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Hypothalamic lipid metabolism and the control of food intake

DOCTORAL THESIS

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This doctoral thesis entitles **"Hypothalamic lipid metabolism and the control of food intake"** developed by **Ahmed Hamed Mohamed Elhady Mosa Arisha**, has been conducted by him at the Department of Physiology of the University of Santiago de Compostela and the Center for Investigations in Molecular Medicine and Chronic Diseases (CIMUS) of the University of Santiago de Compostela, estimating that it is concluded and in conditions to be presented for the eligibility for the degree of Doctor.

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Santiago de Compostela, May 2016

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Abstract

Homeostatic regulation of energy balance is a precise tool concerned with maintaining a stable balance between energy intake and expenditure. Failure in the regulation of this physiological process gives rise to some disorders that are now considered public health problems with their reported increase in prevalence rate such as obesity and cachexia. Obesity is caused by a chronic rise in energy consumption while cachexia results from a long-standing increase expended energy. Brown adipose tissue (BAT) thermogenesis is a crucial component of the energy balance equation. Recently, the hypothalamus and in particular, the ventromedial nucleus of the hypothalamus (VMH), vital role in the regulation of BAT activity has emerged. The hypothalamic AMP-activated protein kinase (AMPK), a sensor of energy levels, not only has a pivotal role in the regulation of both cellular and whole body energy balance but also is crucial in regulating BAT thermogenesis through the sympathetic nervous system (SNS).

The impact of sexual dimorphism on energy homeostasis and fat distribution is well established. Also, the influence of sex steroids on energy balance has been reported. Here, we demonstrate that estrogens and androgens have a major role in the modulation of energy balance through central actions. Central estradiol (E2) treatment inhibits AMPK in the VMH, leading to activation of thermogenesis in BAT in a feeding-independent manner. Moreover, fluctuations in E2 levels during estrous cycle also modulate this integrated physiological network. On the other hand, dihydrotestosterone (DHT)-treatment inhibited BAT thermogenesis so that the central effects of androgens on energy expenditure cannot be attributed to aromatization. Notably, central DHT-treatment demonstrated the ability to modulate the lean mass. Overall, these findings showed that E2 and DHT regulation of the VMH AMPK-SNS-BAT axis is an essential determinant of energy balance and suggest potential targets for treatment of both cachexia and obesity.

Resumen

La regulación homeostática del balance energético es una precisa herramienta que se ocupa de mantener un equilibrio estable entre la ingesta y el gasto energético. Cualquier desequilibrio en la regulación de este proceso fisiológico dará lugar a algunos trastornos que son considerados hoy en día un problema de salud pública con una prevalencia creciente tal como la obesidad y la caquexia. La obesidad está causada por un aumento crónico en consumo de energía y la caquexia es el resultado de un aumento mantenido durante mucho tiempo del gasto energético. La termogénesis del tejido adiposo marrón (Brown adipose tissue; BAT) es un componente crucial en la ecuación del balance energético. Recientemente, el hipotálamo y en particular, el núcleo ventromedial del hipotálamo (ventromedial nucleus of the hypothalamus; VMH) ha surgido como principal regulador de la actividad del BAT. La proteína quinasa hipotalámica activada por AMP (AMP-activated protein kinase; AMPK), un sensor de los niveles de energía, no sólo tiene un papel fundamental en la regulación del balance energético celular y de todo el cuerpo, sino que también es crucial en la regulación de la termogénesis del BAT a través del sistema nervioso simpático (sympathetic nervous system; SNS).

El impacto del dimorfismo sexual en la homeostasis energética y la distribución de la grasa está bien establecido. A su vez, también se ha reportado la influencia de los esteroides sexuales sobre el balance energético. Aquí, se demuestra que los estrógenos y los andrógenos tienen un papel importante en la modulación del balance energético a través de acciones centrales. El tratamiento central con estradiol (estradiol; E2) inhibe la AMPK en VMH, conduciendo a la activación de la termogénesis en el BAT de manera independiente a la ingesta. Además, las fluctuaciones en los niveles de E2 durante el ciclo estral también modulan esta red fisiológica. Por otro lado, el tratamiento central con dihidrotestosterona (Dihydrotestosterone; DHT) inhibe la termogénesis en BAT de manera que los efectos centrales de los andrógenos sobre el gasto energético no se pueden atribuir a la aromatización. De manera notable, el tratamiento central con DHT demostró la capacidad de modular la masa magra. En general, estos resultados demuestran que la regulación del E2 y la DHT sobre el eje AMPK VMH-SNS-BAT es un determinante esencial del balance energético y sugieren dianas potenciales para el tratamiento de la caquexia y la obesidad.

Resumo

A regulación hemostática do balance enerxético é unha ferramenta que se encarga de manter un equilibrio estable entre a inxesta é o gasto enerxético. Calquera desequilibrio na regulación deste proceso vai levar a trastornos fisiolóxicos que se consideran hoxe un problema de saúde pública co aumento da prevalecía de obesidade e caquexia. A obesidade é causada por un aumento no consumo de enerxía crónica e a caquexia é o resultado dun aumento do gasto de enerxía a longo mantida. A termoxéneses do tecido adiposo marrón (Brown adipose tissue; BAT) é un compoñente crucial na ecuación do balance enerxético. Recentemente o hipotálamo e, en particular o núcleo Ventromedial do hipotálamo (ventromedial nucleus of the hypothalamus; VMH), ten emerxido como o principal regulador da actividade de BAT. A proteína hipotalámica quinasa AMP-activada (AMP-activated protein kinase; AMPK), un sensor dos niveis de enerxía, non só ten un papel fundamental na regulación do balance enerxético celular e todo o corpo, senón que tamén é crucial na regulación da termoxénese BAT a través do sistema nervioso simpático (sympathetic nervous system; SNS).

O impacto de dimorfismo sexual na homeostase enerxética e na distribución de graxa está ben establecida. Pola súa banda, tamén foi investigada a influencia dos esteroides sexuais sobre o equilibrio de enerxía. Aquí, móstrase que os estróxenos e andróxenos teñen un papel importante na modulación do balance enerxético, a través de accións centrais. O tratamento central con estradiol (estradiol; E2) inhibe a AMPK no VMH, conducindo á activación da termoxéneses no BAT independentemente da inxesta. Ademais, as flutuacións nos niveis de E2 durante o ciclo estral tamén modulan esta rede fisiolóxica. Ademais, o tratamento central con dihidrotestosterona (dihydrotestosterone; DHT) inhibe termoxénese no BAT de xeito que os efectos centrais dos andróxenos sobre o gasto enerxético non poden ser atribuídos a aromatización. Notablemente, o tratamento central con DHT demostrou a capacidade para modular a masa magra. En xeral, estes resultados demostran que a regulación do E2 e a DHT no eixe AMPK VMH-SNS-BAT é un determinante fundamental do balance enerxético e suxiren obxectivos potenciais para o tratamento da caquexia e a obesidade.

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TO MY FAMILY AND FRIENDS

TO EMAN AND HAMZA

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INTRODUCTION

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1. Obesity epidemic and associated diseases

Obesity has emerged as an issue of considerable concern. The American Medical Association (AMA) in 2013 has declared obesity as a disease (Atkinson, 2014). Obesity occurs due to a chronic imbalance between energy intake and energy expenditure; energy utilized exceeds what is expended causing overgrowth of the adipose tissue to a level that adversely affects the state of health (Sanchez-Gurmaches and Guertin, 2014b). Since measuring the fat mass directly is technically difficult, the Body mass index (BMI; an individual's weight divided by his height in kg/m2) has alternatively been used as an indicator of adiposity (Farooqi and O'Rahilly, 2005). The use of BMI has its limitations; it is a measure of weight, not fat content (Atkinson, 2014). The world health organization (WHO) has defined obese persons as those who have a BMI over 30 kg/m2 (van der Klaauw and Farooqi, 2015) while those with BMI over 25 are considered to be suffering from overweight (Farooqi and O'Rahilly, 2005).

Obesity is linked to comorbidities including insulin resistance, type 2 diabetes (T2D), cardiovascular disease, cancer, a range of other disorders collectively known as the metabolic syndrome (van der Klaauw and Farooqi, 2015). Also, sleep apnea, inflammation, gall bladder disease, breathlessness, gestational diabetes mellitus, and fatty liver disease (Sturm, 2002). Furthermore, high risk of coronary heart disease, back problems, gout, stroke, cancer, cataracts, stroke, osteoarthritis and impaired fertility were reported (Calkins and Devaskar, 2011). These bulk of diseases makes that the medical costs associated with obesity add more than $200 billion annually in the United States alone (Heindel et al., 2015). In 2010, estimated mortality from obesity and overweight was nearly 3.4 million per year (Lim et al., 2012b).

Recently, obesity prevalence level has been elevated in both developed and developing countries (Hebert et al., 2013). A dramatic doubling in the global prevalence of obesity over the past decades from 6.4% in 1980 to 12.0% in 2008 has also been reported (Stevens et al., 2012). In 2014, the WHO showed that the region of the Americas have the highest prevalence of obesity (27% obese) while the South-East Asia region was the lowest (5% obese) (WHO, 2014). Also, differences in prevalence between sexes have been evident, as 11% of men and 15% of women worldwide were

obese (WHO, 2014). Interestingly, in the European and Eastern Mediterranean regions and region of the Americas roughly 25% of women are obese (Figure 1).

