and the treatment length[27]. In addition, many animal studies administrate RSV together with obesogenic diet when the animal is still not obese, producing a preventive effect rather than treating obesity once it has been developed[28]. Thus, from now on, human and animal studies should be designed in agreement to be able to compare the data and to obtain similar results.

Concomitant decrease in plasmatic leptin concentration with increasing hypothalamic pSTAT3 levels is consistent with higher leptin sensitivity in this tissue. Thus, we reasoned that if hypothalamic STAT3 phosphorylation is mainly consequence of leptin-induced activation of ObRb signalling, the levels of hypothalamic pSTAT3 (assessed by immunoblotting) referred to the levels of leptin in plasma could be a more reliable estimation of leptin sensitivity in this tissue. Using this estimation, our results showed for the first time that, at dose of 200 mg/kg/day, RSV consumption restored the hypothalamic sensitivity to leptin appropriately in obese rats. Several authors have indicated that the metabolic disturbances observed in animals fed a highly palatable diet are firmly related to a dysfunctional leptin system in the hypothalamus. Accordingly, our results showed that this restoration of leptin action in obese animals was concomitant to body weight loss that, in turn, was associated with increased 24h-energy expenditure and lipid oxidation but not with a reduction in food intake. Although, it has been reported that RSV is able to decrease food intake in rodents fed chow diet via modulation of melanocortinergic system[35], our results indicate that food intake in obese animals presumably is likely to be regulated not only by leptin signal pathway but also by other mechanisms such as ghrelin, cholecystokinin, glucagon-like-peptide-1 and peptide YY (PYY) signal pathways.

The mechanisms by which RSV increases energy expenditure still need to be clarified. Accordingly, Ramadori et al.[29] showed that RSV was able to mediate anti-diabetic effects in the brain after chronic intracerebroventricular infusion by increasing SIRT1 functionality, and we also observed a significant increase in hypothalamic *Sirt1* expression in our previous study with PACs[31]. In addition, its known that differently genetic overexpression of SIRT1 in mice reduces food intake and energy expenditure[30]. However, our results demonstrated that the consumption of RSV improve leptin sensitivity by probably up-regulating hypothalamic *Obrb* expression without any difference in *Sirt1* gene expression levels in non-obese animals, indicating that RSV is able to enhance hypothalamic response to leptin in a SIRT1-independent manner. In fact, the consumption of 100 mg/kg/day of RSV significantly increased the cellular content of ObRb in both obese and non-obese animals. Further studies are needed to confirm the role of SIRT1 in obesity.

As reported in other medicine fields, RSV could also have beneficial effects at lower doses to maintain health[43-45]. However, in regard to the lower doses of RSV tested in this study, only the consumption of 100 mg/kg/day showed a trend to reduce body weight and body fat content with slight but significant changes in hypothalamic leptin signalling as indicated by increased levels of both pSTAT3 and ObRb**.** In addition, at dose of 100 mg/kg/day, the consumption of RSV also produced higher hypothalamic levels of SOCS3 with respect to untreated obese animals. There is a controversial interpretation of SOCS3 in the literature because it negatively regulates leptin signalling**,** but also its transcription is activated by higher levels of pSTAT3, working as a negative feedback mechanism, and high levels of SOCS3 would indicate enhanced leptin signalling. Nevertheless, further studies are needed to confirm the effect 100 mg/kg/day of RSV on hypothalamic leptin system.

Although high doses of RSV have been a matter of concern, the toxicity and tolerability of doses ranging from 5 mg to 5 g has been widely examined in clinical trials[32]. In our study we worked within a dose range that is acceptable for rodents and that would be tolerated by humans. It was based on Body Surface Area (BSA) calculation[33] and human equivalent dose for an obese adult would not exceed 5 g per day. Studies supplementing high doses of RSV, state that it was well tolerated exempting some intestinal discomfort and that high doses may be needed since RSV is greatly metabolized which limits the availability in certain tissues[34,35]. In fact in rats, it has been reported that doses of 300 mg/kg are the maximum tolerated without detrimental effects[36]. The fact that RSV has different effects in a large range of concentration emphasizes that this compound affect different signalling pathways, and, therefore, it is essential to know the suitable dose in each case to achieve beneficial effects[37].

In conclusion, our study shows that the consumption of a high dose of RSV within a tolerable range in rodents is able to produce beneficial effects decreasing circulating leptin levels, body weight and body fat mass but not food intake in obese animals. These metabolic effects could be partly due to increased hypothalamic leptin sensitivity mediated by increased pSTAT3 signalling and ObRb protein content in the hypothalamus and, in consequence, enhanced 24h-energy expenditure and fat oxidation. Mechanisms of action should be investigated thoroughly to provide more accurate information about the anti-obesity effects of both resveratrol and anthocyanins.

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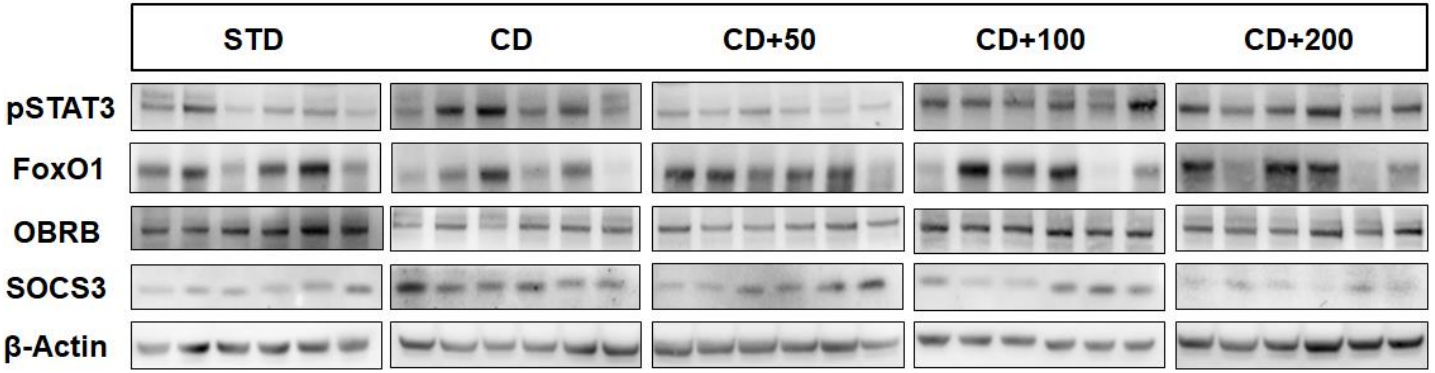
Supplementary materials

Table S1. Primer sequences used in qPCR amplification from mouse genes in hypothalamus.

Primer (Mouse)		Sequence 5'-3'	Product size (bp)	GenBank accession no
Obrb	**Fw**	GGGACGATGTTCCAAACCCC	132	NM_146146.2
	Rw	CAGGCTCCAGAAGAAGAGGAC		
Socs3	**Fw**	ACCAGCGCCACTTCTTCACG	171	NM_007707.3
	Rw	GTGGAGCATCATACTGATCC		
Ptp1b	**Fw**	GTCACCGGCTTCTTTCCTCA	130	NM_011201.3
	Rw	GTCAGCCAGACAGAAGGTCC		
Pomc	**Fw**	CCATAGATGTGTGGAGCTGG	134	NM_008895.3
	Rw	CCAGCGAGAGGTCGAGTT		
AgRP	**Fw**	AGTTGTGTTCTGCTGTTGGC	149	NM_007427.2
	Rw	CTGATGCCCTTCAGTGGAG		
Npy	**Fw**	ATACTACTCCGCTCTGCGAC	143	NM_023456.2
	Rw	GTGTCTCAGGGCTGGATCT		
Sirt1	**Fw**	GATGACAGAACGTCACACGC	110	NM_019812.3
	Rw	ATTGTTCGAGGATCGGTGCC		
Ppia	**Fw**	CTTCTGTAGCTCAGGAGAGCG	117	NM_008907.1
	Rw	CCAGCTAGACTTGAAGGGGAA		

Abbreviations: *Obrb*, leptin receptor isoform b; *Socs3*, suppressor of cytokine signaling 3; *Ptp1b*, protein tyrosine phosphate 1B; *Pomc*, proopiomelanocortin; *Agrp*, agouti-related protein; *Npy*, neuropeptide Y; *Sirt1*, NAD-dependen deacetylase sirtuin 1; *Ppia*, peptidylprolyl isomerase A; Fw, Forward; Rw, Reverse.

Figure. S1. Epression of proteins involved in leptin signalling cascade in the hypothalamus.



Abbreviations: STD, standard diet; CD, cafeteria diet; CD+50; cafeteria diet+50 mg/kg resveratrol; CD+100; cafeteria diet+100 mg/kg resveratrol; CD+200; cafeteria diet+200 mg/kg resveratrol pSTAT3, phosphorylated signal transducer and activator of transcription 3; FoxO1, forkhead box protein O1, OBRB, long form of leptin receptor; SOCS3, suppressor of cytokine signalling 3; β-Actin, beta actin isoform. Effect of different doses of resveratrol treatment on the hypothalamic leptin signalling pathway in animals fed a cafeteria diet. Leptin signalling pathway was assessed by evaluating the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3) and by the determination of the cell surface content of OBRB. Negative regulation of leptin signalling pathway was assessed by determining the expression of FoxO1 and SOCS3. β-Actin expression was used as a loading control.

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CHAPTER 3

Seasonal fruits consumption affects hypothalamic leptin signaling system in a photoperiod dependent mode

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In preparation

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Abstract

Leptin has a central role on the maintenance of energy homeostasis in the hypothalamus by which produces satiety and decreases energy expenditure through the modulation of neuropeptides including POMC, AgRP and NPY. Leptin secretion is influenced by photoperiod which is able to produce a switch on energy balance. For instance, seasonal animals exposed to long-day (LD) photoperiod show a decrease on leptin sensitivity. Similarly, changes in lifestyle in modern society are associated with altered eating patterns and the consumption of energy rich food that contributes to the obesity epidemic and metabolic diseases, in which cases leptin signaling is also attenuated. Dietary strategies to prevent metabolic diseases and promote a healthy life style include the consumption of fruits, since they are a rich source of nutrients and phytochemicals. However, the health implications of consuming fruits out of season have not been evaluated. The goal of this study is to investigate the effect of consuming seasonal fruits in different photoperiods on hypothalamic leptin signaling pathway and overall energy balance in animals fed a balanced or high-energy diet. Grapes and cherries are a rich source of polyphenols and are harvested in autumn (SD) and spring (LD) respectively. These fruits present similar phenolic composition with some differences that in turn can be highly influenced by environmental signals specific of each season. This fact is related to the Xenohormesis Hypothesis and how molecules produced by a mild environmental stress in plants can affect the animals that consume them. Therefore, the potential of this two fruits in modulating hypothalamic leptin signaling in different photoperiods could bring useful information on the regulation of energy balance and how to increase leptin sensitivity to prevent obesity.

1. Introduction

Leptin is a hormone produced by adipose tissue that has a key role in the central regulation of energy homeostasis[1]. The main target is the hypothalamic arcuate nucleus (ARC) where it activates anorexigenic neurons (proopiomelanocortin, Pomc) and inhibits orexigenic neurons (agouti related peptide, AgRP and neuropeptide Y, Npy), modulating second order neuron activity through melanocortin 4 receptor (MC4R)[2] and NPY1 receptor (NPY1R) [3]. As a result, leptin produces a satiating effect as well as an increase on energy expenditure in order to maintain body weight[4].

Remarkably, Leptin secretion follows circadian[5,6] as well as circannual [7] rhythms. Studies with mammals sensitive to photoperiods have shown that animals develop an adaptive leptin resistance in long-day periods to overcome periods of food scarcity[8,9]. This means that, despite presenting high leptin levels in blood, leptin is unable to produce anorectic effects, providing animals with a sufficient energy stores on the upcoming short-day seasons[8]. This has been considered an evolutionary mechanism for survival[7].

Westernized society developed inappropriate dietary patterns which contribute to the obesity epidemic[10] and fruit consumption is advised because fruits are a valuable source of nutrients with health promoting properties which supports their daily consumption[11]. Furthermore, fruit contains phytochemicals that despite not being essential for life can exert long-term beneficial effects. Among them, polyphenols are an important group of compounds present in fruits[12] and some studies demonstrate that specific polyphenols increase leptin sensitivity in obese animals[13–15]. Each fruit has a distinctive polyphenol

composition [16,17] that could determine its capacity to modulate the leptin system.

Notably, nowadays there is a broad fruit offer and people can choose to consume either seasonal or out-of-season fruits. However, the effects regarding the consumption of fruits in- or out-season on leptin sensitivity have not been studied yet. Accordingly, our goal was to mimic seasons by submitting animals to different photoperiods, short-day for autumn and long-day for spring, to investigate the effects of seasonal fruit intake on the leptin system. Because of the importance of leptin maintaining body weight, this study was performed in both lean and dietary-induced obese rats. Numerous studies report the metabolic protective effects of grape, grape by-products, cherries or their pure compounds[18–23], thus we have chosen red grape and cherry as representative fruits of autumn and spring, respectively. This approach might provide valuable information to design strategies of fruit consumption to counteract positive energy balance through the modulation of central leptin signaling pathway and downstream effectors.

2. Materials and Methods

2.1 Fruit characteristics and preparation

Royal Down sweet cherries (*Prunus avium* L.) were original from Argentina and were purchased in Mercabarna (Barcelona, Spain). Grapes (*Vitis vinifera* L.), Grenache variety were from Ribera de l'Ebre (Catalonia, Spain) and were gently gift by the producer. Cherry pits were removed whereas grapes were kept intact. Fruits were frozen in liquid nitrogen and later grinded to achieve homogeneity. Afterwards, the homogenates were lyophilized until reaching

dryness in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Barcelona, Spain) at -55 ºC. Lyophilized fruits were further grinded to obtain a fine powder. Cherry and grape powder was aliquoted and protected from humidity and light.

2.2 Animals

The study was approved by the Animal Ethics Committee of University Rovira i Virgili (reference number 4249 by Generalitat de Catalunya) and carried out according to ethical standards comprised in the Declaration of Helsinki.

2.2.1 Experimental design in lean animals

Male Fisher 344 rats of 8 weeks of age and 186±17g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were paired-housed, distributed in two different rooms, according to photoperiod, and fed a standard chow diet (Panlab 04, Barcelona, Spain) composed by a 72% carbohydrate, 8% lipid and 20% protein and water *ad libitum*. Photoperiod groups consisted in long-day (LD) (18:6h light:dark cycle) and short day (SD) (6:18h light:dark cycle) and kept at 22ºC. After an adaptation period of 4 weeks, animals in each photoperiod were weight-matched and distributed into 3 subgroups of 6 animals, the control group, and 2 groups supplemented with an oral dose of 100mg/Kg (diluted in water) of lyophilized grapes or cherries, for a period of 10 weeks. The control group was supplemented with the same volume of a sugar solution (10mg fructose/10mg glucose) to much the sugar present in lyophilized fruits.

2.2.2 Experimental design in diet-induced obese animals

Male Fisher 344 rats of 8 weeks of age and 216±15g body weight were purchased from Charles River Laboratories (Barcelona, Spain). The animals were paired-housed and distributed in two different rooms, according to the photoperiod, and fed a standard chow diet (Panlab 04, Barcelona, Spain) plus a cafeteria diet, composed by a 65% carbohydrate, 20% lipid and 14% protein and water *ad libitum*. Cafeteria diet consisted of bacon, carrots, cookies, foie-gras, muffins, cheese and milk with 22% sucrose (w/v). Animals were distributed in two photoperiod groups, LD and SD and kept at 22ºC. After an adaptation period of 4 weeks, animals in each photoperiod were weight-matched and distributed into 3 subgroups of 10 animals, the control group, and 2 groups supplemented with an oral dose of 100mg/Kg (diluted in water) of lyophilized grapes or cherries, for a period of 7 weeks. The control group was supplemented with the same volume of a sugar solution (10mg fructose/10mg glucose) to much the sugar present in lyophilized fruits.