Obesity is now known to have multiple etiologies and predisposing factors. Both the changes in our environment, as well as genetic predisposition constitute the major predisposing factors. Changes in our daily lifestyle have stood for promoting obesity; the availability of intense energy palatable food with less physical activity. Also, individuals appear to be genetically more vulnerable to weight gain than others. However, body fat growth and distribution patterns vary according to the gender difference (Geer and Shen, 2009). Women show higher tendencies toward adiposity than men do, men usually accumulate fat centrally while women tend to have more fat on the lower body regions (Geer and Shen, 2009).

All current approaches for treating obesity either with dieting, exercising or changing the lifestyle have proven to be a failure rendering obesity with a temporary relief and no cure (Atkinson, 2014). Developing more efficient anti-obesity drugs is shown itself to be the future of research in this field that should focus on a better understanding of the underlying etiologies and mechanisms of obesity. Recent studies on obesity have drawn much attention toward the impact of the regulatory mechanisms of energy balance on the quality of human life both at individual and community levels. The fact that women are more susceptible to obesity than men has included the sexual dimorphism in the equation. The governing factors of this dimorphism remain not fully understood. Thus, the involvement of sex steroid hormones as a prime candidate to claim a leading role has been suggested.

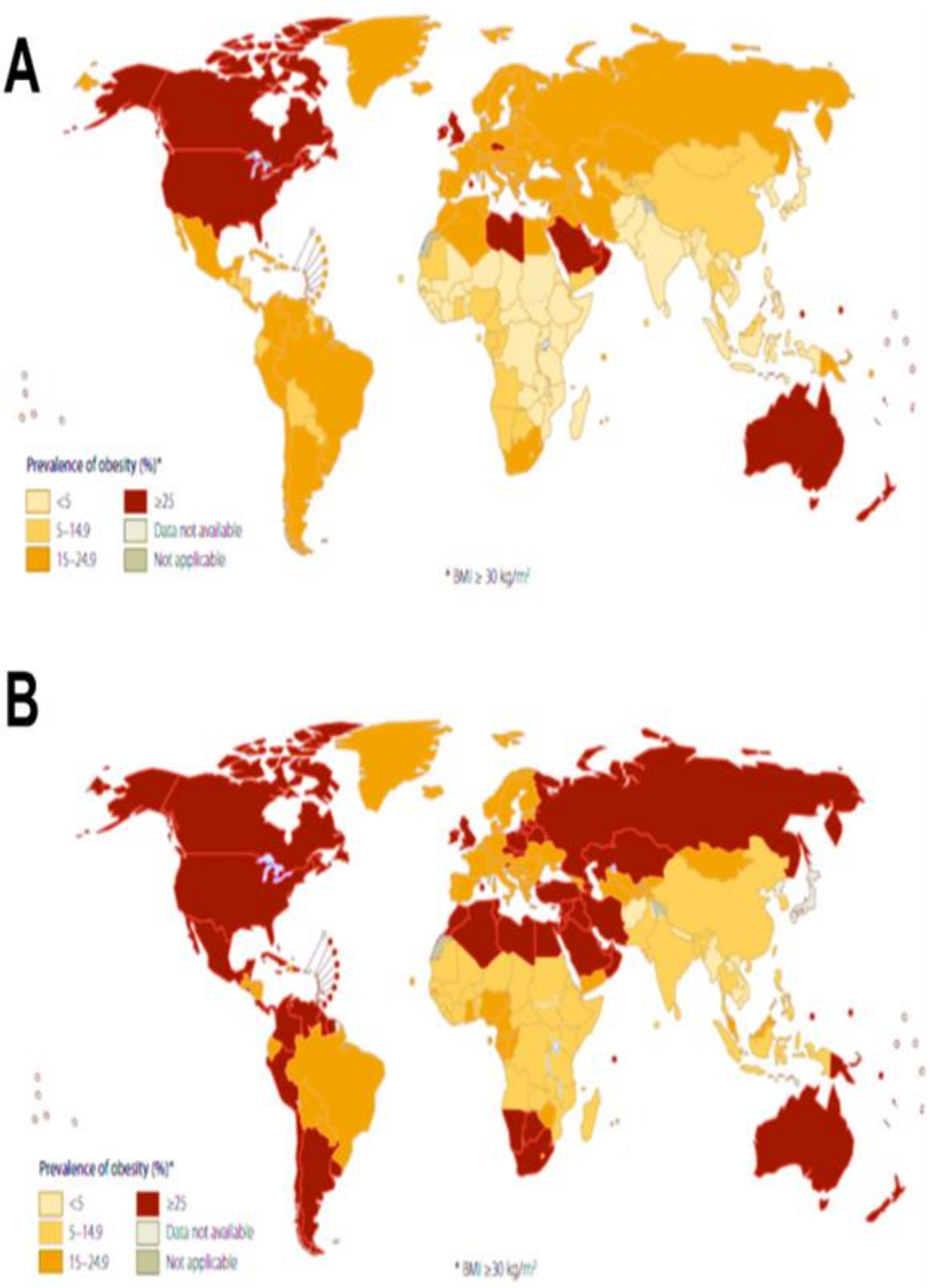

Figure 1: Global prevalence of obesity: (A) among men and (B) in women. The age range for this survey was 18 years and over. Modified from (WHO, 2014).

2. Cachexia and wasting in chronic diseases

Cachexia is a complex metabolic syndrome resulting from a long-standing imbalance between energy intake and expenditure where dietary energy intake is exceeded by the energy expenditure (Evans et al., 2008). Patients suffering from cachexia, usually lose weight mainly muscle with or without loss of fat mass without actively trying (Evans et al., 2008). They also suffer from muscle atrophy associated with weakness, fatigue as well as reduced appetite. Cachexic patients are those who lose more than 5% of their body weight in one year or less (Yoshida and Delafontaine, 2015). It is usually linked to underlying chronic illness such as chronic obstructive pulmonary disease, chronic kidney disease (CKD), congestive heart failure (CHF), and cancer. While the worldwide estimation of cachexia burden remains inconclusive, some studies show that its prevalence ranges from 5–15 % in end-stage chronic heart failure to 50–80 % in case of advanced cancer (Arthur et al., 2014; von Haehling and Anker, 2010).

Also, Cachexia is proven to be a multifactorial syndrome that cannot be entirely reversed by dietary supplementation. In cachexia conditions, the level of muscle protein degradation, myofibrillar protein, exceeds its synthesis, this imbalance results in rapid loss of muscle mass (Yoshida and Delafontaine, 2015). The biggest problem of cachexia is that it can significantly reduce the quality of life or even terminate it; weight loss and decreased muscle mass are associated with increased mortality (Lok, 2015). Thus, cachexia is a significant public health issue, and the development of interventions to block or attenuate it would have notable therapeutic benefits valuable in patients suffering from a variety of chronic diseases. Despite the excitement in laboratory studies, clinical research has so far proved disappointing.

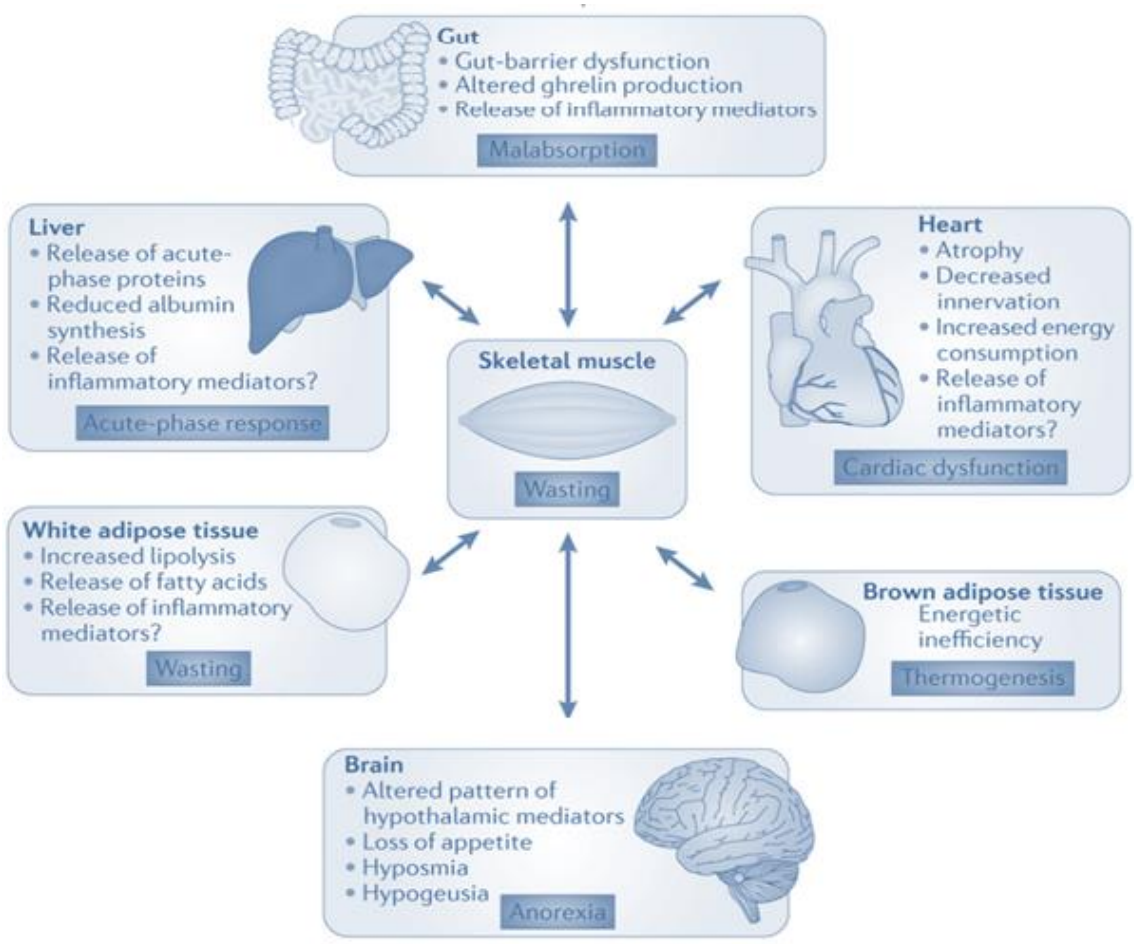

Figure 2: Cachexia and muscle wasting involve multiple organs alterations. Modified from (Argiles et al., 2014).