In the two experiments, the treatment was administered at 09:00 am every day. The animals were sacrificed by decapitation at the start of light cycle (lights on at 09:00 am) taking no more than two hours to complete the sacrifice. Blood was collected and allowed to clot at room temperature. Serum was obtained after centrifugation (2000g, 15min, 4ºC), aliquoted and stored at -80ºC. The hypothalamus was dissected, weighted and frozen immediately in liquid nitrogen. Body weight and food intake were weekly measured.

2.3 Body composition analysis

The last week of the study, animals were echoed by magnetic resonance imaging (MRI) using EchoMRI-700 (Echo Medical Systems, LLC., TX, USA) to determine the lean and fat mass composition. Data is expressed as a percentage of total body weight.

2.4 Indirect calorimetry

After an acclimation period of 3h animals underwent 20h of indirect calorimetry analyses using Oxylet Pro System (Panlab, Cornellà, Spain) performed during the 7th week of treatment. The oxygen consumption (VO2) and carbon dioxide production (VCO2) measures provided information about the energy expenditure. The program software Metabolism 2.1.02 (Panlab) automatically calculated respiratory quotient (RQ) as VCO2/VO2 and energy expenditure as VO2x1.44x[3.815+(1.232xRQ] (Kcal/day/Kg^0.75) according to Weir equation[28]. A nitrogen excretion rate (n) of 135 μg/kg/min was assumed[29].

2.5 Serum leptin levels

Leptin concentration in serum samples was measured using a rat specific Enzyme Immunoassay kit (Millipore, Madrid, Spain), following manufacturer's protocol

2.6 Gene expression analyses

The hypothalamus was processed to extract total RNA using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) followed by RNeasy Mini Kit

(Qiagen, Barcelona, Spain) according to manufacturer's instructions. RNA quantity and purity was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only RNA samples with A260/A280 ratio ≥1.8 and A230/A260 ratio ≥2 were included in the study. Afterwards, RNA quality was assessed on a denaturing agarose gel stained with SYBR Green dye (Bio-Rad, Barcelona, Spain). Reverse transcription was performed to convert RNA to cDNA using the High-Capacity complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain). Gene expression was analyzed by Real-Time PCR, using the iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain), in the ABI prism 7900HT Real-Time PCR system (Applied Biosystems) using primers obtained from Biomers.net (Ulm, Germany) (Supplementary Table 1). Relative expression of each gene was calculated referring to *Ppia* and *Rplp0* housekeeping genes and normalized to Long-day vehicle (LD-VH) control group. □□Ct method was used and corrected for primer efficiency [30].

2.7 Statistical Analysis

The effect of fruit supplementation (F), photoperiod (P) or the interaction between the two variables (F*P) was evaluated by Two-way ANOVA. If a main effect was significant, differences between groups were further assessed using one-way ANOVA Tukey post-hoc, unless specified. In the absence of main effect but with a significant interaction between fruit and photoperiod, pairwise comparisons were calculated among photoperiod groups and fruit groups using a Student's *t* test. GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. The values are expressed as the means ± SEM. Grubb's test was used to determine outliers. $P \leq 0.05$ was considered significant.

3. Results

Photoperiod and fruit consumption modulated energy balance by altering energy expenditure in lean rats

First, we focused on the effect of photoperiod and fruit consumption on body weight, fat mass and energetic homeostasis.

Supplementing animals with 100mg/kg of grape or cherry for 10 weeks did not produce significant changes on body weight in any photoperiod, and the photoperiod itself did not affect this feature in control group (**Table 1**). However, control animals placed at SD had a significant lower fat mass than those placed at LD. Cherry consumption kept this photoperiod effect whereas grape consumption abolished it.

In order to know how efficiently animals used the available energy, cumulative food intake (**Table 1**) and energy expenditure (**Figure 1**) were analyzed. Photoperiod and cherry consumption did not alter food intake. In contrast, grape consumption significantly decreased food intake at SD.

Energy expenditure is expressed as oxygen inspired (VO2) throughout 20h. **Fig. 1A** shows the effect of photoperiod on the VO2 inspired, showing that animals placed in SD significantly increased VO2 inspired at all the times studied, indicating that animals spent more energy in SD than in LD**.** Grape consumption did not alter this pattern (**Fig. 1B and 1C**)**.** In contrast, cherry intake significantly increased the VO2 inspired in the animals placed at LD **(Fig. 1D),** without any alteration at SD (**Fig. 1E**). Thus, cherry consumption modulated energy expenditure in a dependent way of the photoperiod, increasing it only at LD.

Table 1. Effect of different photoperiod and fruits consumption on body weight gain, fat mass, cumulative food intake, energy balance and respiratory quotient of animals fed a standard chow diet.

	Photoperiod	Control	Grape	ANOVA[1]	Cherry	ANOVA[1]
Body weight gain (g)	**LD**	89.17±4.1	91.33±4.7	ns	83.80±5.0	ns
	SD	84.17±4.8	80.67±1.7		90.00±7.0	
Fat mass (%)	**LD**	14.38±0.7	14.40±1.2	ns	15.17±0.7	P
	SD	12.52±0.3	14.98±0.3		13.43±0.6	
Cumulative food intake (MJ)	**LD**	2.26±0.1	2.20±0.0	F	2.21±0.0	ns
	SD	2.26±0.0	2.14±0.0		2.23±0.1	
24h Energy balance (MJ)	**LD**	0.10±0.0	0.09±0.0	F, P	0.07±0.01	F, P
	SD	0.04±0.0	0.01±0.0		0.00±0.01	
24h RQ	**LD**	0.86±0.0	0.86±0.0	P	0.83±0.01	P
	SD	0.80±0.0	0.82±0.0		0.78±0.01	

Abbreviations: LD, long day; SD, short day; RQ, respiratory quotient; Energy balance; (energy intake-energy expenditure); ns, nonsignificant. Animals in each photoperiod were fed with standard chow diet and supplemented with vehicle (Control group), grape or cherry at 100mg/kg for a 10-week-period. Body weight and food intake was monitored weekly. Fat mass was assessed by MRI during the last week together with the indirect calorimetry analyses to determine RQ and energy expenditure. Values are presented as mean±SEM of six animals per group. [1]Denotes two-way ANOVA analysis. P, photoperiod effect; F, fruit effect, assessed by two-way ANOVA ($P \leq 0.05$).

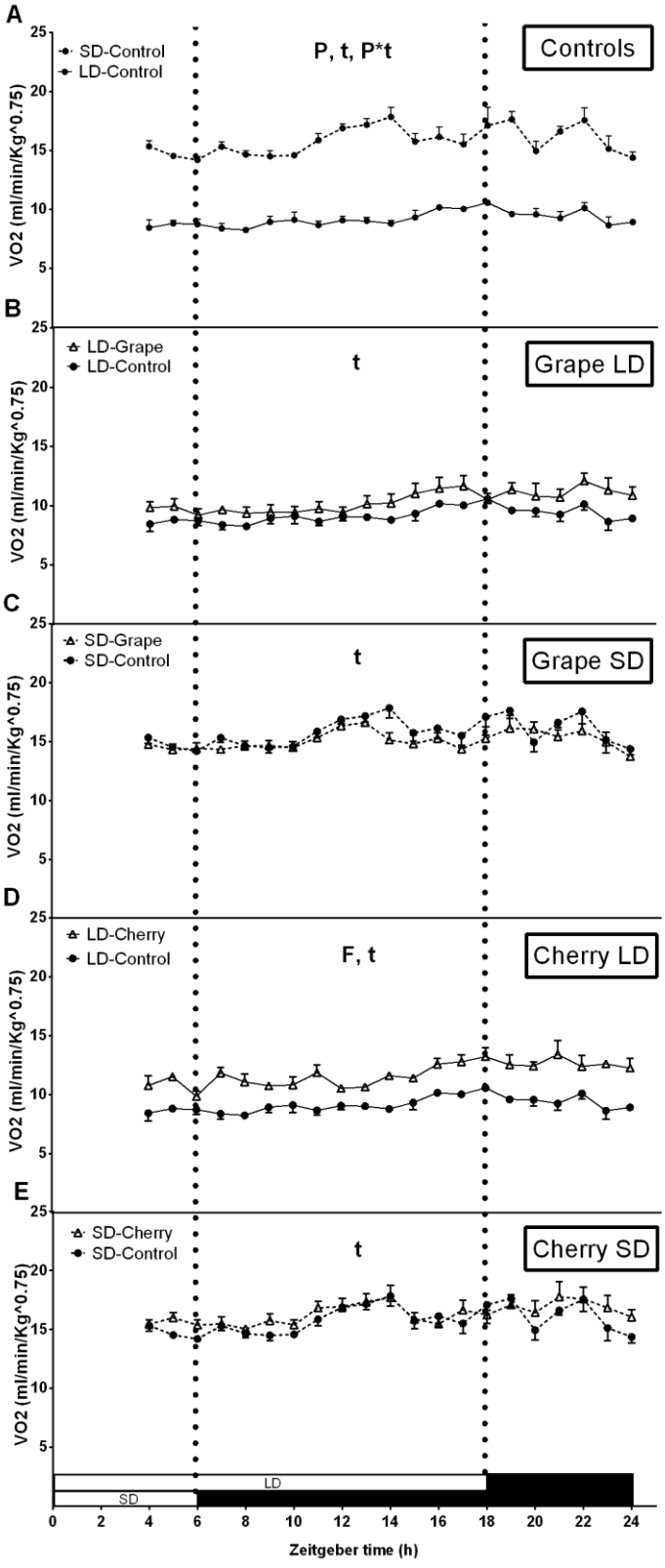
A
SD-Control
LD-Control
P, t, P*t
Controls
B
LD-Grape
LD-Control
t
Grape LD
C
SD-Grape
SD-Control
t
Grape SD
D
LD-Cherry
LD-Control
F, t
Cherry LD
E
SD-Cherry
SD-Control
t
Cherry SD
VO2 (ml/min/Kg^0.75)
LD
SD
Zeitgeber time (h)

Figure 1. Oxygen consumption (VO2) measures assessed by indirect calorimetry analyses. 20 hours of indirect calorimetry were measured in animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A-E).** First, VO2 of the controls in SD and LD are displayed **(A).** Grape effect on VO2 during LD **(B);** Grape effect on VO2 during SD **(C);** Cherry effect on VO2 during LD **(D)** and Cherry effect on VO2 during LD **(E).** Values are mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*t, interaction of fruit treatment and time; P*t, interaction of photoperiod and time, assessed by two-way ANOVA ($P \leq 0.05$).

24-hour energy balance was estimated from the values of 24 hours energy expenditure and food intake (**Table 1**). Animals placed at SD presented a significant reduction in its energy balance, and this effect was magnified by grape or cherry consumption. These results indicate that SD directed energy homeostasis towards a zero balance whereas LD did towards a positive one. Furthermore, animals placed in SD presented a significant reduction on RQ (**Table 1**) meaning that the contribution of carbohydrates and lipids, as energetic substrates, depended of the photoperiod in the sense that LD favored carbohydrate whereas SD favored lipids use.

Altogether, these results indicate that SD, compared to LD, put healthy lean animals on a higher energy expenditure and favoured lipid use as energetic substrate, thus resulting in reduced energy balance and lower fat mass. Fruit consumption kept this global photoperiod pattern. However, cherry consumption also increased energy expenditure when was consumed at LD whereas grape consumption decreased food intake and blocked the fat mass drop when was consumed at SD.

Photoperiod and fruit consumption modulated serum leptin levels and *Pomc* expression in the hypothalamus of lean rats

Leptin controls body fat mass, food intake and energy expenditure. Thus, the next goal was to investigate whether photoperiod and/or fruit consumption could change these parameters through the modulation of leptin levels and/or central leptin sensitivity.

Photoperiod significantly affected serum leptin concentration, increasing its levels at LD (Figure 2A and 2C). Despite no significant effects were observed on leptin levels by fruit intake, grape and cherry consumption magnified the increase induced by the LD photoperiod.

Central leptin sensitivity was predicted by analyzing the expression of the leptin receptor (*Obrb*), the negative regulators *Socs3* and *Ptp1b*, and the neuropeptides regulated by leptin in the hypothalamus.

The expression of *Obrb*, *Socs3* and *Ptp1b* was not modified by photoperiod or grape consumption (Fig. 2B). In contrast, cherry significantly affected *Obrb* expression in a way that was dependent of the photoperiod, increasing or decreasing it at SD or LD, respectively (Fig. 2D). Furthermore, cherry consumption resulted in a significant overexpression of *Socs3* in both LD and SD photoperiods.

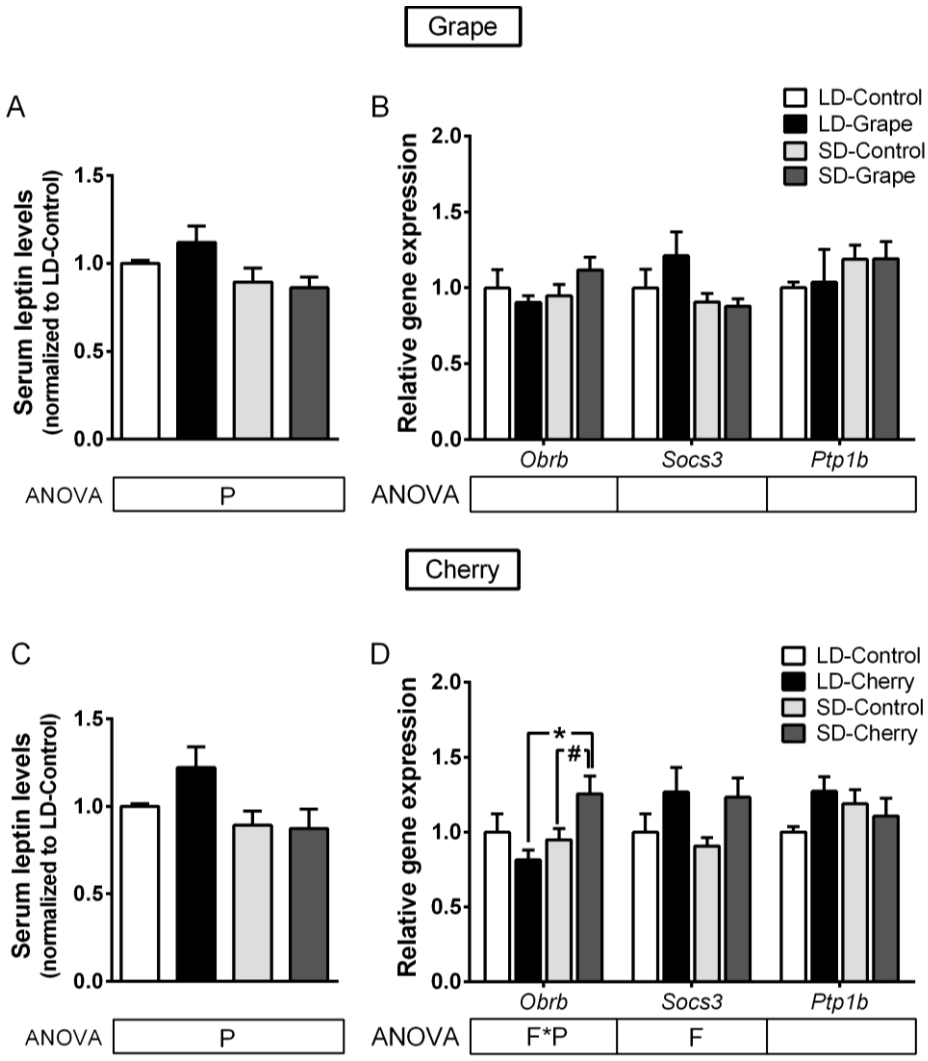

Figure 2. Effect of photoperiod and seasonal fruit supplementation on leptin signaling pathway. Serum leptin was measured in animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle. **(A, C).** Afterwards, gene expression of the long form of leptin receptor (*Obrb*), negative regulator molecules *Socs3* and *Ptp1b* were analysed by qPCR**(B, D).** Values are normalized against the LD-Control group for leptin concentration and gene expression. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA (*P* ≤0.05). *****Effect of photoperiod in the fruit treated groups; **#**effect of fruit in the photoperiod group determined by Student's *t* test (*P*≤0.05).

Further, we determined the expression of *Pomc*, the precursor of the anorexigenic neuropeptide α-MSH, and orexigenic neuroppetides *Agrp* and *Npy*, all of them produced by first order neurons in the ARC under leptin regulation. In addition, the expression of *Mc4r* and *Npy1r* receptors was examined as main targets of α-MSH, AgRP and NPY in second order neurons. Interestingly, grape and cherry intake remarkably increased *Pomc* expression (20 times) only at SD (**Fig. 3A and 3B**). Thus, the effect of grape and cherry was clearly dependent on the photoperiod where they were consumed. In addition, cherry consumption significantly increased *Agrp* expression in both photoperiods (**Fig. 3B**)**.** Photoperiod or fruit consumption did not modify the expression of Npy, *Mc4r* and *Npy1r*.

From these results stand out that SD, compared to LD, was associated to lower serum leptin. Remarkably, the consumption of each fruit strongly increased this photoperiod *Pomc* expression pattern, inducing *Pomc* overexpression when fruits were consumed at SD. In addition, cherry consumption increased *Agrp* expression at both photoperiods. Only cherry consumption was able to modulate the expression of some leptin-signalling components, such as *Obrb* and *Socs3*. Thus, the consumption of cherry modulated the leptin system with higher intensity than grape.

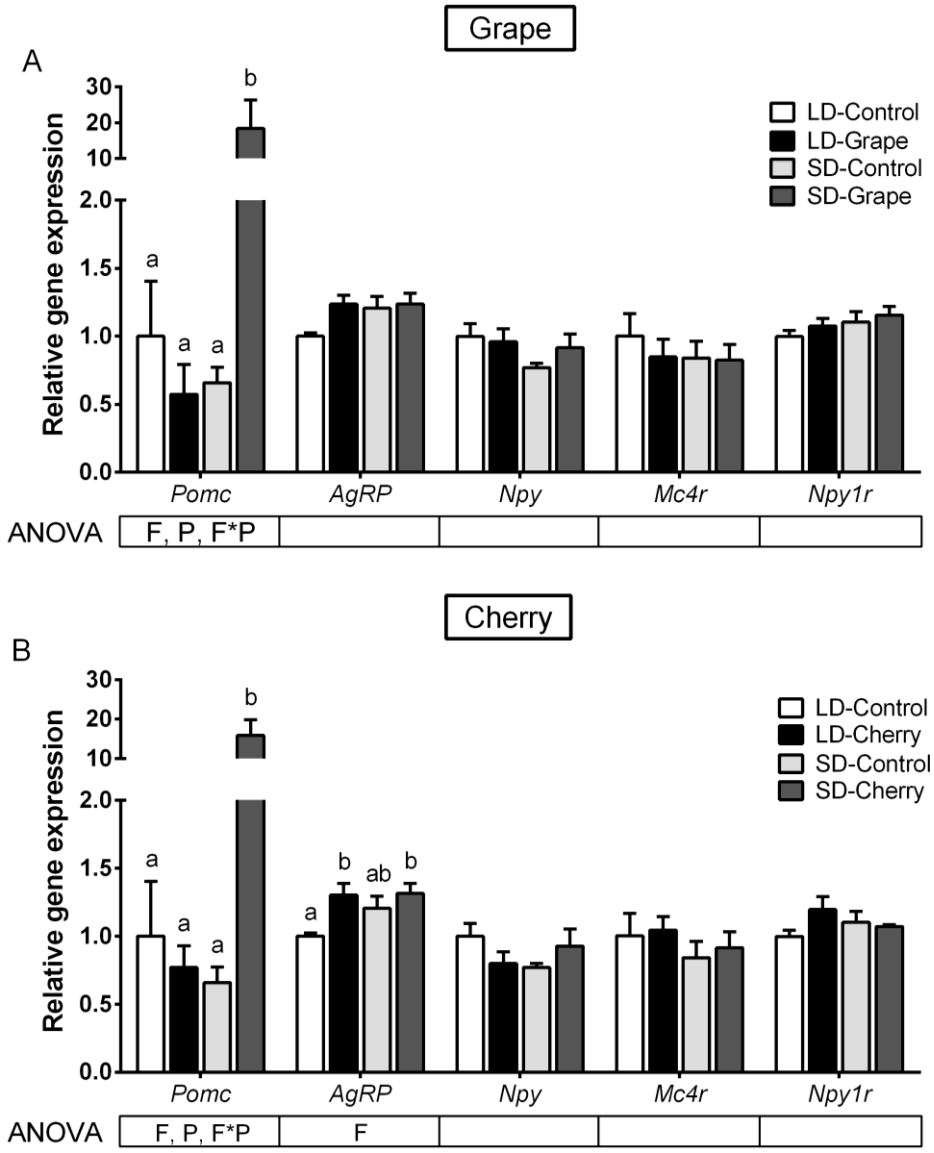

Figure 3. Effect of photoperiod and seasonal fruit supplementation on hypothalamic neuropeptides regulated by leptin. Hypothalamic gene expression of neuropeptides was evaluated by qPCR on animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 10-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, B).** Anorexigenic *Pomc* and orexigenic *Agrp* and *Npy* mRNA levels were determined, together with the mRNA's of receptors involved in energy balance *Mc4r* and *Npy1r* . Values are normalized against the LD-Control group. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of

photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$).

Photoperiod and fruit consumption modulated energy balance by altering energy intake in diet-induced obese rats

The observation that photoperiod and fruit consumption conditioned fat mass and energy balance in healthy and lean rats, prompted us to investigate how fruit and photoperiod could affect energy homeostasis in obesity. Rats were fed a cafeteria diet for 7 weeks together with 100 mg/kg of lyophilized grape or cherry.

Table 2 shows the effect of photoperiod and fruit consumption on body weight, fat mass, cumulative energy intake, energy balance and RQ. Photoperiod or fruit consumption did not modulate body weight, like in lean animals. In addition, body fat mass, which was reduced by SD photoperiod in lean animals, was neither affected by photoperiod or fruit consumption.

Obese rats significantly decreased their cumulative energy intake **(Table 2**) when placed at SD and grape consumption did not modify this photoperiod pattern. Remarkably, cherry consumption even reduced the cumulative energy intake when it was consumed at LD. Furthermore, photoperiod or fruit consumption did not significantly altered energy expenditure, measured as VO2 inspired (**Figure 4**). As expected, 24-hour energy balance **(Table 2)** in obese rats was positive in all conditions, but was lower in animals placed at SD, like in lean animals. Interestingly, cherry also significantly reduced energy balance when was consumed at LD. All these data indicate that the lower 24h-energy balance experimented by obese rats placed at SD was consequence of a reduced

energy intake. This fact totally diverges of that observed in lean animals, in which energy balance was lower at SD as consequence of an increase of energy expenditure.

Table 2. Effect of different photoperiod and fruit consumption on body weight gain, fat mass, cumulative food intake, energy balance and respiratory quotient of animals fed cafeteria diet.

	Photoperiod	Control	Grape	ANOVA[1]	Cherry	ANOVA[1]
Body weight gain (g)	**LD**	113.31±5.4	128.36±4.3	ns	121.09±3.2	ns
	SD	116.74±6.6	117.39±4.3		119.70±5.9	
Fat mass (%)	**LD**	22.03±0.6	22.57±0.6	ns	21.70±0.7	ns
	SD	21.53±0.8	21.10±1.3		21.82±1.0	
Cumulative food intake (MJ)	**LD**	5.79±0.2	5.72±0.2	P	5.13±0.2	F
	SD	5.43±0.2	5.20±0.1		5.01±0.1	
24h Energy balance (MJ)	**LD**	0.83±0.1	0.79±0.0	P	0.40±0.1[#]	F, F*P
	SD	0.60±0.1	0.46±0.1		0.55±0.1	
24h RQ	**LD**	0.78±0.0	0.78±0.0	P	0.79±0.0	F*P
	SD	0.81±0.0	0.80±0.0		0.78±0.0[#]	

Abbreviations: LD, long day; SD, short day; RQ, respiratory quotient; Energy balance; (energy intake-energy expenditure); ns, nonsignificant. Values are presented as mean±SEM of six animals per group. [1]Denotes two-way ANOVA analysis. P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Pairwise comparisons for interactions were determined by Student's *t* test. #effect of fruit in the photoperiod group determined by Student's *t* test ($P \leq 0.05$).

In addition, photoperiod also modulated RQ in obese animals. Specifically, obese animals placed at SD presented a significant increase on RQ (**Table 2**), indicating that LD photoperiod favored lipid whereas SD favored carbohydrate use as energetic substrates in obesity, a situation totally opposed as those observed in lean rats. In addition, in obese animals, cherry consumption at SD significantly decreased RQ.

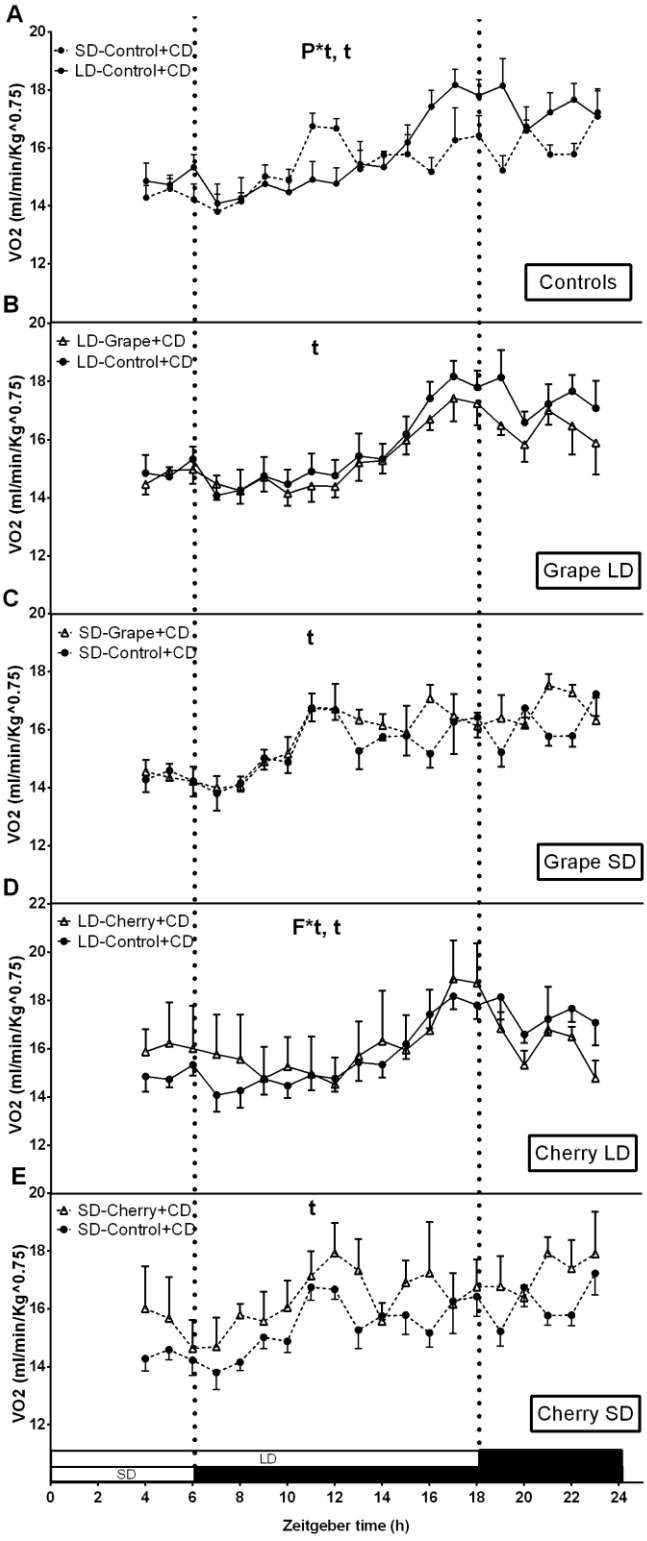
A
SD-Control+CD
LD-Control+CD
P*t, t
Controls
B
LD-Grape+CD
LD-Control+CD
t
Grape LD
C
SD-Grape+CD
SD-Control+CD
t
Grape SD
D
LD-Cherry+CD
LD-Control+CD
F*t, t
Cherry LD
E
SD-Cherry+CD
SD-Control+CD
t
Cherry SD
VO2 (ml/min/Kg^0.75)
SD
LD
Zeitgeber time (h)

Figure 4. Oxygen consumption (VO2) measures assessed by indirect calorimetry analyses. 20 hours of indirect calorimetry were measured in animals fed cafeteria diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A-E).** First, VO2 of the controls in SD and LD are displayed **(A).** Grape effect on VO2 during LD **(B);** Grape effect on VO2 during SD **(C);** Cherry effect on VO2 during LD **(D)** and Cherry effect on VO2 during LD **(E).** Values are mean±SEM (n=6). F*t, interaction of fruit treatment and time; P*t, interaction of photoperiod and time, assessed by two-way ANOVA ($P \leq 0.05$).

All these results indicate that SD, compared to LD, put obese rats on a lower energy intake and favored carbohydrate use as energetic substrate, resulting in reduced energy balance that was not reflected on body weight or fat mass. Grape consumption kept this photoperiod pattern, but cherry consumption decreased RQ at SD and even reduced the cumulative energy intake when it was consumed at LD. Comparing these data with that of lean animals, it is evident that the response of obese animals to photoperiod and fruit consumption strongly diverged from that of lean animals.

Photoperiod and food consumption modulated Socs3 and *Agpr* expression in the hypothalamus of diet-induced obese rats

Next, we studied whether photoperiod and/or fruit consumption modulated leptin system in obesity, and thereby the cumulative food intake.

First, we analyzed serum leptin. All obese rats showed the same levels. Thus, remarkably, feeding animals with the cafeteria diet eliminated the photoperiod serum leptin response observed in lean animals **(Fig. 5A and 5C).**

The potential of leptin signaling pathway in the hypothalamus of obese rats was evaluated by determining the expression of *Obrb* as well that of *Socs3* and *Ptp1b*, the negative regulators. Interestingly, *Socs3* was sensitive to photoperiod, increasing its expression in obese rats placed at SD (**Fig. 5B and D**). Grape consumption magnified (**Fig. 5B**) whereas cherry consumption softened (**Fig. 5D**) this overexpression of *Socs3* when consumed at SD. Furthermore, cherry consumption significantly repressed *Ptp1b* expression when it was consumed at SD. Thus, the gene expression of the leptin-signalling cascade was more sensitive to photoperiod and fruit consumption in obese than in lean rats.