3. Homeostatic regulation of energy balance

Living organisms always strive to maintain their internal environment and systems in a state of equilibrium i.e. homeostasis to survive. Energy balance is no exception; our body struggles to provide a stable balance between energy consumed and expended. The peripheral organs and tissues including the gut, white adipose tissue (WAT), gonads, muscles, and thyroid continuously supply signals about the metabolic and nutritional status to the CNS. The CNS then integrates them and provoke the proper response that allows efficient energy homeostasis (Flier, 2004; Fruhbeck and Gomez-Ambrosi, 2003; Lopez et al., 2007b; Medina-Gomez and Vidal-Puig, 2005; Wren and Bloom, 2007). This complex homeostatic mechanism can be assorted to signal emission from the periphery, integration, and the proper response.

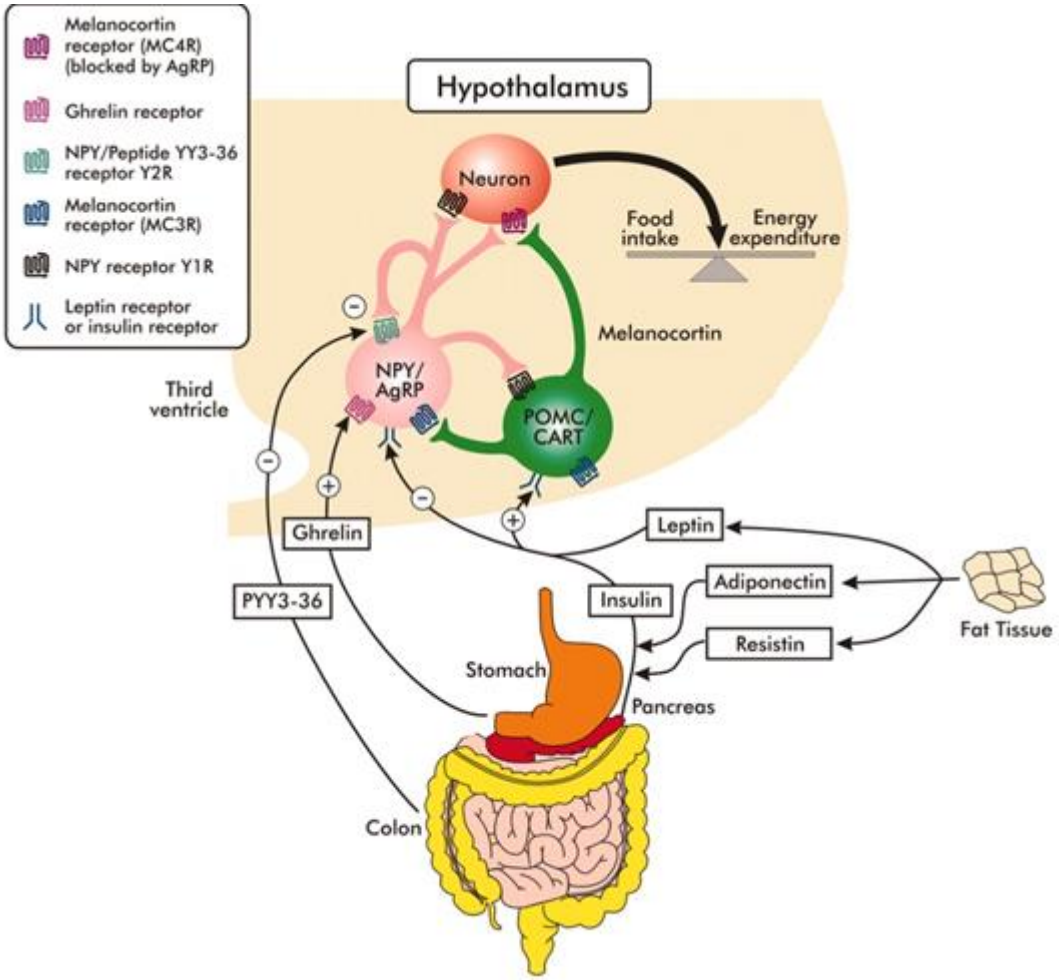

Figure 3. The homeostatic pathways of energy balance. Modified from (Gale et al., 2004).

3.1. Peripheral signals:

Different peripheral signals are implicated in the regulation of energy balance including hormonal signals; the gut hormones such as cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide (GLP-1), mechanical signals; gastric distension, chemical signals: ingested food components and nerve signal; vagal afferent stimulation. All of these signals collaborate on maintaining the CNS informed about the changes in hunger/satiety condition as well as the status of the different energy stores. Peripheral signals can be classed as long-acting adiposity signals and short-acting gastrointestinal factors. Though, some evidence on appetite-regulating hormones including ghrelin and PYY showed that they are involved in the regulation of appetite on both short and long-term basis rendering the margin between both categories very dim (Wren and Bloom, 2007).

The long-acting signals such as insulin and leptin are secreted in proportion to the levels of energy stored as fat and regulate body weight and fat stores over the long term. Short-acting gastrointestinal signals including gut hormones suppress food intake through the sensation of fullness promoting meal termination and satiation (Wren and Bloom, 2007).

3.1.1. Adipose tissue signals (Adipokines).

Since *Zhang et al.* discovered that adipocytes are capable of secreting a hormone called leptin (Zhang et al., 1994). There has been a complete change in the perception of WAT, not only energy stores but also an endocrine organ, various adipokines, adipocytes derived proteins, including adiponectin, has been identified (Bouloumie et al., 2005). Adipokines have pleiotropic activity, acting both locally and systemically, their effect involves the brain, and sympathetic nervous system (SNS), skeletal muscle, immune system, adrenal cortex. They are involved in the regulation of appetite, feeding behaviour, fat metabolism, and insulin sensitivity (Trayhurn et al., 2006). The synthesis and secretion of adipokines are an active process; their level depends on the quantity and distribution of fat mass as described in both human and animal models (Berryman et al., 2004; Staiger et al., 2003; Vidal, 2001). For instance, leptin and adiponectin levels correlate with the degree of adiposity, leptin positively while adiponectin is irreversibly correlated (Bouloumie et al., 2005).

Adipokine	Metabolic Function
Leptin	Appetite control through direct effects on the hypothalamus Increases lipolysis and decreases lipogenesis Improves insulin sensitivity and increases glucose metabolism Stimulates fatty acid oxidation and increase energy expenditure.
Adiponectin	Decreases plasma FFA levels and increases fatty acid oxidation Decreases plasma glucose levels and Increases insulin sensitivity
Resistin	Induces severe hepatic insulin resistance and increased rate of glucose production in rat
Adipsin	Stimulates triglyceride storage and inhibits lipolysis
RBP4	Promotes insulin resistance and the type 2 diabetes
IL-6	Decreases insulin and leptin signaling. Induces fatty acid oxidation and lipolysis
TNFα	Decreases insulin signaling, Increases cellular responsiveness to growth factors Decreases adiponectin and increases IL-6 expression
Apelin	Reduces food intake Inhibits glucose-induced insulin secretion
Vaspin	Improves insulin sensitivity Suppresses the production of resistin, leptin, and TNF-α

Table 1. Metabolic functions of key adipokines.

3.1.2. Gastrointestinal signals

The gastrointestinal tract secretes multiple peptides that have been implicated in energy balance regulation. The main peptide signals are ghrelin secreted from gastric mucosa, CCK from duodenum and jejunum as well as PYY and GLP-1 from the distal small intestine and colon. In addition to these, oxyntomodulin, a cleavage product of proglucagon is known to be secreted in response to food and reduce gastric motility and secretion as well as food intake (Cohen et al., 2003; Dakin et al., 2004; Wynne et al., 2005). Pancreatic polypeptide (PP) and amylin secreted from the endocrine pancreas have also been involved in the control of food intake. PP decreases food intake and may delay gastric emptying and enhances energy expenditure (Batterham et al., 2003). Amylin released from the beta cells, together with insulin,

participates in the control of glucose homeostasis and can also suppress food intake, at least at high levels (Morley and Flood, 1991; Roth et al., 2007).