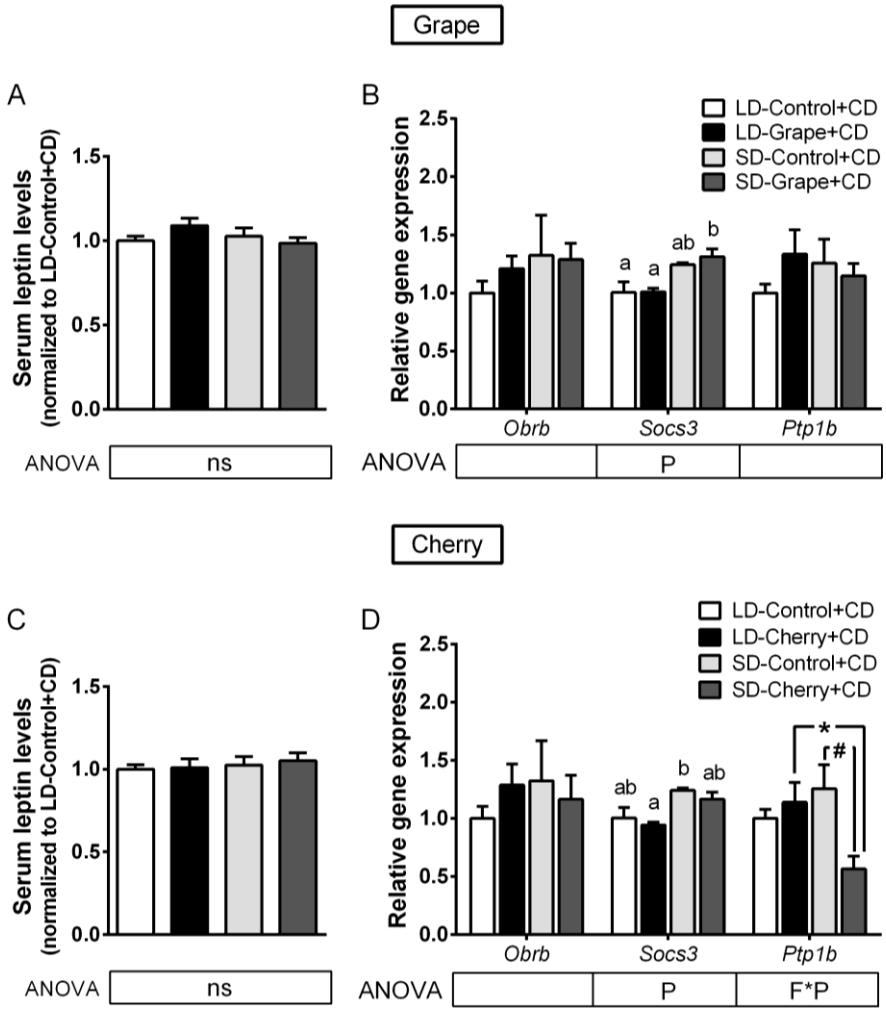

Figure 5. Effect of photoperiod and seasonal fruit supplementation on leptin signaling pathway in DIO rats. Serum leptin was measured in animals fed a cafeteria diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, C).** Afterwards, gene expression of the long form of leptin receptor (*Obrb*), negative regulator molecules *Socs3* and *Ptp1b* were analysed **(B, D).** Values are normalized against the LD-Control group for leptin concentration and gene expression. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$). *****Effect of photoperiod in the fruit treated groups; **#**effect of fruit in the photoperiod group determined by Student's *t* test ($P \leq 0.05$).

After analyzing the expression of the main factors involved in leptin signaling, we determined the hypothalamic mRNA levels of downstream neuropeptides and their receptors. Placing rats at SD induced a marked overexpression of the orexigenic *Agrp* neuropeptide (**Figure 6A and B**). However, the consumption of both fruits at SD prevented this *Agrp* overexpression. Besides, cherry consumption also modulated the expression of Mc4r and Npy1r, the neuropeptide receptors in second order neurons, repressing their expression when cherry was consumed at SD (**Fig. 6B**).

These results point out that SD, compared to LD, was linked to the overexpression of *Socs3* and *Agrp* in the hypothalamus. In contrast, the consumption of each fruit strongly reversed this photoperiod expression pattern of *Agrp*, repressing its overexpression when fruits were consumed at SD. The comparison of these figures with those of lean animals evidences that photoperiod and fruit consumption modulated the leptin system in both obese and lean states, but in a different way that was by altering *Agrp* expression in obesity whereas by modulating *Pomc* expression in lean animals. Like in lean

animals, cherry consumption was more powerful than grape consumption modulating leptin system in the hypothalamus.

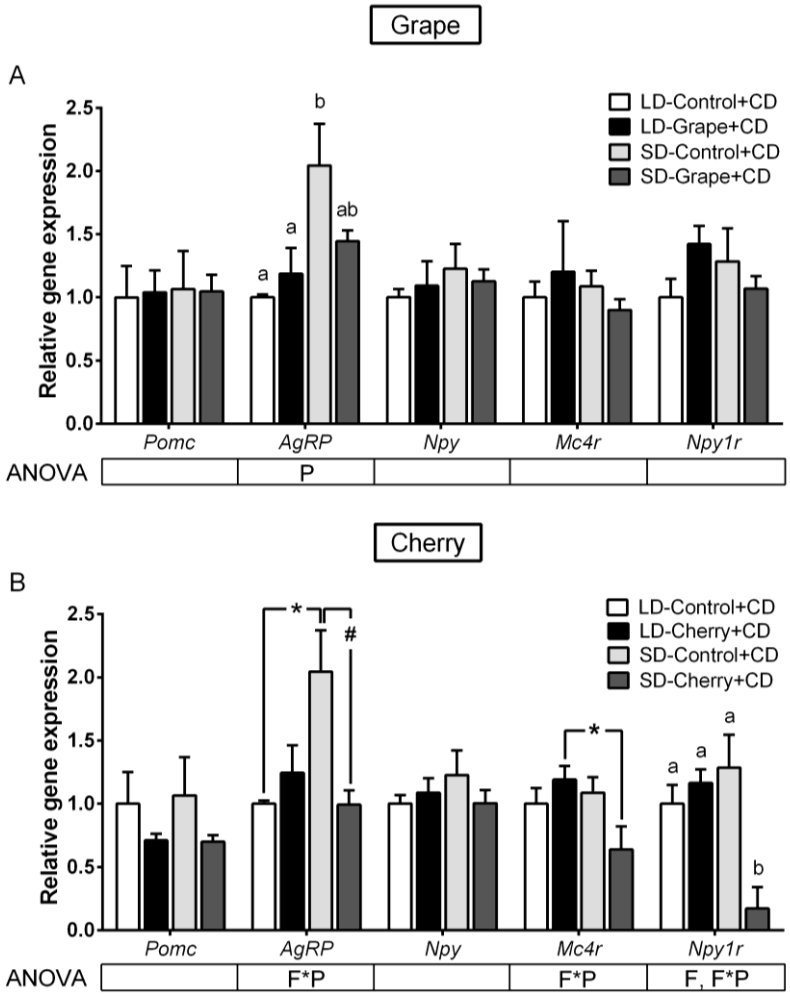

Figure 6. Effect of photoperiod and seasonal fruit supplementation on hypothalamic neuropeptides regulated by leptin. Hypothalamic gene expression of neuropeptides was evaluated by qPCR on animals fed standard chow diet + vehicle or 100 mg/kg of lyophilized grapes or cherries for a 7-week-period and submitted to long-day (LD) or short-day (SD) light cycle **(A, B).** Anorexigenic *Pomc* and orexigenic *Agrp* and *Npy* mRNA levels were determined, together with the mRNA's of receptors involved in energy balance *Mc4r* and *Npy1r* . Values are normalized against the LD-Control group. Data represents the mean±SEM (n=6). P, photoperiod effect; F, fruit effect; F*P, interaction of

photoperiod and fruit treatment assessed by two-way ANOVA ($P \leq 0.05$). Different letters denote significant changes assessed by one-way ANOVA and Tukey post-hoc test ($P \leq 0.05$). ***** Effect of photoperiod in the fruit treated groups; **#** effect of fruit in the photoperiod groups determined by Student's *t* test ($P \leq 0.05$).

4. Discussion

Great attention has been paid to calorie consumption and diet composition in order to improve metabolic health and decrease obesity[24]. Hitherto, there is a lack of studies focusing on seasonal foods and their detrimental or beneficial effects when they are consumed in or out-of-season. Seasonality plays an important role in all the organisms and the environment to maintain the life balance[25]. Here, we present the results of this novel approach showing that seasonal fruits can modulate the leptin system depending on the photoperiod where they are consumed, both in healthy and obese animals.

Siberian Hamster (*Phodopus sungorus*) is the most widely used animal model to study photoperiod responses[26]. However, in this study we have opted for the Fisher 344 strain rat because rat metabolism is closer to humans[27] and this strain is sensitive to photoperiods[28].

Lean animals presented the characteristic phenotype described for Fisher344 rats placed at LD or SD photoperiods, displaying lower fat mass[29,30] and leptin serum[29,31] levels at SD. However, these rats did not present the significant reduction of body weight described as typical for the SD photoperiod[29,30]. But, remarkably, animals can become refractory to SD after a long exposure to this photoperiod in order to attain reproduction[32]. Specifically, this time is considered 10 weeks for Fisher344 rats[33] and rats in our experiment were kept 14 weeks at SD, thus they could be in a refractory state. However, we selected 4

weeks of adaptation to the photoperiod plus 10 weeks of fruit treatment because we aimed to find out the long-term effect of seasonal fruit consumption in animals already adapted to a specific photoperiod.

The fat mass reduction observed in lean rats placed at SD was associated with a lower energy balance (near to zero) that can be ascribed to increased energy expenditure rather than reduced food intake, such as has been described by other authors[29,33]. Studies analyzing energy expenditure in response to photoperiod exposure are scarce and mainly in Siberian hamster. In this rodent spices, energy expenditure increases during the first 2 weeks whereas decreases after 8 weeks of exposure to SD [34]. Leptin is essential on the regulation of central energy homeostasis[35]. However, any of the leptin-signaling components studied, the neuropeptides regulated by leptin or their receptors, in second order neurons, altered their expression in the hypothalamus of lean animals at SD. Thus, other hormones, such as prolactin[36], or an increase of leptin sensitivity[37,38], induced by other leptin signaling components not determined in this study, could participate in this higher energy expenditure observed in SD. In this sense, small rodents, such as hamster or the field vole (*Microtus agrestis*), develop leptin resistance in LD by decreased pSTAT3[38] that has not been quantified in the present study.

Our objective is not to directly compare lean versus obese animals rather than describe fruit and photoperiod effects in the two models separately. However, it is important to stand up that the response of obese animals to photoperiod quite diverged from that of lean animals. Few studies have focused on the photoperiod effects on animals fed an obesogenic diet. However, a study using Fisher 344 rats fed with a high-fat diet during 4 weeks[29] agrees with our findings referring to both, the loss of photoperiod regulation of fat mass and the photperiod regulation of food intake, decreasing it in SD. Thus, obese rats in the

present study reduced their energy balance at SD as consequence of a reduced cumulative energy intake, without any modification on energy expenditure. In addition, the photoperiod effect on serum leptin levels was blunted when animals were on the obesogenic diet despite the leptin system was sensitive to photoperiods in obese animals, in the sense that SD was associated to *Socs3* and *Agrp* overexpression in the hypothalamus. Remarkably, it has been described that leptin resistance associated to LD is caused by high serum leptin levels together with Socs3 overexpression[38] whereas leptin sensitivity accompanying to SD is associated to *Agrp* downregulation in the arcuate nucleus[36]. Thus, the pattern of the leptin system in rats fed the cafeteria diet, together with the high serum leptin levels in SD, indicate that obese rats have lost the capacity to modulate their leptin sensitivity according to photoperiods and strongly suggest that obese rats were even more leptin resistant in SD that in LD.

From all seasonal fruits, red grape was selected as representative fruit of autumn (SD) and cherry as representative of fruit of spring (LD) because their high content on polyphenols[39–41]. Red grapes contain mainly flavonoids including anthocyanins, flavanols (monomers and proanthocyanidins), flavonols and non-flavonoids such as phenolic acids and stilbenes (mainly resveratrol)[40,41]. Cherries also contain flavonoids which include anthocyanins, flavanols (monomers and proanthocyanidins) and non-flavonoids such as phenolic acids (mainly hydroxycinnamates)[42,43]. Evidently, grape and cherry contain similar classes of polyphenols; however cherries contain larger amounts of anthocyanins and hydroxycinnamic acids compared to grapes, but lack the flavonols and stilbenes[17]. Moreover, red grape and cherry also diverge in the individual polyphenol compounds of each polyphenol class[17].

The consumption of both fruits induced a lower energy balance in lean animals placed both at SD or LD, indicating that this fruit attribute is photoperiod

independent. Remarkably, grape and cherry consumption reduced the energy balance through different ways, grape reducing food intake whereas cherry increasing energy expenditure. The anorexigenic effect of grape consumption agrees with previous reported results demonstrating that a grape seed proanthocyanidin extract reduces food intake in obese animals[13].

Interestingly, both cherry and grape intake resulted in the overexpression of *Pomc in* the hypothalamus when they were consumed at SD. Thus, this enhanced anorexigenic effect of both fruits could account, at least partially, for the lower energy balance observed in rats consuming fruits at SD. However, the fact that *Pomc* was only modulated in SD points out the photoperiod dependent capacity of these fruits to modulate leptin signaling. In addition, cherry consumption also modulated hypothalamic *Obrb* expression in a photoperiod dependent manner, increasing its expression in SD. Thus, cherry clearly increased central leptin sensitivity out of season in lean animals. In contradiction, cherry intake was associated with *Socs3* and *Agrp* overexpression in the hypothalamus at both photoperiods, both markers of leptin resistance. The increase of the last could be due to higher sensitivity to cherry polyphenols since the majority of AgRP neurons are found outside of the blood brain barrier[44].

Remarkably, neither the energy balance reduction nor the leptin sensitivity modulation induced by fruits were reflected in the body weight or fat mass accretion, which were not affected or even increased such as fat mass after grape consumption at SD. Thus, cherry and mainly grape should modulate other mechanisms that, in turn, counteract the expected fat mass reduction. In this sense, grape seed proantocyanidins have adipogenic activity, overexpressing PPARγ, increase adipocyte number and reduce adipocyte hypertrophy in visceral and subcutaneous fat pads[45]. Thus, grape could act at adipocyte level,

counteracting the expected response of white adipose tissue to a lower energy balance sate. In addition, grape seed proanthocyanidins increase mitochondrial oxidative capacity in skeletal muscle and brown adipose tissue[46]. Thus, the increase of energy expenditure in these organs also could contribute to the lower energy balance observed in rats fed grapes. There are not studies about the effects of cherry extracts on energy expenditure, but cherry effect increasing energy expenditure by activating brown adipose tissue thermogenesis, and/or the mitochondrial activity in other organs, cannot be ruled out.