Peptide	Primary sites of synthesis	Main receptor	Effect on food intake
Ghrelin	A-cells of gastric fundus, small and large intestine	GHS receptor	Increases food intake
Cholesystokinin (CCK)	I-cells of duodenum, jejunum	CCK1 receptor	Inhibits food intake
Pancreatic polypeptide (PP)	pancreatic islets of Langerhans	Y4 receptor	Inhibits food intake
Peptide YY (PYY)	L-cells of distal small and large intestine	Y2 receptor	Inhibits food intake
Oxyntomodulin	L-cells of distal small and large intestine	GLP-1 receptor	Inhibits food intake
Glucagon-like peptide 1 (GLP1)	L-cells of distal small and large intestine	GLP-1 receptor	Inhibits food intake
Apolipoprotein A-IV	the small intestine and liver	Unknown	Inhibits food intake
Amylin	pancreatic β-cells	RAMPs receptors	Inhibits food intake

Table 2. Gastrointestinal peptides and energy balance

3.2. Central regulation of energy balance

Neuronal circuits directly or indirectly regulate all physiological activities. Experimental studies pointed out that organs involved in energy homeostasis, such as liver, muscle, adipose tissue, gut or the pancreas, are under direct control of the CNS (Kleinridders et al., 2009; Plum et al., 2007; Pocai et al., 2005). The emerging facts about obesity, diabetes, and other metabolic comorbidities epidemics has directed considerable efforts to understand the exact mechanisms involved in both the influence of the different homeostatic signals as well as the central responses to regulate feeding and glucose metabolism. Experimental evidence vastly points out to the pivotal role of the hypothalamus in energy homeostasis regulation (Dieguez et al., 2009; Plum et al., 2006; Schwartz et al., 2000).

3.2.1. Hypothalamic homeostatic regulation of energy balance

The hypothalamus is considered the primary regulator of food intake and energy homeostasis. It consists of a collection of distinct hypothalamic nuclei such as ARC, VMH, LHA, DMH, and the PVH.

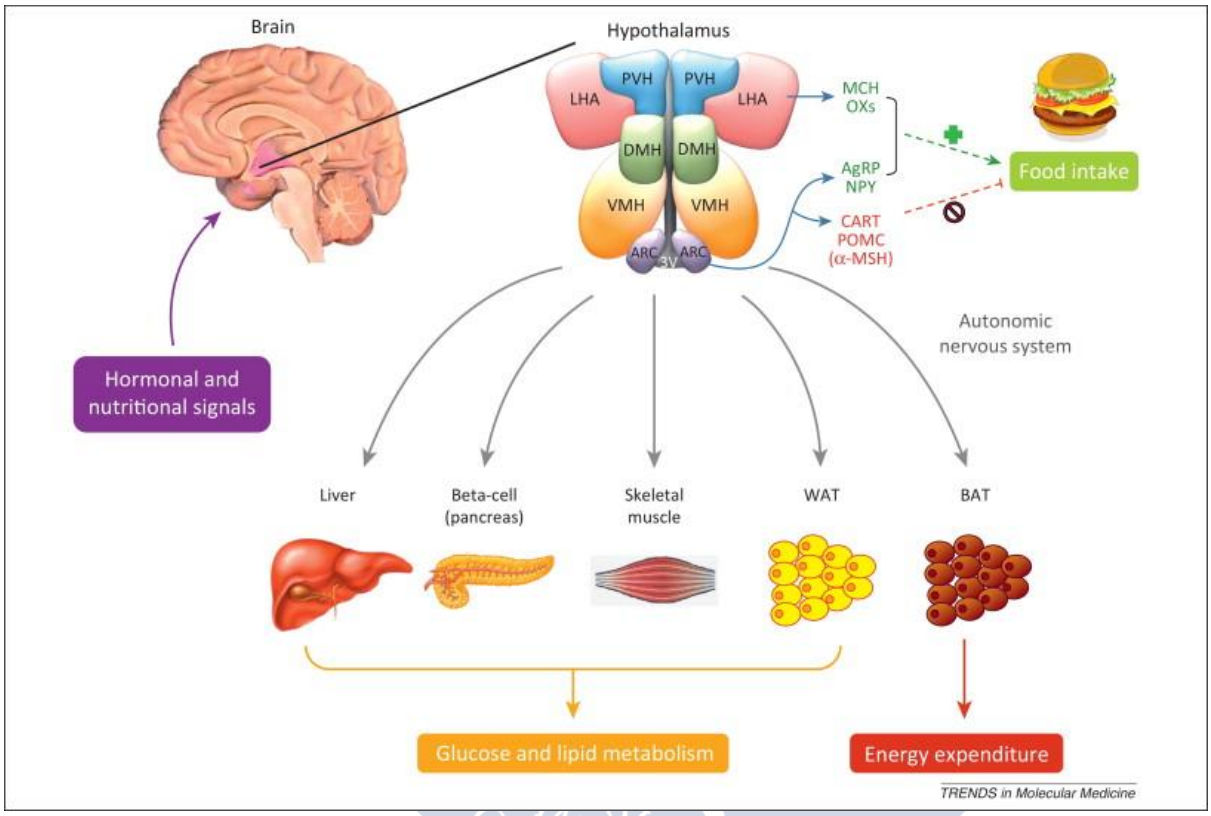

Figure 4. Hypothalamic regulation of energy balance (Lopez et al., 2013).

3.2.1.1. Arcuate nucleus (ARC)

The ARC is a key site in the control of energy homeostasis. Its anatomical location, ventral to the VMH on both sides of the third ventricle immediately adjacent to the median eminence (ME), is advantageous for sensing the hormonal and chemical changes in the bloodstream through the blood–brain barrier (BBB) (Broadwell and Brightman, 1976). The ARC contain two key neuronal populations; a) Orexigenic neurons: express neuropeptide Y (NPY) and agouti-related peptide (AgRP), b) Anorexigenic neurons: express the cocaine and amphetamine regulated transcript (CART) and proopiomelanocortin (POMC) that is a precursor of α-melanocyte stimulating hormone (α-MSH).

POMC is the key element of the central melanocortin system; that is involved in the proper energy homeostatic mechanism through sensing and integration of

peripheral signals consists of these two neuronal populations and the downstream target neurons expressing the melanocortin receptor 3 (MC3R) and the melanocortin receptor 4 (MC4R).

NPY, a 36 amino acid peptide (Tatemoto, 1982), is widely expressed in the CNS predominantly in the ARC (Gehlert et al., 1987). NPY expression and release vary depending on the changes in energy status, being decreased under feeding conditions and increased under fasting conditions (Beck et al., 1990). NPY is a powerful central appetite-stimulant and acts upon its receptors, Y1, Y2, Y4, Y5, but exerts its orexigenic effect predominantly via the Y1 and Y5 receptors (Nguyen et al., 2012; Sohn et al., 2013). ICV administration of NPY stimulated food intake in a dose-dependent manner (Clark et al., 1985; Levine and Morley, 1984). Chronic administration of NPY produced a sustained hyperphagic effect and increased body weight (Beck et al., 1992; Zarjevski et al., 1993).

Most of the NPY neurons also co-express AgRP, also, an orexigenic neuropeptide expressed in the ARC (Broberger et al., 1998; Campbell et al., 2001). AgRP is the endogenous antagonist of the melanocortin receptors (Cone, 2005; Flier, 2006; Williams and Schwartz, 2005). Of particular convincing nature was genetic overexpression or central administration of AgRP studies showing hyperphagia and reduced energy expenditure resulting in obesity (Graham et al., 1997; Ollmann et al., 1997; Small et al., 2003). The orexigenic effect of central AgRP administration lasted for few days (Hagan et al., 2000). Conversely, *Agrp* and *Npy* knockout (KO) mice showed no changes in feeding or body weight (Corander et al., 2011). However, ablation studies of AgRP neurons in adults results in hyperphagia, weight loss, and starvation (Gropp et al., 2005; Luquet et al., 2005). In contrast, neonatal loss of AgRP or AgRP neurons does not produce any effects regarding food intake or body weight (Luquet et al., 2005).

CART is co-expressed by the POMC neurons in the ARC (Elias et al., 1998). CART is involved in the regulation of food intake, maintenance of body weight, reward and addiction, stress response, psychostimulant effects and endocrine functions (Lau and Herzog, 2014; Rogge et al., 2008; Subhedar et al., 2014). Fasting reduces CART expression while feeding enhances it (Kristensen et al., 1998). CART

enhances BAT thermogenesis of (Kotz et al., 2000). Central administration of CART decreases food intake, whereas that of CART antiserum increases food intake (Kristensen et al., 1998). Also, no changes in food intake or body weight were noticed in Cart-pt-deficient mice supplied with a standard diet, although high-fat diet (HFD) induced obesity was used (Asnicar et al., 2001). The role of CART may be more complicated than initially expected since injections of CART in some specific nuclei may increase food intake (Abbott et al., 2001; Kong and Carter, 2003).

POMC neurons are laterally located in the ARC (Mizuno et al., 1998). Cleavage of POMC by endoproteases yields bioactive peptides including α -MSH, that has anorexigenic effects by binding to MC3R and MC4R thus essential for normal energy homeostasis (Mercer et al., 2013; Schwartz et al., 2000). Feeding augment the expression of POMC as well as α -MSH while fasting reduces them (Schwartz et al., 1997). Central administration of α -MSH reduced feeding and caused weight loss (Wirth et al., 2001). Obese mice exhibit decreased hypothalamic POMC mRNA expression (Mizuno et al., 1998). Also, animal models lacking POMC developed hyperphagia and obesity (Coll et al., 2004). Anti-obesity effect of the α-MSH has been reported in genetically manipulated POMC genes so that α-MSH is overexpressed, also reported in rat models fed on HFD (Lee et al., 2007; Mizuno et al., 2003; Savontaus et al., 2004). Human studies have reported that POMC gene mutations were associated with morbid obesity (Lee et al., 2006).