In obese rats, grape seed proanthocyanidins also stimulate thermogenesis in brown adipose tissue[47] and increase hypothalamic leptin sensitivity[13], associated with the overexpression of *Pomc*. However, no effects of grape intake were observed in obese animals in either photoperiod. In contrast, cherries were very effective modulating the leptin system in obese rats in a photoperiod dependent mode. Specifically, cherry repressed the expression of *Agrp* and *Ptp1B* in SD. Interestingly, *Ptp1b* is a negative regulator of the leptin signaling pathway and its inhibition increases leptin sensitivity in obese animals[48], thus cherry consumption at SD clearly increased leptin sensitivity in obese animals. Furthermore, *Mc4r* and *Npy1r* were also repressed in SD, indicating that cherry was also effective modulating the response in second order neurons. Altogether these results indicate that cherry intake modulated central leptin system out of season in obese animals, like did in lean animals. Moreover, these results strongly suggest that cherry has anorexigenic effects in a photoperiod dependent mode, but independently of the fat stores and body weight. This reduction of the orexigenic signal *Agrp* by cherry agrees with the decreased cumulative food intake in obese rats that consumed cherry in SD. In contrast, the photoperiod dependent effect of cherry on leptin sensitivity, increasing it in SD, disagrees with the lower energy balance and RQ in obese

rats consuming cherry at LD. Thus, like in lean animals, a cherry effect increasing energy expenditure in brown adipose tissue when it is consumed in-season cannot be excluded.

The xenohormesis hypothesis[49], defined by Howitz and Sinclair, proposes that animal uses chemical signals from plants, mainly polyphenols, to know about the environmental status or food supply. This acknowledgment would allow animals to respond in advance to environmental alterations, thus increasing their probability of survival. Therefore, the specific polyphenol content in grape and cherry could act as a distinctive mark, informing rats of the different environmental conditions such as photoperiod. Reinforcing this idea, previous studies of our group have demonstrated that grape proanthocyanidins modulate circadian rhythms at hypothalamic level[50]. Nevertheless, cherries are also rich on melatonin[51] a hormone that informs about day length, controlling seasonal phenotypic adjustments[52]. Interestingly, melatonin reduces the energy expenditure induced by cold exposition in the Siberian hamster placed at LD[53] and stimulates *Pomc* expression in mice[54]. Therefore, the high melatonin contained in cherries could significantly contribute to the effects of induced by cherry intake observed in this study.

Summing up, fruit consumption decreased energy balance in lean animals in a photoperiod independent manner. Grape reduced cumulative food intake whereas cherry increased energy expenditure. However, rats fed cherry did not display any fat mass reduction and rats fed grape even increased it when placed at SD. In contrast, both fruits modulated the leptin system in a photoperiod dependent way, increasing leptin sensitivity in SD. In obese animals, only cherry intake modulated the leptin system, repressing *Agrp* and *Ptp1B* in SD. Thus, cherry intake modulated central leptin system when was consumed out of season both in obese animals and lean animals.

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Supplementary materials

Table S1. Primer sequences used in qPCR amplification from rat genes in hypothalamus.

Primer (Rat)		Sequence 5'-3'	Product size (bp)	GenBank accession no/reference
Obrb	**Fw**	CCAGTACCCAGAGCCAAAGT	122	NM_012596.1
	Rw	GGATCGGGCTTCACAACAAGC		
Socs3	**Fw**	CTGGACCCATTCGGGAGTTC	148	NM_053565.1
	Rw	CTGGGAGCTACCGACCATTG		
Ptp1b	**Fw**	CCCTTTTGACCACAGTCGGA	119	NM_012637.2
	Rw	TTGGTAAAGGGCCCTGGGTG		
Pomc	**Fw**	GAGGCGACGGAGGAGAAAAG	98	NM_139326.2
	Rw	TGAGGCTCTGTCGCGGAAA		
AgRP	**Fw**	GAGAACTCTGGGAACAGGGC	140	NM_033650.1
	Rw	CAAGCAAAGGCCATGCTGAC		
Npy	**Fw**	CTATCCCTGCTCGTGTGTTTGG	136	Sun B et al. 2014
	Rw	TGGTGATGAGATTGATGTAGTGTCG		
Mc4r	**Fw**	CAACTCCTTTGCAAGCTCCG	129	NM_013099.3
	Rw	TCCAACCTCCTAGGTCAGGG		
Npy1r	**Fw**	TCTTCTCTGCCCTTCGTGATC	73	NM_001113357.1
	Rw	TGAACGCCGCAAGTGATACA		
Ppia	**Fw**	CTTCGAGCTGTTTGCAGACAA	138	NM_017101.1
	Rw	AAGTCACCACCCTGGCACATG		
Rplp0	**Fw**	GGACCTCACCGAGATTAGGG	225	NM_022402.2
	Rw	CCCACCTTGTCTCCAGTCTT		

Abbreviations: Obrb, leptin receptor isoform b; Socs3, suppressor of cytokine signaling 3; Ptp1b, protein tyrosine phosphate 1B; Pomc, proopiomelanocortin; Agrp, agouti-related protein; Npy, neuropeptide Y; Mc4r, melanocortin 4 receptor; Npy1r, neuropeptide Y receptor Y1; Ppia, peptidylprolyl isomerase A; Rplp0, ribosomal protein lateral stalk subunit P0.

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CHAPTER 4

The adenosine A2B receptor contributes to AgRP neuronal activation

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Abstract

Adenosine signaling has an important role in the central nervous system modulating neuronal activity. However, little is known about the function of the adenosine A_{2B} (A2B) receptor. Recently, it has been reported that AgRP neurons can be activated by G protein-coupled receptors (GPCR) linked to G stimulatory protein (Gs-GPCR), leading to a maintained increase in feeding. A2B receptors are linked to Gs proteins that activate intracellular signaling pathways. These evidences suggest a link between adenosine signaling and AgRP neurons that has not been investigated in physiological conditions. First, we show that mice that lack the A2B receptor in the nervous system exhibit reduced food intake after a 24-hour fast-refeed test, consistent with a decrease in *AgRP*/*Npy* and *AgRP/Pomc* mRNA levels. Further, mice that lack A2B receptors specifically in AgRP neurons do not display a feeding phenotype. However, after a short fast they show a decrease in the activation of AgRP neurons in the arcuate nucleus. In summary, we report that the A2B receptor signaling plays a role in the nervous system food intake regulation and in AgRP neurons under physiological conditions.

1. Introduction

Adenosine is a purine nucleoside that acts as a constituent of nucleic acids and when phosphorylated is the metabolic energy currency ATP[1]. Adenosine is found in the majority of mammalian cells and its levels depend on the export and uptake or on the release of adenine nucleotides that are hydrolyzed to adenosine in the extracellular space. The production of extracellular adenosine from ATP, ADP and AMP is performed by ectonucleotidases such as CD39 (nucleoside triphosphate diphosphohydrolase) and CD37 (5'-ectonuclotidase)[2]. Similarly when intracellular adenosine concentrations are high due to ATP degradation it can be released through the equilibrative nucleoside transporters (ENT1 and ENT2)[3].

Adenosine signaling plays an important role in the central nervous system (CNS) and it is considered a modulator of neurotransmitters and neuronal activity[4]. Extracellular adenosine plays a variety of physiological roles by binding different adenosine receptors which are G protein-coupled receptors (GPCR): $A_{1,}$ A_{2A}, A_{2B}, A_3. Each receptor has specific tissue distribution, distinct abundance and affinity[5]. Particularly, A2B receptors are stimulatory G proteins (Gs), that increase adenylate cyclase activity, triggering a variety of effects depending on the cell type[6].

The role of A2B receptors in the brain and nervous system is still not well understood because of the low abundance and low affinity of A2B for adenosine and most agonists[4,7]. However, in the periphery, it has been shown to be involved in metabolic effects such us the regulation of glucose homeostasis and lipid metabolism[8]. Metabolism is centrally regulated by the brain which receives and integrates peripheral signals. In particular, the hypothalamus is a brain area of key importance in metabolic regulation[9]. Proopiomelanocortin

(POMC) and AgRP neurons are found in the arcuate nucleus (ARC) of the hypothalamus and are key regulators of energy balance, promoting satiety and food intake respectively[10]. It is remarkable that the majority of AgRP neurons are found outside the blood-brain barrier (BBB) and are more sensitive to peripheral signals and susceptible to changes[11]. Recently, it has been reported that AgRP neurons express Gs-GPCR and the activation of this receptors increases food intake[12]. We hypothesize that AgRP neurons express A2B receptors and have physiological effects in the modulation of AgRP activity.

To address this we determined the gene expression profile of neuropeptides found in the ARC in mice with A2B (*Adora2b*) deletion in the nervous system. We also studied AgRP neuronal activation in the arcuate nucleus (ARC) and dorsomedial hypothalamic nucleus (DMH) of mice with A2B deletion specifically in AgRP neurons.

2. Materials & Methods

2.1 Animals

Mice were housed in barrier facility under a 12-hour light cycle (lights on from 7 AM to 7 PM) with ad libitum access to water and a standard mouse chow (21.6%, 23.2% and 55.2% kcal from fat, protein and carbohydrate, respectively; Purina mouse diet #5058). Transgenic mice expressing Cre recombinase under the *Nestin* promoter [B6.Cg-Tg(Nes-cre)1Kln/J] or the *AgRP* promoter [Agrptm1(cre)Lowl/J] were purchased from Jackson Laboratory. To generate mice lacking the A2B receptor in the central and peripheral nervous system (*Nestin-Cre/+; Adora2b*$^{fl/fl}$ mutants) and their littermate controls (*Nestin-Cre/+; Adora2b*$^{fl/+}$*), Adora2b*$^{fl/fl}$ females were mated with *Nestin-Cre/+; Adora2b*$^{fl/+}$

males. Since expression of *Nestin-Cre* alone presents a body weight phenotype[13,14], control mice expressing Nestin-Cre were chosen. Mice of 23 weeks of age under were sacrificed under *ad libitum* conditions before the onset of the dark cycle. Only male mice are used for experiments.

To generate mice lacking the A2B receptor in AgRP neurons, *Adora2b*$^{fl/fl}$ females were mated with *Adora2b*$^{fl/+}$*; Agrp-Cre* males. This breeding pair resulted in control (*Agrp*+/+*; Adora2b*$^{fl/fl}$ or *Agrp*+/+; *Adora2*$^{fl/+}$*)* and AgRP-specific A2B receptor mutant (*Agrp-Cre*/+; *Adora2b*$^{fl/fl}$) animals. Mice of 23 weeks of age were fasted at 10:00 and sacrificed at the onset of the dark phase at 19:00, at a time when AgRP neurons show high activity levels[15]. Only male mice are used for experiments.

All experiments were carried out under a protocol approved by the University of California at San Francisco Institutional Animal Care and Use Committee.

2.2 Immunofluorescence

AgRP-Cre; Adora2b mice were anesthetized with ketamine/xylazine solution and perfused with 4% (wt/vol) paraformaldehyde (PFA). Brain was excised and post-fixed in 4% PFA solution for 2 hours at 4 ºC. After this, brain samples were transferred to a 30% sucrose solution for cryoprotection and kept overnight at 4 ºC. Samples were embedded in Shandon M-1 embedding matrix (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and stored at -80 ºC. Brain samples were cut using a cryostat to obtain 10 µm-thick coronal sections. Double staining was performed by simultaneous incubation with primary antibodies, rabbit anti-cFos (1:4000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-AgRP (1:1000, Neuromics, Minneapolis, MN,

USA) overnight at 4 ºC. Sections were then washed and incubated at room temperature for 1 hour with secondary donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488 (1:200, Thermo Fisher Scientific Inc.) Nuclear counterstain was performed by 4',6-diamidino-2-phenylindole (DAPI, 1:2000) incubation together with the secondary antibody. Sections were prepared using Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting media.

2.3 Images quantification

Images were captured using an Olympus BX51WI microscope equipped with a QImaging Retiga 2000R digital camera. Images were taken with the same exposure, avoiding pixel saturation and merged using QCapture Pro 6 (Qimaging, Surrey, BC, Canada). Only sections processed in the same experiments were compared for analysis. Images were viewed using Adobe Photoshop (San Jose, CA, USA) and Image, (NIH, Bethesda, MD, USA) was used for the quantification of cFos-positive cells. Images were blinded for the cell counting analysis. Sections were matched by anatomical landmarks according to Bregma (anterior, Bregma: -1.84 mm; medial, Bregma -1.94 mm; posterior, Bregma -2.06 mm) using the Mouse Brain Atlas[16]. At least two sections per mouse per region were quantified and then averaged. Quantification of cFos-positive cells was performed normalizing the background intensity with ImageJ. Only signals clearly positioned overlapping the cell nuclei, which is visible by DAPI staining, is taken as true cFos signal and quantified. In the ARC, only cFos-positive cells located together with the pre-synaptic AgRP fibers were counted, and in the DMH only cFos-positive cells located together with the AgRP projections in post-synaptic neurons.

2.4 Gene expression studies

Hypothalamic and liver RNA isolation from *Nestin-Cre; Adora2b* mice was performed using the RNeasy Lipid Tissue Mini Kit and RNeasy plus mini kit respectively (Qiagen, Valencia, CA, USA). Gene expression was assessed by qPCR using the following Taqman gene expression assay probes (Thermo Fisher Scientific Inc.): *Pomc*, mm00435874_m1; *Agrp*, Mm00475829_g1; *Npy*, Mm00445771_m1 for hypothalamus and *Fasn*, mm00662319_m1*; Acaca*, mm01308023_m1for liver. Relative expression was referred to *Rn18S*, mm03928990_g1.

2.5 Liver triglyceride levels

Homogenization of 40-50 mg of liver was done in 500 µl buffer (250 mM sucrose, 50 mM Tris-HCL pH 7.4) and determined using an enzymatic (Serum) Triglyceride Determination Kit (TR0100, Sigma-Aldrich, St. Louis, MO, USA). Samples were diluted 1:5. Serial dilutions of the standard known concentrations were placed in the same microplate and all samples were incubated with Free Glycerol Reagent and Triglyceride reagent 1:4 mixture for 15 minutes at room temperature. Absorbance was measured at 540 nm. Triglyceride levels of each sample were normalized to total protein content. Protein quantification was measured with Bradford Reagent (B6916, Sigma-Aldrich, St. Louis, MO, USA).

2.6 Body composition and metabolic analysis

Body composition was measured using EchoMRI-700 (Echo Medical Systems, LLC., HoustonTX, USA). Data is expressed as grams of total body weight. After sacrifice, gonadal white adipose tissue was excised for weight determination. Indirect calorimetry, locomotor activity and food intake were measured over 5 days at room temperature (23.5 ºC) using a comprehensive lab animal monitoring system (CLAMS) (Columbus instruments, Inc., Columbus, OH, USA). Mice were allowed to acclimate in the first day and data from this period were excluded from analysis. Respiratory exchange ratio (RER) was calculated as (VCO_2/VO_2) and energy expenditure calculation was performed using the following formula: (3.815 + 1.232 x RER) x VO_2.

2.7 Statistical analysis

Data is expressed as mean ± SEM. Two-tailed Student's *t*-test was used to compare two independent groups of mice. Under conditions where the same animals were analyzed over time, repeated-measures two-way ANOVA was used. Statistical analyses were performed using Prism software (GraphPad Software, Inc, La Jolla, CA, USA). Differences were considered as statistically significant when $P \leq 0.05$.

3. Results

Mice lacking the A2B receptor in the nervous system maintain normal body weight, body composition and hepatic metabolism.

To investigate the role of the A2B receptor in the nervous system, we studied the effect of A2B deletion using *Nestin-Cre/+; Adora2b*$^{fl/fl}$ (*Nestin-Cre;A2B*$^{fl/fl}$) mice and their littermate controls (*Nestin-Cre/+; Adora2b*$^{fl/+}$). Since expression of Nestin-Cre alone presents a body weight phenotype[13,14], heterozygous mice expressing Nestin-Cre were chosen as controls. Both groups of mice were fed a standard chow diet. Body weight, whole-body lean and fat masses as well as gonadal white adipose tissue (WAT) at 23 weeks of age **(Fig. 1A, B)** were not significantly different between mutants and control animals, indicating that there is not a major role for the A2B receptor under physiological conditions.