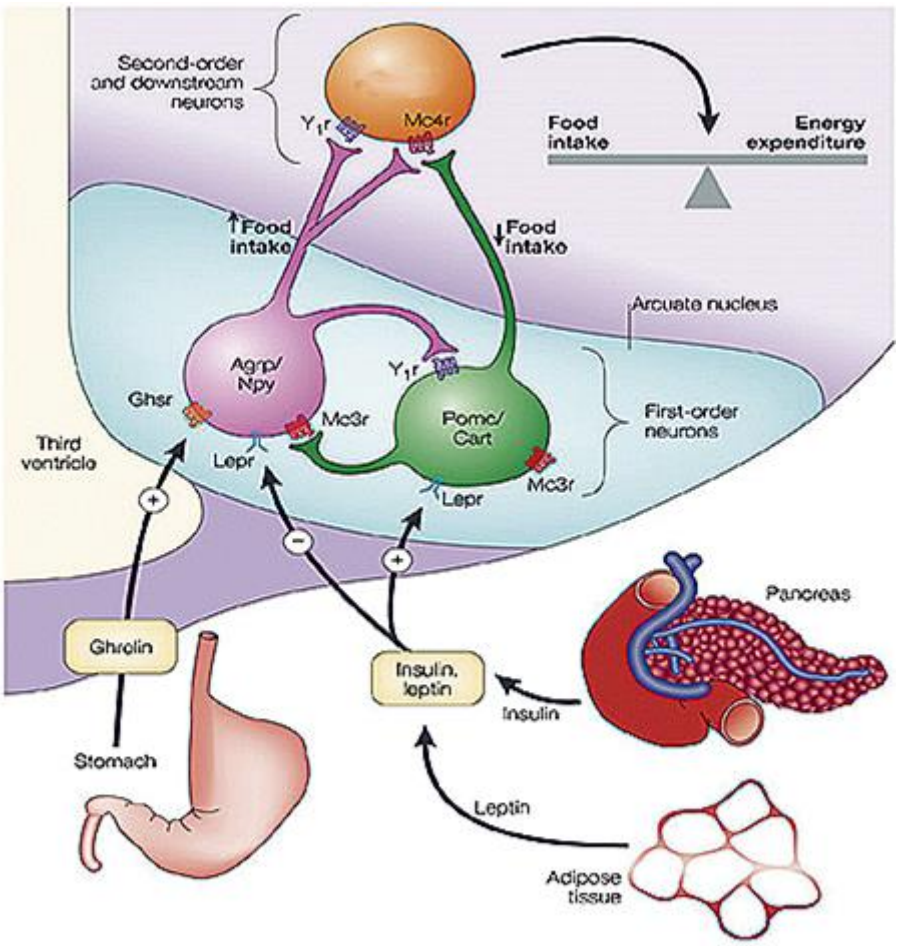

Figure 5. AgRP/NPY and POMC/CART neurons of the ARC. Modified From (Barsh and Schwartz, 2002).

3.2.1.2. Paraventricular nucleus (PVH)

The PVH is located on either side of the superior part of the third ventricle in the anterior hypothalamus. Its magnocellular neurosecretory neurons project directly to the posterior pituitary where they release oxytocin or vasopressin into the general circulation (Russell et al., 1988). Also, The parvocellular neurosecretory neurons project to the ME and release CRH, TRH, growth hormone-releasing hormone (GHRH), and dopamine into the portal blood vessels of the anterior pituitary (Whitnall, 1993). The PVH also has neurons that expressed neuropeptides such as CART, (Hillebrand et al., 2002; Leibowitz and Wortley, 2004). The PVH expresses high levels of MC3R/MC4R. Regarding the energy homeostasis, the PVH is considered a valuable integration center as it receives several afferent inputs such as those from the AgRP and POMC neurons of the ARC or even from nucleus tractus solitarius (NTS) (Kim et al., 2000; Stanley et al., 1986). The NPY/AgRP neurons of

the ARC innervate TRH neurons in the PVH and inhibit pro-TRH gene expression, whereas α-MSH/POMC projections stimulate TRH (Fekete et al., 2000; Fekete et al., 2002; Legradi and Lechan, 1999). Specific nucleus administration of some neuropeptides such as NPY, AgRP, and a-MSH has proven its involvement in the regulation of food intake (Kim et al., 2000; Stanley et al., 1986).

3.2.1.3. Lateral hypothalamus area (LHA)

The LH extends rostrally from the mesencephalic tegmentum to the lateral preoptic area in dorsal and lateral aspect to VMH (de Lecea et al., 1998). It contains sparsely distributed subpopulation of neurons expressing melanin-concentrating hormone (MCH) and orexins (orexin A and B) (de Lecea et al., 1998; Ferno et al., 2015; Lopez et al., 2010a; Sakurai et al., 1998) thus it is involved in the mediation of orexigenic responses. Orexin neurons produce orexin A and orexin B from preproorexin, with an increase in expression under fasting conditions (Sakurai et al., 1998). Central administration of orexins to rodents elicited hyperphagia (Dube et al., 1999; Lopez et al., 2002; Sakurai et al., 1998). Orexin neurons have extensive communication with other brain centers, it receives many projections from NPY/AgRP and α-MSH/POMC neurons from the ARC, also from LHA, PVH, and NTS (de Lecea et al., 1998; Peyron et al., 1998). Orexins have been reported to play a significant role in sleep regulation; orexin deficiency causes a sleep disorder termed narcolepsy (Chemelli et al., 1999; Hara et al., 2001). Recently, a role for orexin in the control of BAT thermogenesis in the rostral raphe pallidus (rRPa) was reported (Tupone et al., 2011). Orexin null mice suffered from a failure of BAT thermogenesis owing to the inability to differentiate in brown pre-adipocytes (Sellayah et al., 2011). MCH expression is enhanced by fasting while its ICV administration or genetic overexpression causes hyperphagia (Ludwig et al., 2001; Qu et al., 1996). Also, central MCH directly controls hepatic and adipocyte metabolism through different pathways (Imbernon et al., 2013).

3.2.1.4. Dorsomedial nucleus (DMH)

The DMH receives projections from the ARC NPY and α-MSH/POMC neuronal terminals (Broberger et al., 1998), whereas it sends projections to communicate with VMH, PVH and LHA (Elmquist et al., 1998; Kalra et al., 1999).

Electrolytic destruction of the DMH causes hyperphagia and obesity (Bernardis and Bellinger, 1987). It has been noted that the DMH expresses neuropeptides such as NPY and CRH as well as receptors for other peptides involved in the control of appetite and energy balance (Chan et al., 1996; Kamegai et al., 1996). NPY gene expression is increased in the DMH in case of diet-induced obesity and genetic obese mice (Elmquist et al., 1998; Li et al., 1998), as well as during pregnancy and lactating periods. The DMH is also involved in the regulation of brown adipose tissue (BAT) thermogenesis (Chao et al., 2011).

3.2.1.5. Ventromedial nucleus (VMH)

The VMH of the hypothalamus, located adjacent to the ARC, receives NPY/AgRP and POMC/CART projections from the ARC (Koylu et al., 1997; Kristensen et al., 1998). Also, it sends efferent projections to the DMH, PVH, ARC and brain stem regions such as NTS (Cheung et al., 2013). The neurons in the VMH have long been hypothesized to play a major role in metabolic regulation since bilateral VMH lesions cause hyperphagia and obesity (Weingarten and Powley, 1980). Brain-derived neurotropic factor (BDNF) is highly expressed in the VMH, and central or peripheral administration of BDNF reduces feeding and causes body weight loss (Lapchak and Hefti, 1992; Pelleymounter et al., 1995) through MC4R signalling (Xu et al., 2003). Also both humans and mice studies have demonstrated that lacking BDNF or its receptor leads to hyperphagia and obesity (Lyons et al., 1999; Yeo et al., 2004). Also, the identification of the steroidogenic factor 1 (SF1) in the VMH (Davis et al., 2004; Parker et al., 2002) was valuable in production of conditional KO mice proving the critical role of VMH in energy homeostasis (Bingham et al., 2008; Kim et al., 2011; Zhang et al., 2008). Recent findings have promoted the role for AMPK in the VMH on the regulation of thermogenesis (Lopez et al., 2010b; Martinez de Morentin et al., 2012; Whittle et al., 2012).

3.3. Energy expenditure (EE)

Maintaining relatively stable body weight is an active process that includes a balance between food intake and energy expenditure. Under normal physiological conditions, food is used to provide energy that is usually used in resting metabolic rate (RMR), physical activity and thermoregulation. The brain controls these three parts of

energy expenditure. RMR refers to the amount of energy the body uses at rest to maintain the basic cellular metabolic activities. RMR varies according to body size, fat mass, age, gender (Tataranni and Ravussin, 1995). It makes for a large portion of the energy expanded nearly 60% to 70% of total daily EE (Ravussin et al., 1986). In minimal activity people, RMR represents about two-thirds of their total daily EE; in higher activity people, RMR may represent only half of their daily EE (Speakman and Selman, 2003). The hypothalamus controls RMR mainly through neuroendocrine systems, particularly the hypothalamus pituitary thyroid (HPT) axis (Silva, 2003). Regarding physical activity, it is the most variable component; it may account for up to 40% of the total daily EE (Westerterp and Kester, 2003). The CNS controls the skeletal muscle contraction in exercise, intrinsic spontaneous physical activity, or shivering after cold exposure (Rui, 2013). As for thermogenesis, it involves brown adipose tissue (BAT) activity (Cannon and Nedergaard, 2004; Contreras et al., 2015; Whittle et al., 2011). The brain, mainly the hypothalamus and the brainstem, are responsible for the control of BAT thermogenesis through sympathetic projections. For example, E2, nicotine, GLP-1R and BMP8B signaling in the brain increases thermogenesis by enhancing the sympathetic outflow to BAT (Beiroa et al., 2014; Lockie et al., 2012; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012).