It has been reported that the A2B receptor has a role on the development of fatty liver[17]. Specifically, this receptor promotes the accumulation of hepatic triglyceride through the decrease in peroxisome proliferator-activated receptor alpha (PPARα) and AMP-activated protein kinase (AMPK) phosphorylation, factors involved in fatty acid oxidation[5]. AMPK regulates lipid metabolism by inhibiting acetyl CoA carboxylase (ACC)[18,19], the rate limiting enzyme for *de novo* synthesis of fatty acids in liver[20]. Furthermore, the A2B receptor also regulates sterol regulatory element-binding protein 1 (SREBP1), a fundamental transcription factor on the regulation of fatty acid synthesis genes in the liver which include fatty acid synthase (*Fasn)* and acetyl-CoA carboxylase (*Acaca*)[21,22]. Further, It has been shown that there is a neural regulation of liver metabolism through the autonomic nervous system[23,24] Together, these findings suggests that the A2B receptor may play a role in the brain which could

influence hepatic metabolism. To investigate this, we measured the liver triglyceride content and the gene expression of *Fasn* and *Acaca* under baseline conditions in mutant and control mice. Triglyceride levels in the mutants are comparable to the ones in the controls **(Fig. 1C)**. The mRNA expression of *Fasn* and *Acaca*, did not reach statistical significance although the mutants show a trend to reduction of its levels **(Fig. 1D, E)**.

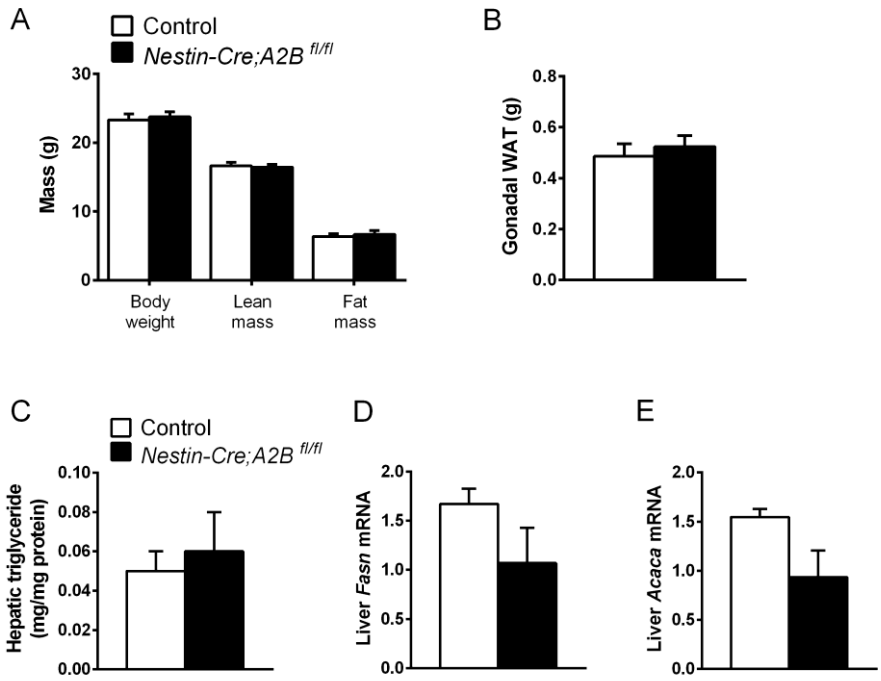

Figure 1. Mice that lack A2B in central and peripheral nervous system present similar body weight, fat mass and hepatic triglyceride compared to control mice. *Nestin-Cre; A2B*$^{fl/fl}$ mice show similar body weight and body composition **(A)** compared to control mice. Gonadal WAT remains unchanged between groups **(B).** Triglyceride levels **(C)** and mRNA expression of *Fasn* **(D)** and *Acaca* **(E)** in the liver are not significantly different in *Nestin-Cre; A2B*$^{fl/fl}$ mice relative to the controls. However, gene expression in mutant mice shows a trend to reduction. Mice were weighed at 20-23 weeks of age and body composition was assessed using EchoMRI one day before the sacrifice. Fat pads and liver were excised and weighted after the sacrifice**.** Data are mean ± SEM of n=5-7/group. *P≤0.05 as determined by Student's *t*-tests.

Deletion of the A2B receptor in nervous system promotes an anorectic effect after food deprivation

To determine if deletion of A2B receptors from the nervous system has an impact on metabolism and energy utilization, animals were placed in CLAMS. Following one day of acclimation period, we measured respiratory exchange ratio (RER), energy expenditure and food intake under baseline condition and following a 24-hour fast **(Fig. 2A-F).** Both RER **(Fig. 2A)** and energy expenditure **(Fig. 2C)** were comparable in control and mutant mice within the four-day period of measurement. Further, no difference was observed in these parameters during the day and night **(Fig. 2B, D)**. Under baseline condition, food intake under baseline condition is not different in control versus *Nestin-Cre; A2B*$^{fl/fl}$ animals **(Fig. 2E, F).** However, following a 24-hour fast, *Nestin-Cre; A2B*$^{f/f}$ mutant animals showed a marked reduction in hyperphagia relative to control mice **(Fig. 2G).** This change in food intake after food deprivation is consistent with a decrease in *Agrp/Npy* mRNA ratio, since *Agrp* expression in the mutants is decreased compared with *Npy* expression which is a component of the same cells. Furthermore, there is a trend on the reduction of *Agrp/Pomc* mRNA ratio in the mutants which indicates that the overall melanocortin tone is different, with higher *Pomc* expression compared to *Agrp.* AgRP is an antagonist of melanocortin-4 receptor (MC4R), whereas POMC acts as agonist activating the same receptors and producing an anorexigenic effect[25]**(Fig. 2H, I)**. This result suggests that the reduction of food intake could be driven by a decrease in *Agrp* expression, leading to a reduction in its orexigenic effect that is exerted through MC4R signaling pathways.

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Figure 2. Food intake is decreased in *Nestin-Cre; A2B$^{fl/fl}$* after a fast-refeed challenge consistent with the modulation of gene expression of orexigenic and anorexigenic neuropeptides. Metabolic analysis of RER **(A)**, energy expenditure **(C)** and food intake **(E)** during four full days measurement in CLAMS are similar between controls and mutant mice except for the 6-hour refeeding period were mutants show a decrease on food intake **(E).** Shaded areas in A, C and E represent dark cycle (19:00-07:00). The average of hourly daily (07:00-07:00h), day (07:00-19:00) and night (19:00-07:00) of RER **(B)**, energy expenditure **(D)** and food intake **(F)** CLAMS analysis do not show differences between groups. The average of 6 hours refeeding measurements after 24-hour fast **(G)** further confirms the reduced fasting-induced feeding of mutant mice compared to controls. Accordingly, the mutant mice showed decreased *Agrp* ratio compared to *Npy* and *Pomc* mRNA levels in the hypothalamus **(H, I).** Animals were fasted towards the dark phase (18:00) and food was introduced again after 24 hours. (n=5-7, 12-15 weeks of age). Hypothalamus was excised at the end of the experiment (n=5-7, 20-23 weeks of age). Data are mean ± SEM *$P \leq 0.05$ as determined by two-way repeated measures ANOVA (A, C, E) and Student *t*-tests (B, D, F, G).

Physiological parameters are maintained after deletion of A2B in AgRP neurons

To determine if the reduction in fasting-induced food intake in *Nestin-Cre; A2B$^{fl/fl}$* animals could be attributed to AgRP neurons, an important group of neurons that regulate feeding[26], we next investigated the effect of AgRP-specific A2B deletion. *AgRP-Cre; A2B$^{fl/fl}$* mice show similar body weight, body composition and hepatic triglyceride levels relative to control animals **(Fig. 3A-C).** Further, analysis of RER, energy expenditure and food intake measured in CLAMS shows no difference between controls and *AgRP-Cre; A2B$^{fl/fl}$* mice **(Fig. 4A-F)**. In contrast to the *Nestin-Cre; A2B$^{fl/fl}$* mutant, *AgRP-Cre; A2B$^{fl/fl}$* mice do not show a defect in mounting a hyperphagic response to fasting **(Fig. 4G).** Indeed, the amount of food consumed in the first 6 hours following the reintroduction of food was similar between *AgRP-Cre; A2B$^{fl/fl}$* mice and

controls. Together, these data suggest that there are no differences in metabolism between control and *AgRP-Cre; A2B*$^{fl/fl}$ animals. We next investigated the possibility that there are alterations at a neuronal level that might underlie a pathophysiological role of A2B receptor in AgRP neurons.

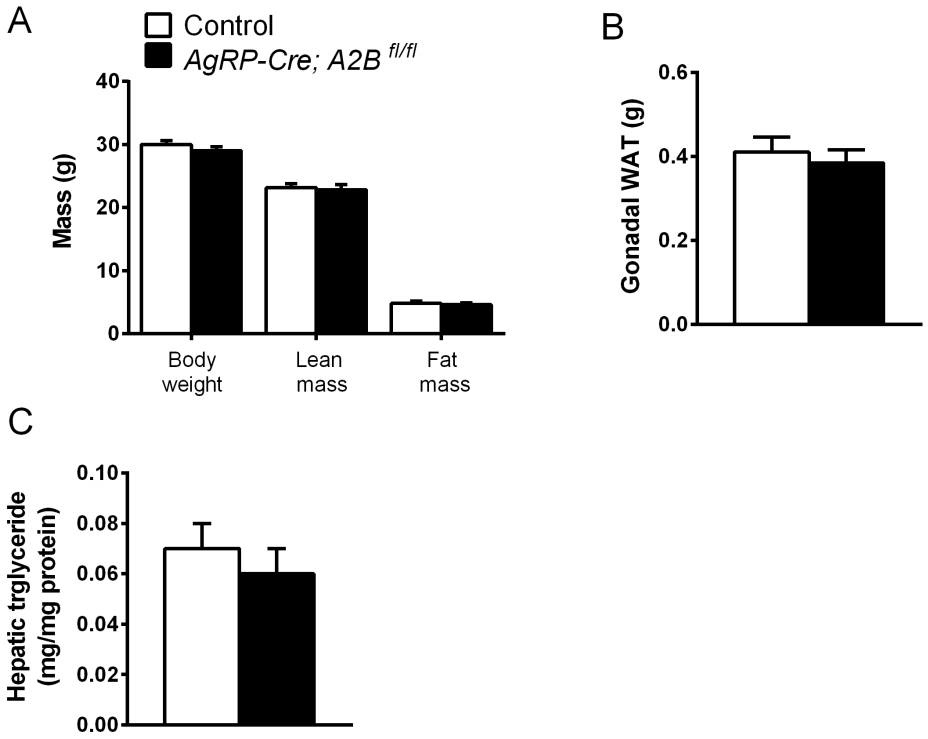

Figure 3. Deletion of the A2B receptors in AgRP neurons does not alter body weight, body composition and hepatic triglyceride levels. *Agrp-Cre; A2B*$^{fl/fl}$ mice show similar body weight and body composition **(A)** compared to control mice. Specifically, gonadal WAT remains unchanged between groups **(B)** as well as the hepatic triglyceride content **(C)**. Mice were weighted and echoed the day before the sacrifice using RMI to assess body composition. Fat pads and liver were excised and weighted after the sacrifice (n=9-10, 20-23 weeks of age). Data are mean ± SEM. **P*≤0.05 as determined by Student's *t*-tests.

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Figure 4. Metabolic measurements of RER, energy expenditure and food intake are similar in both controls and *AgRP-Cre; A2B*$^{fl/fl}$ and fast-refeed challenge does not show a different phenotype. Metabolic analysis of RER **(A)**, energy expenditure **(C)** and food intake **(E)** during four full days measurement in CLAMS are similar between controls and mutant. Shaded areas in A, C and E represent dark cycle (19:00-07:00). The average of hourly daily (07:00-07:00h), day (07:00-19:00) and night (19:00-07:00) of RER **(B)**, energy expenditure **(D)** and food intake **(F)** CLAMS analysis do not show differences between groups. The average of 6 hours refeeding measurements after 24-hour fast **(G)** is not different between mutant and control mice. Animals were fasted towards the dark phase (18:00) and food was introduced again after 24 hours. (n=9-10, 12-15 weeks of age). Data are mean ± SEM *$P \leq 0.05$ as determined by two-way repeated measures ANOVA (A, C, E) and Student *t*-tests (B, D, F, G).

A2B is involved in the activation of AgRP neurons in the arcuate nucleus

We next investigated the neuronal characteristics of these mice in the ARC where AgRP neurons are found. Apart from fasting, AgRP neurons exhibit the highest level of activity towards the dark phase[27]. Activation of AgRP neurons in this case requires a large amount of energy for ATP production, which leads to the generation of adenosine that is then released from the neurons. We hypothesize that extracellular adenosine will bind the A2B receptor to sustain neuronal firing in a positive feedback loop. Further, intracellular adenosine will produce cAMP that will activate intracellular signal transduction pathways. To examine the activity of ARC neurons under a setting of high energy demand, we sacrificed control and *AgRP-Cre; A2B*$^{fl/fl}$ mice at the onset of dark phase following a 10-hour daytime fast (09:00-19:00). AgRP fibers were localized in brain sections by staining with an AgRP antibody. cFos staining in the ARC was used as a marker of neuronal activity[28,29].

Our result demonstrates a typical nuclear cFos staining that overlaps with nuclear marker, DAPI **(Fig. 5A, B).** Merged images demonstrate that cFos-positive cells are found alongside with AgRP fibers. Interestingly, mice that lack A2B in AgRP neurons show a clear reduction in cFos-positive cells than controls in the ARC **(Fig. 5A),** particularly in the mediobasal area where AgRP neurons predominate. AgRP neurons are known to project to downstream neurons, including those in the DMH (dorsomedial hypothalamus)[30], where they exert an inhibitory effect **(Fig. 5B)**. We investigated whether mutant mice show a greater abundance of cFos-positive cells in DMH in association with reduced ARC neuronal activity. Quantification of cFos-positive cells in the DMH reveals that cFos is expressed similarly in both groups **(Fig. 5C).** Together, these data indicate that ARC neurons, likely AgRP neurons, are less active in animals without A2B receptor.

Figure 5. A2B deletion in AgRP neurons decreases the number of cFos-positive cells in the ARC. Immunostaining for AgRP fibers (green), cFos (red) and DAPI (blue) in the arcuate nucleus and merged images **(A, B)**. cFos positive cells in the mediobasal hypothalamus adjacent to the third ventricle in the arcuate nucleus are decreased in mutant mice compared to control mice **(A).** cFos positive cells in the DMH are expressed equally in mutant and control mice **(B)**. Quantification of cFos positive cells in the arcuate and DMH is consistent with the immunofluorescence images, showing a significant decrease in mutant mice compared to the controls in the arcuate nucleus whereas DMH cFos positive cell counting is similar in both groups **(C)**. Mice were fasted 10h before sacrifice to produce a fasting-activation of AgRP. Activation of AgRP neurons is visualized by

the indirect marker of neuronal activity, cFos in the ARC. Mice were sacrificed towards the dark phase (19:00). Data are mean ± SEM of at least 4 mice. 2 sections per mouse per Bregma were quantified. *$P \leq 0.05$ as determined by Student's *t*-tests.