3.3.1. BAT thermogenesis

BAT is valuable for its role in adaptive thermogenesis (Cannon and Nedergaard, 2004). BAT cells are entirely distinct from white adipose tissue (WAT) not only histologically and functionally but also at the level of origin (Sanchez-Gurmaches and Guertin, 2014a, c; Shan et al., 2013). Histologically, BAT adipocytes are characterized by the presence of small lipid droplets and numerous mitochondria that contain cytochromes pigment, responsible for the brownish colour of BAT (Lim et al., 2012a). Functionally, WAT can be considered an energy store in the form of triglycerides (TG), BAT burns TG to produce heat. Brown fat is located in different body parts in rodents and human. In rodents, it is found in interscapular, periaortic, axillary, subscapular, and perirenal regions (Cannon and Nedergaard, 2004). In humans, BAT is not only located in subscapular, periaortic, pericardial, periadrenal,

cervical, perispinal and mediastinal regions (Cannon and Nedergaard, 2004; Lidell et al., 2014) but also was reported as functional in adults (Ouellet et al., 2012; Virtanen et al., 2009).

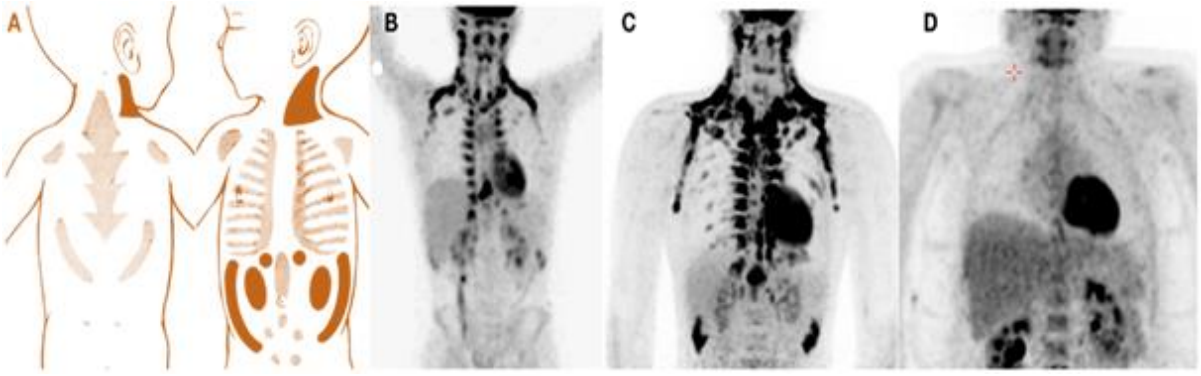

Figure 6. Human BAT distribution: (A) new-born, (B) 13-year-old boy, (C) adult male. Modified From (van Marken Lichtenbelt, 2011).

Recently, a phenomenon called the browning of WAT has been reported. It involves the appearance of a particular type adipocytes called beige, brite, or recruitable brown adipocytes in anatomically new locations corresponding to WAT (Lee et al., 2014; Lidell et al., 2013; Sharp et al., 2012). Although different in origin from the classical BAT adipocytes, they are a true functional adipocytes regarding their role in thermogenesis (Petrovic et al., 2010; Seale et al., 2008; Shabalina et al., 2013) but with lesser capacity *in vivo* (Keipert and Jastroch, 2014; Nedergaard and Cannon, 2013; Shabalina et al., 2013).

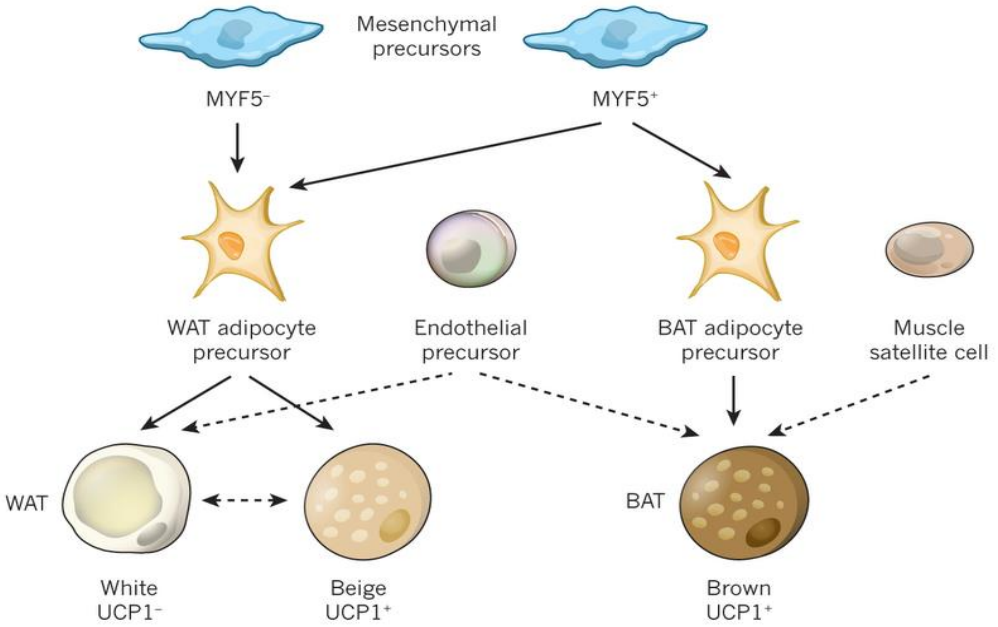

Figure 7. Origins of adipocytes. Modified from (Peirce et al., 2014).

In rodents, BAT provides an extra-thermogenic mechanism (non- shivering thermogenesis) in cold environment that gives them an alternative to shivering thermogenesis of muscles (Cannon and Nedergaard, 2004). The sympathetic nervous system (SNS) is essential to activate BAT thermogenesis as reflected by a high density of nerve endings in the tissue (Cannon and Nedergaard, 2004; Cao et al., 2001). Norepinephrine activates an adrenergic receptor that generates signals to enhance thermogenesis and stimulate intracellular lipolysis (Cannon and Nedergaard, 2004). The lack of β1- or β3-adrenergic receptors diminishes BAT thermogenesis in mice (Penfornis et al., 2000; Zennaro et al., 1998), and β-adrenergic receptor-deficient mice cannot tolerate cold (Kharitonenkov et al., 2005). The thermogenesis of BAT is associated with the presence of a mitochondrial membrane protein called the uncoupling protein-1 (UCP-1), or thermogenin, that is present in the inner mitochondrial membrane of BAT adipocytes. This protein enhances thermogenesis through uncoupling between respiratory chain complexes and the adenosine triphosphate (ATP) production (Cannon and Nedergaard, 2004; Whittle et al., 2011; Zingaretti et al., 2009) promoting the free movement of protons into the mitochondrial matrix, thus generating heat instead of ATP (Cannon and Nedergaard, 2004; Garlid et al., 2000; Jaburek et al., 2001; Whittle et al., 2011).

Many hypothalamic nuclei are associated with the regulation of thermogenesis, reports using retrograde viral tracing by using trans-neuronal pseudorabies virus in rat BAT area revealing the potential connection with the preoptic area (POA), PVH, DMH, and LHA (Cano et al., 2003; Oldfield et al., 2002). The POA is considered the classical center responsible for thermoregulation; it was thought to be the only brain center involved in the process due to the presence of temperature-sensitive neurons (Boulant, 2000). The POA receives incoming signals from cold- and warm-sensitive receptors over the body (Bratincsak and Palkovits, 2004). It has been reported that both glutamatergic and electric stimulation of the POA results in enhanced BAT thermogenesis (Holt et al., 1987; Nakamura and Morrison, 2008; Thornhill and Halvorson, 1994). Regulation of brown fat activity also involves the central melanocortin system. The central administration of MC3-R or MC4-R agonists enhanced SNS signalling and activated BAT thermogenesis (Enriori et al., 2011;

Muller et al., 1997; Vaughan et al., 2011), the opposite response resulted from their inhibition (Kooijman et al., 2014).

In regarding the crosstalk between VMH and BAT, it has been known for a long time despite the fact that the specific molecular mechanisms have been recently reported. Electrical stimulation of the VMH was associated with an increase in interscapular BAT temperature that was blocked by β–adrenergic antagonists (Holt et al., 1987; Hugie et al., 1992; Kelly and Bielajew, 1991; Perkins et al., 1981). Stereotaxic specific administration of glutamate (Amir, 1990; Hugie et al., 1992; Yoshimatsu et al., 1993), hydroxybutyrate (Sakaguchi et al., 1988), norepinephrine, serotonin, and tryptophan (Sakaguchi and Bray, 1989) into VMH activated BAT. More recently, genetic evidence has also supported the role of the VMH in the modulation of BAT thermogenesis. For example, VMH- SF-1 knockout reported reduced EE and BAT expression of UCP1 (Jo, 2012; Kim et al., 2011).