4. Discussion

AgRP neurons play a key role in hypothalamic regulation of food intake and energy expenditure through the mealnocortin system. These neurons produce AgRP, an orexigenic neuropeptide that antagonizes melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R). AgRP cell bodies are found in the ARC and exert their effect via projections to DMH and PVN among other areas[31]. Interestingly, unlike other hypothalamic neurons, the majority of AgRP neurons are located outside the blood-brain barrier, allowing them to sense and respond to circulating metabolic signals[11,32]. Indeed, circulating hormones such as ghrelin, a gut hormone that informs the brain about energy availability is known to activate AgRP neurons by binding to the growth hormone secreatagogue receptor (GHSR) to produce an increase on caloric intake and adiposity[33]. Aside from ghrelin, other factors can also activate AgRP neurons in a different manner. Among them, acute ethanol dose increases AgRP immunoreactivity in the ARC of C57BL/6J mice. Ethanol consumption leads to the production of acetate in neurons and increases extracellular adenosine levels in the brain[34]. The mechanism by which alcohol triggers the increase of AgRP signaling is yet to be determined, but it is plausible that there is a link between adenosine levels and AgRP activity. We hypothesize that extracellular adenosine might bind the A2B, which is known to be a stimulatory GPCR, to sustain the firing in a positive feedback loop and to activate cellular responses

in AgRP neurons. In this study, our goal is to determine the function of A2B in the nervous system and in AgRP neurons.

The use of two genetic mice models, one with a the deletion of A2B in the central and peripheral nervous system and the other with the deletion of A2B only in AgRP neurons allows distinguishing between the effects produced by A2B in the nervous system or in AgRP neurons alone. *Nestin-Cre; A2B$^{fl/fl}$* mice maintained at physiological conditions and fed a normal chow diet did not show changes on body weight, body composition, nor liver triglyceride levels. Fatty acid synthase and acetyl CoA-carboxylase, genes involved in hepatic lipid accumulation showed a trend to be reduced although it did not reach statistical significance**.** Hepatic analyses are relevant since it has been reported that whole body A2B knockout mice are protected from fatty liver after chronic alcohol consumption[5]. Further metabolic analyses were performed in CLAMS and animals were challenged with a 24-hour fast-refeed test to examine metabolic adaptation of the mutant mice. RER and energy expenditure remained the same among groups. Nevertheless, food intake analysis showed a meaningful decrease in refeeding after a 24-hour fast in *Nestin-Cre; A2B$^{fl/fl}$* mice**.** This phenotype indicates that A2B could have a role on food intake regulation. Then, gene expression of the neuropeptides that control food intake was assessed. Consistent with the previous results *Agrp* gene expression is reduced compared to other neuropeptides in mutant mice, meaning that they receive a lower degree of orexigenic signals. Previous studies reported that after consuming high-fat diet (HFD) A2BKO mice show elevated fat to lean ratio and higher leptin levels compared to WT mice[35]. This data further supports a connection with the regulation of energy homeostasis although no mechanisms have been described.

Subsequently, we focused on the specific population of AgRP neurons. In contrast to our previous results with *Nestin-Cre; A2B$^{fl/fl}$* deletion of A2B

receptor in AgRP neurons does not show significant phenotypical changes on the metabolic factors analyzed. Thus, we proceed to analyze more extensively the hypothalamic nucleus where AgRP neurons are localized. The approach we used to study AgRP neuronal activity consisted in achieving a situation where AgRP neurons are activated such in fasting[27]. Furthermore, it has been demonstrated that AgRP stimulates feeding at the onset of dark-phase[31,36]. Therefore, these two stimuli were taken into account to provide the right conditions. To detect AgRP neuronal activity we used cFos as indirect marker. We show that control mice have higher cFos expression in the arcuate nucleus surrounded by AgRP fibers and particularly there are more abundant in the area adjacent to the third ventrical in the medibasal hypothalamus. cFos signal is specific since it colocalizes with nuclear DAPI staining. As reported by Olofsson et al. the predominant neuronal type in the mediobasal hypothalamus situated outside of the blood-brain barrier are AgRP neurons. Accordingly, they are exposed to peripheral signals making them more sensitive to metabolic changes[11]. This data supports that the majority of neurons seen in the images that express cFos are truly AgRP neurons, and this are more active when A2B is present. On the contrary, *AgRP-Cre; A2B*$^{fl/fl}$ mice immunofluorescence shows fewer cFos positive cells in the arcuate which is confirmed with the quantification analysis. Additionally, cFos expression can also be seen in neurons located in the DMH, where AgRP fibers project. In this case, the images show slightly fewer cFos signal in the control mice compared to mutants. However, the posterior quantification indicates that there are no differences between mice groups. Even though ARC neurons are less activated, neurons that receive inhibitory inputs from AgRP neurons such as those in the DMH are not affected. Additional investigations should be carried out to study effects in downstream neurons.

In summary, mice with a deletion of A2B in the nervous system show a reduced refeeding phenotype, whereas deletion of A2B in AgRP neurons does not produce changes during refeeding compared to their controls. This could mean that A2B exerts effects through neurons other than AgRP that also regulate food intake such us neuropeptide Y (NPY) neurons or POMC neurons among others. Relative to AgRP, we suggest that during fasting, which is characterized by a state of energy deficiency, AgRP neurons increase their firing rate and produce ATP which is hydrolyzed to adenosine to obtain energy. Adenosine is transported outside of the cell through ENT1 and subsequently extracellular adenosine levels rise. In this situation adenosine may bind A2B and activate the transcription of AgRP and contribute to a positive feedback. Potentially, the same pathway that modulates AgRP activity through A2B might be high-jacked by ethanol producing AgRP neuron activation. Future approaches include to carry out these experiments using a female cohort and the study of the effects of ethanol in both mice lines using mice of both genders, to better define the A2B signaling and its pathophysiological role.

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POLYPHENOL EFFECTS ON CENTRAL LEPTIN SENSITIVITY IN OBESITY
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IV. GENERAL DISCUSSION

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Dietary polyphenols have been widely studied due to its beneficial effects on health[1,2]. Furthermore, data regarding the effects of polyphenols on metabolism is promising for the prevention and treatment of cardiovascular disease, diabetes type II and obesity[3]. It is of great interest to establish new approaches to decrease obesity since this condition is accompanied by increased health risk factors[4]. In this sense, *in vitro*, *in vivo* and some human clinical trials tested the potential of polyphenols on obesity treatment as compounds with the ability to increase energy expenditure[5]. Up to now, the vast majority of studies have focused on studying the effects of polyphenols improving obesity by its action in peripheral organs such as liver, adipose tissue and muscle. For instance, green tea catechins, resveratrol and curcumin *in vivo* may improve obesity by increasing fat utilization, decreasing plasma triglycerides, improving glucose homeostasis, reducing inflammation and activating Sirt1[5,6]. However, few studies investigated the effects of polyphenols on the regulation of energy homeostasis through their action in the central nervous system.

As mentioned in the introduction section, leptin is a key hormone on the regulation of energy homeostasis by signaling first order neurons in the arcuate nucleus of the hypothalamus to [7–9]. Leptin activates POMC neurons and suppresses AgRP/NPY neurons activity producing satiety and increased energy expenditure[10]. In obesity, leptin action is blunted which is reflected by increased circulating leptin levels that fail to counteract the increase in body weight, which is known as leptin resistance[11–13]. Several mechanisms are suggested to be involved in the development of leptin resistance[14]. Recent data challenged the classic idea that there is no response to leptin in obesity, In fact it has been shown that obese animals are able to respond to endogenous leptin, despite not responding to exogenous administration of this hormone[15]. Therefore, this points out that the so called “leptin resistance” does not occur at the level of

leptin receptor rather than altered downstream signaling, that might involve the overexpression of negative regulators such as SOCS3 among other mechanisms[16]. Therefore, the aim of this thesis was to evaluate the potential of polyphenols to improve leptin sensitivity in the hypothalamus in obesity and determine by which mechanisms these compounds modulate leptin signaling pathway.

Previous research demonstrated that a high, pharmacologic, dose of grape seed proanthocyanidins (GSPE) is able to reduce body weight by inhibiting food intake and boosting energy expenditure. The described mechanisms by which GSPE exerts these effects include the upregulation of β-oxidation and lipolylitic genes, particularly in subcutaneous WAT[17] and the satiating effects of GSPE involving the action of the enterohormones together a delayed gastric emptying[18]. These are promising results for the use of GSPE as anti-obese compounds. However, further studies limiting the dose of GSPE to dietary doses are still necessary. Moreover, the satiating effects may be produced by additional mechanisms that regulate food intake and energy homeostasis such hypothalamic leptin signaling. Hence, we performed an experiment that consisted on the first induction of obesity to animals using a cafeteria diet and after 10 weeks, when animals achieved at least a 10% increase on body weight, were supplemented with 25 mg/kg for a total of 3 weeks **[Chapter 1]**. This dose corresponds to a human intake of 284 mg GSPE/day by converting animal doses to human equivalent doses (HED) using the body surface area (BSA) normalization method and estimating the daily intake for a 60 kg adult[19]. The human intake of proanthocyanidins ranges from 95 to 200 mg/day[20–22]. Thus, GSPE doses administered in this study simulate the human proanthocyanidin intake.

The effect of GSPE on central leptin signaling was evaluated using rat hypothalamus and determining the expression of genes that regulate leptin signaling pathway and downstream effectors. Firstly, hypothalamic pSTAT3 protein levels were determined as one of the gold standard to assess leptin cascade activation *in vivo* (besides responses to food intake and body weight after leptin administration)[23]. The results show that obese animals supplemented with GSPE increased pSTAT3 levels compared to the cafeteria control group reaching similar values to the ones depicted by the lean group. Furthermore, *Socs3* levels were also normalized as well as *Ptp1b*. According to these changes *Pomc* levels were remarkably enhanced by GSPE treatment,. Therefore, the data obtained suggest that GSPE treatment increased leptin signaling in the cafeteria-diet fed rats. Importantly, POMC expression is linked to the induction of anorexigenic signals[24] and animals supplemented with GSPE showed a significant reduction on food intake and a tendency to reduce adiposity after the treatment. This increased of leptin sensitivity in the hypothalamus of obese rats supplemented with GSPE was not explained by higher *Obrb* expression. Therefore other mechanisms were assessed. Diet induced obesity and leptin resistance is characterized by increased hypothalamic inflammation and ER stress[25,26]. Notably, in our experiment GSPE reduced hypothalamic inflammation, depicted by the decrease in *inos mRNA* levels, suggesting this effect could be one of the mechanisms involved in the improvement of leptin signaling induced by GSPE. Furthermore, it has been reported that Sirt1 activity is able to increase leptin sensitivity[27,28] which lead us to evaluate *Sirt1* expression in the hypothalamus. Interestingly, *Sirt1* was overexpressed by GSPE treatment meaning that proanthocyanidins could also improve leptin sensitivity by this mechanism. The fact that proanthocyanidins are able to cross the BBB[29] strengthens the idea that GSPE may have a direct action in the hypothalamus.

Additionally, leptin actions in the periphery play an important role on the development of leptin resistance[30]. Hence, we further investigated the capacity to modulate the leptin signaling cascade in these animals. Remarkably, GSPE supplementation normalized the expression levels of the leptin cascade components in mesenteric white adipose tissue and muscle. Therefore, proantohocyanidins were also able to improve leptin sensitivity in the periphery.

Altogether these results indicate that GSPE increased central leptin sensitivity that was associated to a significant reduction of food intake. However, GSPE supplementation did not correct hyperleptinemia, body weight and adiposity at this dose and time length.

For this reason we next focused on other phenolic compounds that could complement GSPE effects modulating leptin signaling and successfully reducing body weight **[Chapter 2].** Many studies indicate that resveratrol and anthocyanins have the capacity to reduce body weight (cites). Thus, we planned a first preliminary study to test the capacity of these types of polyphenols to modulate the leptin signaling pathway in the hypothalamus using healthy mice. The results showed that the daily treatment of mice with resveratrol, at a dose of 100 mg/kg body weight for 15 days, increased the energy expenditure in mice and overexpressed *Obrb* in the hypothalamus whereas an anthocyanin rich extract did not produce significant changes. According, some *in vivo* studies show that anthocyanins have anti-obesity properties by decreasing circulating leptin levels[31] through the modulation of adipocytokine secretion and lipid metabolism in the adipose tissue[32,33].

These results indicate a higher potential of resveratrol over the anthocyanins to improve leptin signaling in the hypothalamus and to reduce energy

homeostasis. Moreover, resveratrol was an interesting candidate since, besides some reported effects on the reduction of body weight and fat mass through the modulation of lipid metabolism in the periphery[34], one study[35] also indicate its ability to modulate hypothalamic leptin signaling in . Specifically, Franco et al. [35] reported an increase of pSTAT3 levels in the hypothalamus and a reduction of hyperleptinemia in maternally programed HFD animals at 150 days of age after consuming a high-fat diet supplemented with 30 mg resveratrol /kg body weight for 30 days. Despite these effects, only adiposity was reduced due to resveratrol treatment whereas body weight and food intake remained unaltered[35].

Due to the wide range of resveratrol doses used in animal studies studies[36], we decided to test the effect of a low, moderate and high dose of resveratrol using a diet-induced obesity model. Therefore, obese animals were supplemented with 50, 100 and 200 mg/kg of resveratrol which in HED correspond approximately to 486 mg, 970mg and 1.95 g resveratrol/day for an individual of 60 kg[19]. These doses have previously been tested in human clinical trials and were generally well tolerated[37,38]. The results demonstrated that the highest dose of resveratrol effectively corrects hyperleptinemia produced by the cafeteria diet and increased hypothalamic pSTAT3, suggesting that leptin sensitivity was improved. Taking into account that in normal conditions leptin signaling through ObRb produces the phosphorylation of STAT3[39], we found out that pSTAT3 levels in the hypothalamus referred to plasma leptin levels is a good ratio for leptin sensitivity estimation . Interestingly, the highest dose of resveratrol produced a significant reduction of body weight, increased 24h energy expenditure and lipid oxidation without decreasing food consumption. Therefore, the high dose of resveratrol clearly exerted anti-obesity effects that were mediated, at least partly, by the restoration of leptin sensitivity in the

hypothalamus of obese rats. Although *Sirt1* expression in lean mice was not affected by resveratrol supplementation, further investigations in this obesity model are needed to clarify the mechanism by which leptin sensitivity is improved by this polyphenol.

The findings obtained in chapter 1 and 2 suggest that a mixture of 25 mg GSPE with 200 mg resveratrol by kg of body weight has the potential to successfully reverse obesity in obese animals since both types of polyphenols act as leptin sensitizer in the hypothalamus and GSPE modulate satiety whereas resveratrol induced energy expenditure, all of them key factors for body weight regulation.

Fruits are an important polyphenol source in the human diet[20–22] and their consumption is highly recommended to maintain health. Nowadays the consumption of seasonal and out-of season foods has generated some debate related to health effects and ecosystem sustainability[40]. In this sense, the Xenohormesis Hypothesis states that plant molecules, such as polyphenols, are able to modulate mammalian physiology when consumed. These cues warn about environmental conditions where the plant developed allowing animals to adapt to the situation[41].. Thus, we consider relevant to assess the effects produced by seasonal fruits rich in polyphenols when they are consumed out of season regarding their impact in the central leptin system and obesity **[Chapter 3]**.

Leptin secretion is influenced by the light-cycle in seasonal animals which means the levels of this hormone fluctuate in accordance to a circannual rhythm[42]. It is common that seasonal mammals develop leptin resistance as an adaptive response to survive to changes on food availability[43]. Therefore, leptin resistance is not an isolated phenomenon that occurs in obesity, it is rather related to several changes that occur at hypothalamic level in different

situations such as malnutrition, seasonal changes and reproduction, some of them affected by photoperiod[44]. Seasonal animals increase their body stores and develop hyperleptinemia during summer and spring and gain less weight and decrease serum leptin during winter and autumn [43,45].