Recently, the impact of AMPK in the VMH emerged as a part of the mechanisms that regulates BAT activity. There is an inversely correlation between VMH AMPK activity and BAT thermogenesis as reported in different studies including thyroid hormone, estradiol (E2), BPM8b, and nicotine (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012).

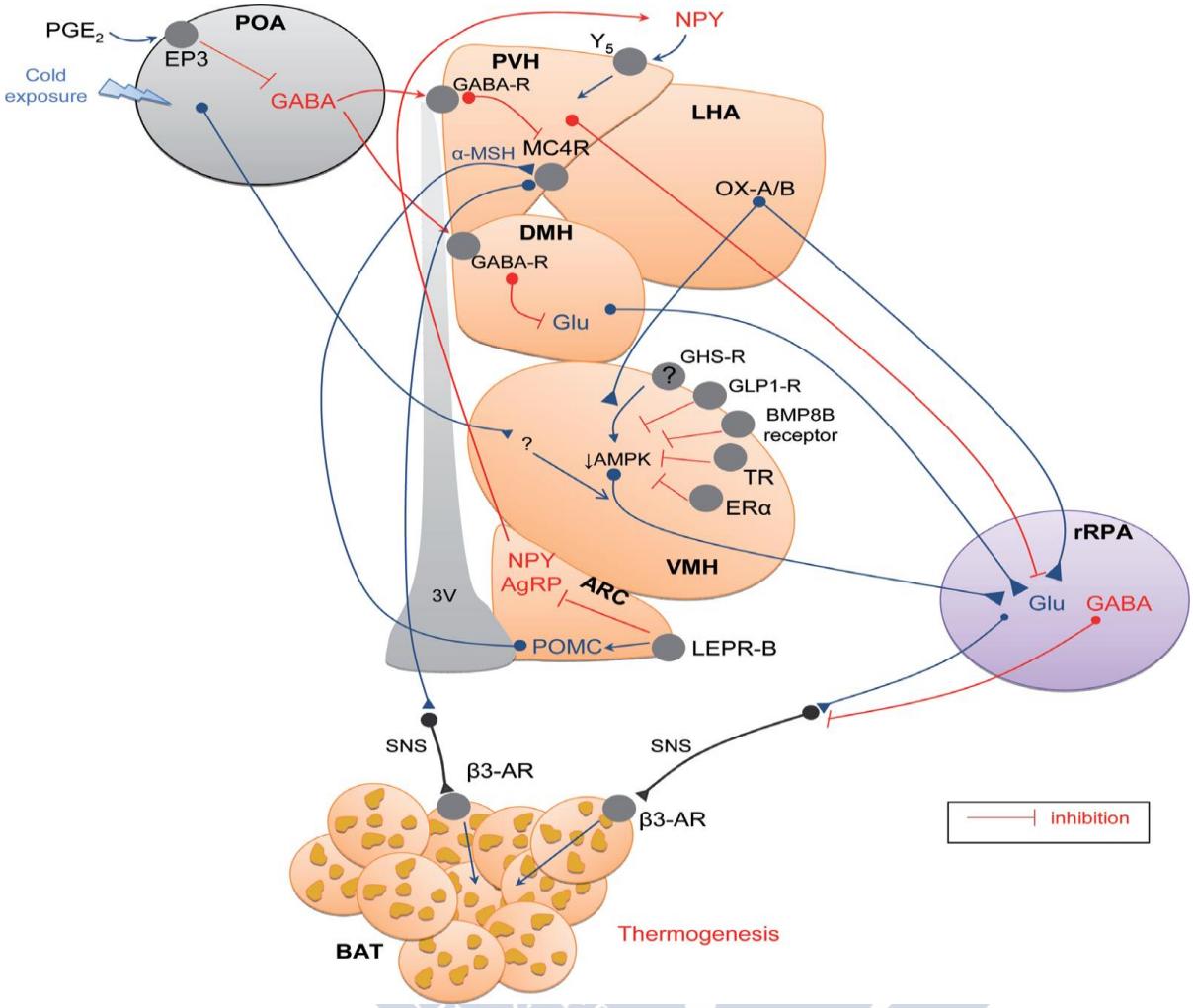

Figure 8. Hypothalamic regulation of BAT thermogenesis (Contreras et al., 2015).

BAT expresses estrogen, progesterone, and testosterone receptors (Rodriguez-Cuenca et al., 2007). Many studies support the difference in the effect of sex steroids on BAT thermogenesis between genders both in human and animals. In humans, computed tomography scans showed that active BAT is more abundantly present in women than men (Au-Yong et al., 2009; Cypess et al., 2009; Perkins et al., 2013) that may result from their greater sensitivity to cold (McArdle et al., 1984). In small animals, females appear to have larger BAT (Justo et al., 2005; Rodriguez-Cuenca et al., 2002; Rodriguez et al., 2001), larger mitochondria (Rodriguez-Cuenca et al., 2002), as well as higher GDP-binding indicator of increased UCP1 activity (Quevedo et al., 1998) than males. Regarding the metabolic and thermogenic activity of BAT, the lipid droplets in BAT adipocytes become more abundant and larger with estradiol and progesterone compared to testosterone, suggesting higher cellular metabolic capacity (Rodriguez et al., 2002). Also, BAT thermogenesis is downregulated during

pregnancy and lactation to preserve energy (Frontera et al., 2005). VMH silencing of the ERα resulted in weight gain, visceral adiposity, increased food intake, and a decrease in BAT thermogenesis (Xu et al., 2011b). On the contrary, many *in-vitro* scientific studies reported that testosterone was associated with a reduction in thermogenic capacity. It resulted in a decreased in the expression of UCP-1 (Rodriguez et al., 2002), as well as a reduction in both the abundance of lipid droplets (Rodriguez et al., 2002) and lipolytic activity (Monjo et al., 2003). Rodent models showed that testosterone was associated with decreased body mass with increased RMR (Abelenda et al., 1992; LeBlanc et al., 1998).

4. Hypothalamic lipid metabolism

As previously illustrated, the regulation of energy balance regulation is an interaction between many factors including the circulating nutrients, different hormones and peptides, and neuropeptides. Recently some basic cellular metabolic pathways have proven to play a remarkable part in this physiological network. Current evidence supports a potential critical role for the hypothalamic malonyl-CoA/carnitine-palmitoyl transferase 1 (CPT1)/long chain fatty acyl-CoA (LCFAs-CoA) axis, *de novo* lipolysis and fatty acid oxidation pathways, in regulating body mass and peripheral glucose homeostasis (Yue and Lam, 2012). This pathway can be concise into the hypothalamus sensing a physiological rise in fatty acids content in the circulation to regulate glucose homeostasis.

Malonyl-CoA is derived from acetyl-CoA by the catalytic enzyme acetyl-CoA carboxylase (ACC) (Dieguez et al., 2009; Lam et al., 2005). Excess glucose elevates pyruvate that is converted into acetyl-CoA through a serious of enzymatically catalyzed reactions in the cytoplasm and mitochondria (Dieguez et al., 2009). Both Acetyl-CoA and malonyl-CoA are substrates for the enzymatic reactions catalyzed by fatty acid synthase (FAS) to produce palmitate that is stored depending on the metabolic demands of the cell as a de novo synthesized fatty acids (Dieguez et al., 2009). Neurons use them mainly for the membrane synthesis and rarely as a fuel (Yue and Lam, 2012). Fatty acids are also derived from the diet. Circulating long-chain fatty acids (LCFAs) gain entrance in the brain by either passive diffusion (Hamilton and Brunaldi, 2007) or translocation (Mitchell et al., 2009). AcylCoA synthetase

(ACSs) esterifies these LCFAs into LCFA-CoAs. CPT-1 imports the LCFA-CoAs into the mitochondria to undergo β-oxidation (Caspi et al., 2007). Malonyl-CoA inhibits CPT-1 activity endogenously (McGarry et al., 1977; McGarry et al., 1983). Fatty acid oxidation is regulated by the availability of malonyl-CoA, which inhibits CPT-1 activity.

Malonyl-CoA is believed to be the main substrate in this axis as it is involved in both de novo lipogenesis and fatty acid oxidation pathways. Any alterations in the activities of ACC, MCD and FAS, will subsequently affect the level of malonyl-CoA. The level of phosphorylation of AMPK regulates the activities of ACC and MCD. Activation of AMPK phosphorylates and inhibits ACC, activates MCD (Dowell et al., 2005; Lage et al., 2008; Lopez et al., 2007a; Ruderman et al., 2003), and inhibits FAS (Lopez et al., 2008; Zhou et al., 2001). With hypothalamic AMPK activation, hypothalamic glucose sensing fail to inhibit glucose production while AMPK inhibition suppressed glucose production (Yang et al., 2010). The hypothalamic level of malonyl-CoA is dynamically altered by fasting/feeding conditions and subsequently changes the feeding behaviour (Hu et al., 2003; Wolfgang and Lane, 2006). Centrally or peripherally treated mice with FAS inhibitors exhibited an increase in the hypothalamic malonyl-CoA and hypophagia (Hu et al., 2003; Loftus et al., 2000). Conversely, administration of ACC inhibitor decreases hypothalamic malonyl-CoA reverses inhibition by FAS inhibitors; resulting in hyperphagia (Hu et al., 2003). Furthermore, pharmacological inhibition or genetic ablation of hypothalamic CPT1c activity decreased food intake (Obici et al., 2003; Wolfgang et al., 2006). While activation of hypothalamic CPT1 alongside the reduction in malonyl-CoA after treatment with ghrelin causes an increase in intake (Lopez et al., 2008).