For this study we used the Fisher-344 rat strain because its sensitivity to photoperiods (cita). Rats were placed in long-day (LD) (18:6h light:dark cycle) or short day (SD) (6:18h light:dark cycle) to simulate spring or autumn, respectively. Numerous studies report the metabolic protective effects of grape, grape by-products, cherries or their pure compounds[33,46–50], thus we have chosen grape and cherry as representative fruits of autumn and spring, respectively. In addition, this study was performed in both lean and dietary induced obesity models.

Our results show that lean animals placed in SD photoperiod displayed decreased fat mass and serum leptin, despite no changes in body weight were observed. This data agrees with other studies using the same rat strain[51,52]. Moreover, animals in SD showed a lower energy balance attributed to increased energy expenditure since no changes in cumulative food intake were detected. The gene expression of the components involved in leptin signaling pathway in the hypothalamus were not affected by photoperiod in lean animals. On the other hand, animals that consumed an obesogenic diet loose the photoperiodic regulation of fat mass but also showed a decreased energy balance in SD, in this case as a consequence of a reduction on the cumulative food intake. Contrarily to lean animals, in the obese model the photoperiod did not affect circulating leptin. However, central leptin system was affected, overexpressing *Socs3* and *Agrp* during SD. Seasonal leptin resistance in seasonal rodents and sheep is characterized by increased SOCS3 during LD[43,44] and some studies indicate a downregulation of AgRP using Fisher 344 rats[51]. Thus, these discrepancies

between our and other results could be mainly due to different time length to photoperiod exposition that was longer in our experiment.

In view of the previous results, we attempted to understand how the consumption of seasonal fruits in different photoperiod could affect central leptin system and energy balance in both lean and obese animals. In lean rats, grape and cherry consumption decreased the energy balance in a photoperiod independent manner. Interestingly, this effect was consequence of a reduced cumulative food intake in rats consuming grape whereas an increased energy expenditure was observed in animals consuming cherry. Furthermore, both fruits increased *Pomc* mRNA levels in SD, which could explain the drop in energy balance observed in animals exposed to SD. Besides, cherry intake increased *Obrb* expression in SD suggesting that cherry consumption increases the central leptin sensitivity. However, Socs3 and *Agrp,* which are markers of attenuated leptin signaling, were overexpressed by cherry consumption in both LD and SD photoperiods. Thus, further studies are needed in order to clarify the exact effect of cheery consumption on leptin sensitivity.

No effects of grape intake were observed in obese animals in either photoperiod. In contrast, cherries were very effective modulating the leptin system in obese rats in a photoperiod dependent mode. Specifically, cherry consumption produced an anorexigenic response in SD depicted by reduced cumulative food intake as well as downregulation of *Agrp* and *Ptp1b* meaning that leptin sensitivity was increased by this fruit in a photoperiod dependent manner. In addition, cherry intake effectively modulated receptors expressed in second order neurons, outside of the arcuate nucleus, *Mc4r* and *Npy1r* when was consumed at SD Therefore, cherry modulated leptin system when it was consumed out of season in both lean and obese animals . Despite cherry consumption did not modulate the central leptin system when was consumed at

LD, its intake also reduced the energy balance and RQ in this photoperiod. Thus, other mechanism, instead leptin system modulation, could be induced when cherry is consumed in season such as enhanced energy expenditure at brown adipose tissue level. Importantly, in this study cherry modulated *Agrp* gene expression in photoperiod independent manner in both lean and obese animals, which could mean that AgRP neurons were more sensitive to cherry polyphenols compared to Pomc neurons.

Interestingly, it has recently been demonstrated by researchers in Xu Laboratory that around 60-70% of AgRP neurons are located outside the blood brain barrier which makes them more susceptible to metabolic changes, since they are expose to blood borne substances[53,54]. Then, we hypothesize that although polyphenols are able to cross the BBB[29], AgRP neurons are a good putative target for polyphenols since polyphenols circulating in systemic blood can directly interact with these neurons. In order to go in depth in the regulation of AgRP neurons, this part of the thesis **[Chapter 4]** was performed in Professor Allison W. Xu laboratory, in the Diabetes Center at University of California, San Francisco.

AgRP neurons are activated by fasting[55,56]. However the specific mechanisms that modulate AgRP activity are not completely clarified. Recent data showed that AgRP neurons are activated by Gs protein-coupled receptors (GPCRs) which triggers a sustained increase in food intake[57]. Adenosine signaling plays an important role in the CNS modulating neuronal activity[58] by specifically binding to its receptors. Interestingly, adenosine receptor 2B (A2B receptor) is a Gs-coupled GPCR, which means that adenosine binding will produce stimulatory effects[59,60]. Little is known about the role of this receptor in the CNS. However, it has been reported that an acute ethanol dose promotes an increase of AgRP immunoreactivity in the ARC of C57BL/6J mice[61] and, at the

same time, ethanol intake raises the extracellular adenosine levels in the brain[62]. The mechanism by which alcohol triggers AgRP activation has not been explained yet, but the data suggest a possible link between adenosine levels and AgRP activity. Hence, we hypothesize that extracellular adenosine might bind the A2B receptor to sustain the firing in a positive feedback loop and to activate cellular responses in AgRP neurons.

For this reason, we aimed to determine the role of A2B receptor in the nervous system and in AgRP neurons. The results obtained show that mice with a deletion of A2B in the nervous system reduced refeeding phenotype after 24-hour fasting, whereas deletion of A2B in AgRP neurons did not produce any change during refeeding. This could mean that A2B receptor exerts its effects through other pathways that also regulate feeding, such as neuropeptide Y (NPY) release or POMC neurons activity because of the reduced *Agrp/Npy* and *Agrp/Pomc* gene expression ratios observed in these mice.

Furthermore, mice with A2B deletion in AgRP neurons and challenged with 8-hour fasting at the beginning of the dark-phase, showed reduced cFos expression. Remarkably, AgRP neurons are activated by fasting and stimulate feeding at the beginning of the dark phase [55,63,64]. Therefore, our results indicate that mutant mice presented a reduced neuronal activity in the ARC nucleus, where AgRP cell bodies are located. In addition, AgRP neurons may exert their effects through inhibitory projections to the dorsomedial hypothalamus (DMH) and the paraventricular nucleus (PVN)[63]. In this study, cFos expression is also observed in neurons located in the DMH. Assuming that the control mice may have increased AgRP activity, since they show increased cFos expression, in turn they could exert a inhibitory effect to DMH neurons. However, quantification of cFos positive neurons in the DMH did not differ between mutant and control mice. Therefore, despite AgRP neurons in the ARC nucleus

are less activated, neurons that receive inhibitory inputs from AgRP neurons are not affected. Further research should be performed to study the effects in downstream neurons such as the ones in the DMH.

Altogether the results obtained in this study allows to suggest that during fasting AgRP neurons increase their firing rate and produce ATP in order to obtain energy. Adenosine is transported outside of the cell through the ENT1 transporter and, subsequently, extracellular adenosine levels rise. In this circumstance adenosine may bind A2B receptor and activate the transcription of AgRP gene which, in turn, leads to a positive feedback loop that keeps these neurons activated. This study brings novel insights about the modulation of AgRP neurons through A2B receptor which is a potential target to prevent or correct metabolic diseases. Future research on the field of bioactive compounds such as polyphenols may take advantage of these findings.

In summary, different classes of polyphenols showed the ability to ameliorate energy homeostasis in obesity partly through the modulation of leptin signaling and improving leptin sensitivity in the hypothalamus. Therefore, these compounds are promising candidates for the design of functional foods that help to reduce obesity and the associated risk factors.

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V. CONCLUSIONS

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1. **Chronic consumption of a dietary dose of proanthocyanidins normalizes pSTAT3 levels and overexpresses *Pomc* in the hypothalamus of obese rats indicating that proanthocyanidins sensitize first-order neurons to leptin in obesity.** This improvement of leptin signaling emerges, at least to a certain extent, from the neuroprotection against inflammation and enhanced *Sirt1* expression induced by proanthocyanidins in the hypothalamus of obese animals.

2. **Chronic consumption of a dietary dose of proanthocyanidins produces an anorexigenic response in obese rats that is associated to the overexpression of *Pomc*.** Thus, the anorexigenic effect of proanthocyanidins is mediated, at least partly, by the activation of POMC neurons in the hypothalamus.

3. **Chronic consumption of a pharmacological dose of resveratrol represses SOCS3 expression in the hypothalamus and increases the ratio pSTAT3/serum-leptin indicating that resveratrol is able to partially recover central leptin sensitivity in obesity.** This improvement of central leptin sensitivity could be behind the decreased body weight and fat mass as well as the increased energy expenditure observed in obese rats treated with this pharmacologic dose of resveratrol.

4. **The consumption of grape, a seasonal fruit representative of autumn, modulates hypothalamic *Pomc* expression in a photoperiod-dependent manner in lean animals, overexpressing *Pomc* in the hypothalamus of rats placed at short-day photoperiod.** Remarkably, this overexpression of

Pomc is associated to lower cumulative food intake and reduced energy balance in lean rats consuming grape in short-day. Thus, grape consumption improves leptin sensitivity in lean rats when it is consumed in season

5. **Grape consumption together with an obesogenic diet prevents the overexpression of AgRP induced by this obesogenic diet in rats placed at short-day photoperiod**. However, this protection exerted by grape consumption was not reflected in the energy balance or food intake.

6. **The consumption of cherry, a seasonal fruit representative of spring, modulate hypothalamic *Pomc* and *Obrb* gene expression in a photoperiod-dependent manner in lean animals, increasing their expression in the hypothalamus of lean rats placed at short-day photoperiod.** Notably, this overexpression of *Pomc* and *Obrb* is associated to higher energy expenditure and reduced energy balance in lean rats consuming cherry in short-day. Thus, cherry consumption improves leptin sensitivity in lean rats when it is consumed out of season.

7. **Cherry consumption together with an obesogenic diet modulates the central leptin system in a dependent-photoperiod manner, decreasing hypothalamic *Socs3, Ptp1b, AgRP, Mc4r* and *Npy1r* gene expression in short-day photoperiod.** Thus, cherry consumption regulates leptin sensitivity in obese rats when it is consumed out of season, like in lean animals. However, cherry consumption decreases energy balance in both long- and short-day photoperiods in rats fed the obesogenic diet, pointing out that other mechanisms contribute to the stimulation of energy expenditure induced by cherry consumption.

8. **Adenosine receptor 2B plays a significant role on the activation of AgRP neurons. This fact can be concluded because:**

   a. **Deletion of A2B receptor in the nervous system reduces the refeeding phenotype in association with the decreased *Agrp/Npy* and *Agrp/Pomc* ratios.**

   b. **Deletion of A2B receptor in AgRP neurons does not produce a refeeding phenotype. However, arcuate neurons in the mediobasal hypothalamus, where AgRP neurons are abundant, are less activated.**

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VI. APPENDICES

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ABBREVIATIONS

A2B	Adenosine receptor 2B
AGRP	Agouti-related protein
ARC	Arcuate nucleus
BBB	Blood brain barrier
CD	Cafeteria diet
CNS	Central nervous system
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamic nucleus
ER	Endoplasmic reticulum
GSPE	Grape seed proanthocyanidin extract
HFD	High-fat diet
LD	Long-day photoperiod
MC4R	Melanocortin 4 receptor
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y receptor Y1
OBRB	Long form of the leptin receptor
PAC	Proanthocyanidin
POMC	Proopiomelanocortin
PTP1B	Protein-tyrosine phosphatase 1B
SD	Short-day photoperiod
SIRT1	NAD-dependent deacetylase sirtuin-1
SOCS3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3

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ABOUT THE AUTHOR

Maria Ibars Serra was born on the 9th of March, 1989, in Sabadell, Catalonia. After completing secondary school studies in 2008, she chose the bachelor's degree in Biology and Biomedical Sciences at Universitat Autònoma de Barcelona (Barcelona). Her main field of interest was the clinical part of biochemistry, endocrinology and neurobiology. During her bachelor's she did three internships. One at CERBA-Keynova (Barcelona) under supervision of Dr. Pilar Grao, one at the Clinical Immunology Department in KCUS in Bosnia and Herzegovina under supervision of Prof. Jasenko Karamehic and the last one at Ulster University in the Northern Ireland Centre for Food and Health (UK) supervised by Dr. Chris Gill . There, she participated for a period of one year in Ileostomy Berry project which was related with the study of polyphenols from raspberries in ileostomy patients and assessment of antigenotoxic activity in colon cancer cells and SWAFAX project which studied the seaweed derived anti-inflammatory agents and antioxidants. In September 2013 she undertook the MSc degree in Nutrition and Metabolism at Universitat Rovira i Virgili (Tarragona) in the Department of Biochemistry and Biotechnology and she was awarded a research grant to study the effect of proanthocyanidins on the modulation of hypothalamic leptin signaling in obesity in the Nutrigenomics Research Group supervised by Prof. Cinta Bladé and Dr. Gerard Aragonès. After completing her Master's thesis she was selected as a PhD student as a continuation of the project started during the Master's and was awarded a FPI predoctoral fellowship by the Spanish Government. During her PhD, she was awarded a Mobility grant by the Spanish Government and she was involved in a project at University of California San Francisco in Professor Allison W. Xu laboratory where she studied the regulation of neurons involved in feeding and energy homeostasis. The results of this research are presented in this thesis.

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LIST OF PUBLICATIONS

Full papers

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Ardid-Ruiz A, **Ibars M**, Bladé C, Aragonès G, Suárez M. Resveratrol rescues hepatic leptin signal transduction via STAT3 pathway in a cellular model of fat accumulation induced by palmitic acid. Presented at XXVIIIth International Conference on Polyphenols. Vienna, Austria (2016).

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RESUM

L'obesitat és un problema de salut en augment i suposa un risc per al desenvolupament de malalties cròniques. Les estratègies per reduir i prevenir l'obesitat no han sigut satisfactòries el que fa necessari el desenvolupament d'alternatives terapèutiques. Nombrosos estudis en animals i humans demostren que els polifenols tenen propietats protectores en front a trastorns metabòlics per la qual cosa aquests compostos bioactius poden ser útils per a reduir l'obesitat i malalties metabòliques associades. La leptina és una hormona encarregada de la regulació del balanç energètic al sistema nerviós central on activa les neurones POMC i inhibeix les AgRP produint sacietat i promovent la despesa energètica. No obstant això, l'acció de la leptina en l'obesitat es troba afectada. L'objectiu principal d'aquesta tesi és identificar polifenols que millorin la sensibilitat a la leptina en situacions d'obesitat i que tingui com a resultat la pèrdua de pes. En aquesta tesi demostrem com el consum crònic d'un extracte de pinyol de raïm ric en proantocianidines millora la senyalització de la leptina a través de l'augment de l'expressió gènica del neuropèptid POMC i redueix la ingesta energètica sense mostrar canvis al pes corporal. A més, s'ha investigat el potencial d'altres polifenols amb efectes complementaris a les proantocianidines per tal d'estimular la pèrdua de pes. Els resultats presentats mostren que el resveratrol és efectiu reduint el pes i el greix corporal i la hiperleptinèmia en animals obesos, actuant com a agent sensibilitzador de la leptina. D'altra banda, es demostra el potencial de fruites estacionals riques en polifenols en la modulació de la senyalització de la leptina en condicions normals i d'obesitat. Finalment, s'explica el rol d'una nova diana per modular l'activitat neuronal de les neurones AgRP. Els resultats d'aquesta recerca aporten nous coneixements pel disseny d'aliments funcionals que combinin diferents compostos bioactius amb el potencial de poder ser utilitzats com a teràpia anti-obesitat.

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