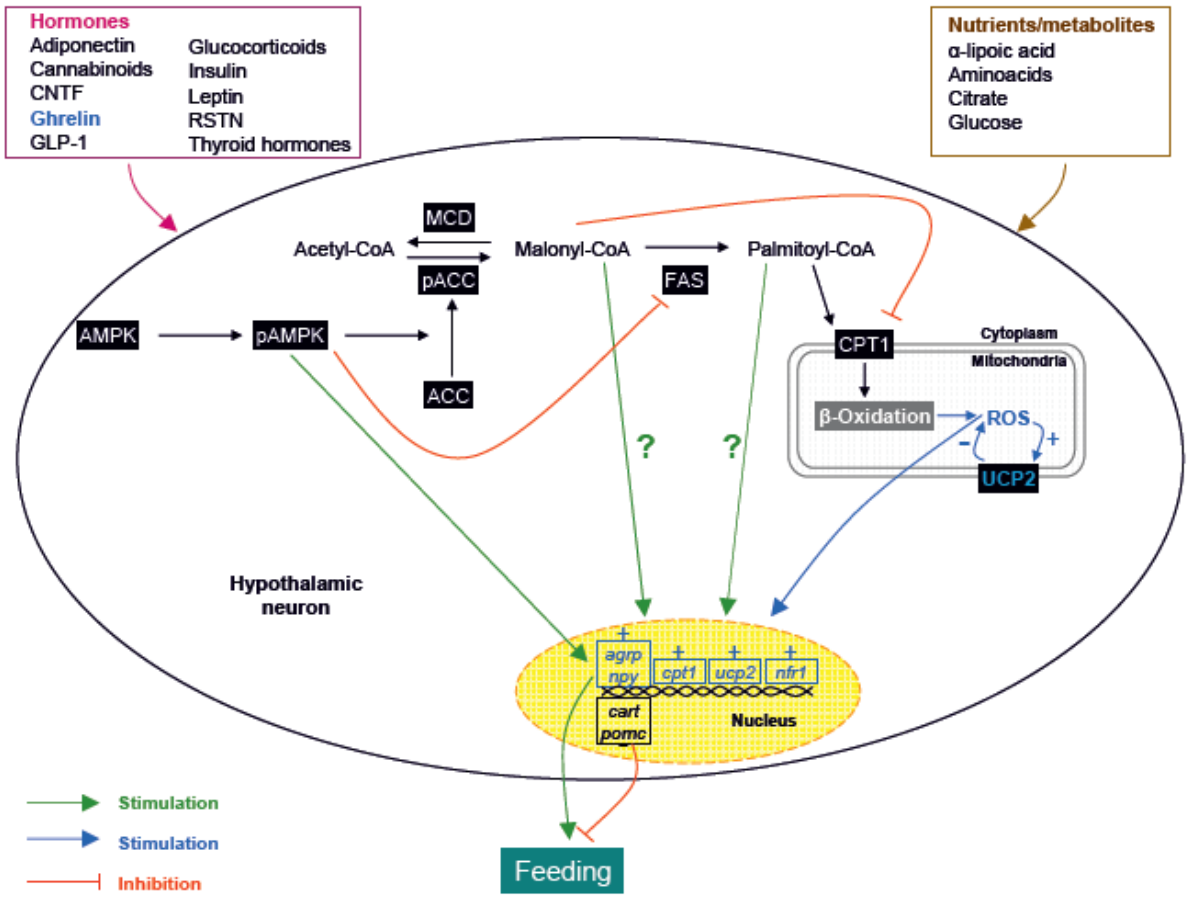

Figure 9. Hypothalamic lipid metabolism and energy homeostasis (Dieguez et al., 2009).

5. AMP-activated protein kinase (AMPK); the master energy sensor

AMPK, an enzyme that belongs to kinase family, is expressed in eukaryotic cells. It is abundantly expressed in different brain areas including the hypothalamus and hindbrain (Kola, 2008; Turnley et al., 1999). Purification and cloning of the mammalian AMPK showed that it consists of a heterotrimeric complexes of a single catalytic subunit with two isoforms (α1 or α2) and two accessory subunits termed β with two isoforms (β1 or β2) and γ with three isoforms (γ1, γ2, or γ3) (Davis et al., 2004; Hardie, 2008, 2013; Mitchelhill et al., 1994). The β and γ subunits are indispensable for the α-catalytic subunits optimal activity (Chen et al., 1999). AMPK is a sensor of the cellular energy status; it become activated by high AMP: ATP or ADP: ATP ratios following metabolic stresses that lower ATP production or raise its

consumption as hypoxia and hypoglycemia (Carling et al., 2011; Hardie, 2014; Hardie et al., 2012). The activated AMPK strives to restore energy balance by enhancing all catabolic processes producing ATP such as fatty acid oxidation while hindering ATP consuming activities such as fatty acid synthesis (Hardie, 2007; Hardie et al., 2012).

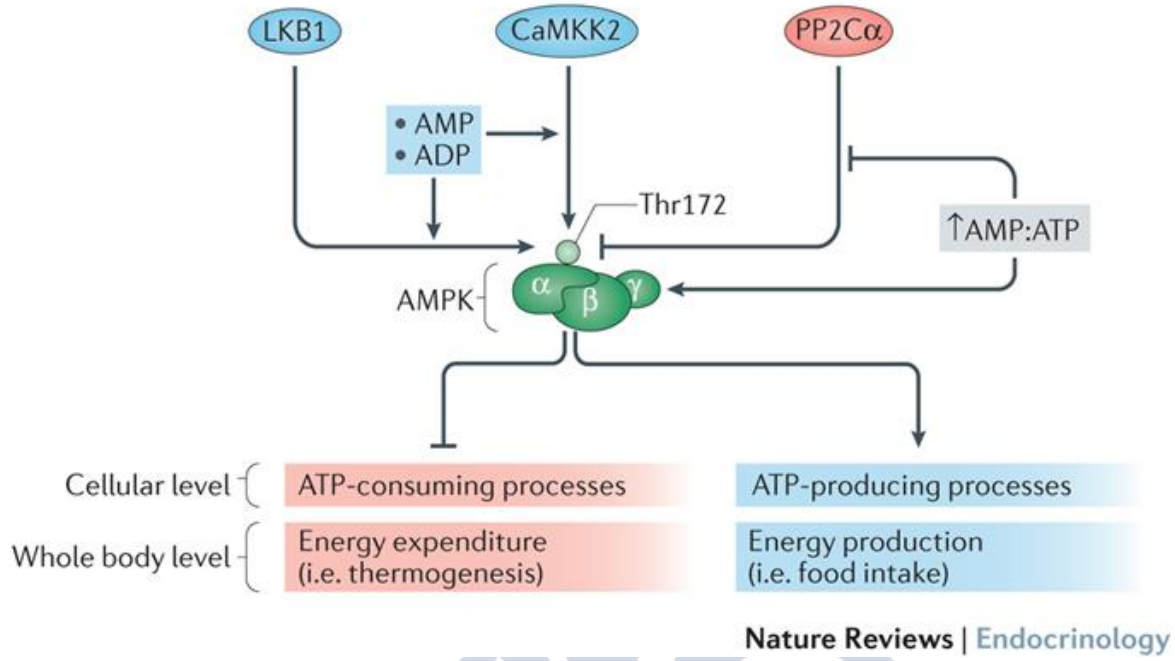

Figure 10. Structure and regulation of AMPK. Modified from (Lopez et al., 2016).

Recent reports showed the pivotal role of AMPK in the control of the whole body energy balance through its central action in the hypothalamus, aside from its role at the cellular level. AMPK is expressed in some hypothalamic nuclei involved in the central regulation of energy balance including the ARC, VMH, PVH and LHA (Minokoshi et al., 2004). Its level fluctuated with energy status, fasting enhances AMPK activity, while refeeding inhibits it in many hypothalamic regions (Andersson et al., 2004; Minokoshi et al., 2004). Also, hypothalamic AMPK activation results in hyperphagia and weight gain, while its inhibition reduces feeding and weight loss (Andersson et al., 2004; Lopez et al., 2008; Minokoshi et al., 2004).

Hormonal regulation of hypothalamic AMPK activity also has been reported. It is activated by orexigenic signals such as ghrelin, glucocorticoids, adiponectin (Gao et al., 2013; Guillod-Maximin et al., 2009; Kubota et al., 2007; Lopez et al., 2008; Shimizu et al., 2008). It is also inhibited by anorexigenic signals such as leptin, estradiol, insulin, and GLP-1 (Gao et al., 2007; Martinez de Morentin et al., 2014;

Minokoshi et al., 2004; Namkoong et al., 2005; Seo et al., 2008). Genetic animal models have also enforced the role of hypothalamic AMPK in energy homeostasis. Global AMPKα2 knockout resulted in weight gain and adiposity under HFD condition (Viollet et al., 2003). Also, neuron-specific knockout of AMPK α2 such as POMC neurons (POMCα2KO) resulted in an increase in body weight and adiposity under standard and HFD feeding conditions (Claret et al., 2007). Conversely, mice with AMPKα2 deficient AgRP neurons had age-dependent leanness (Claret et al., 2007).

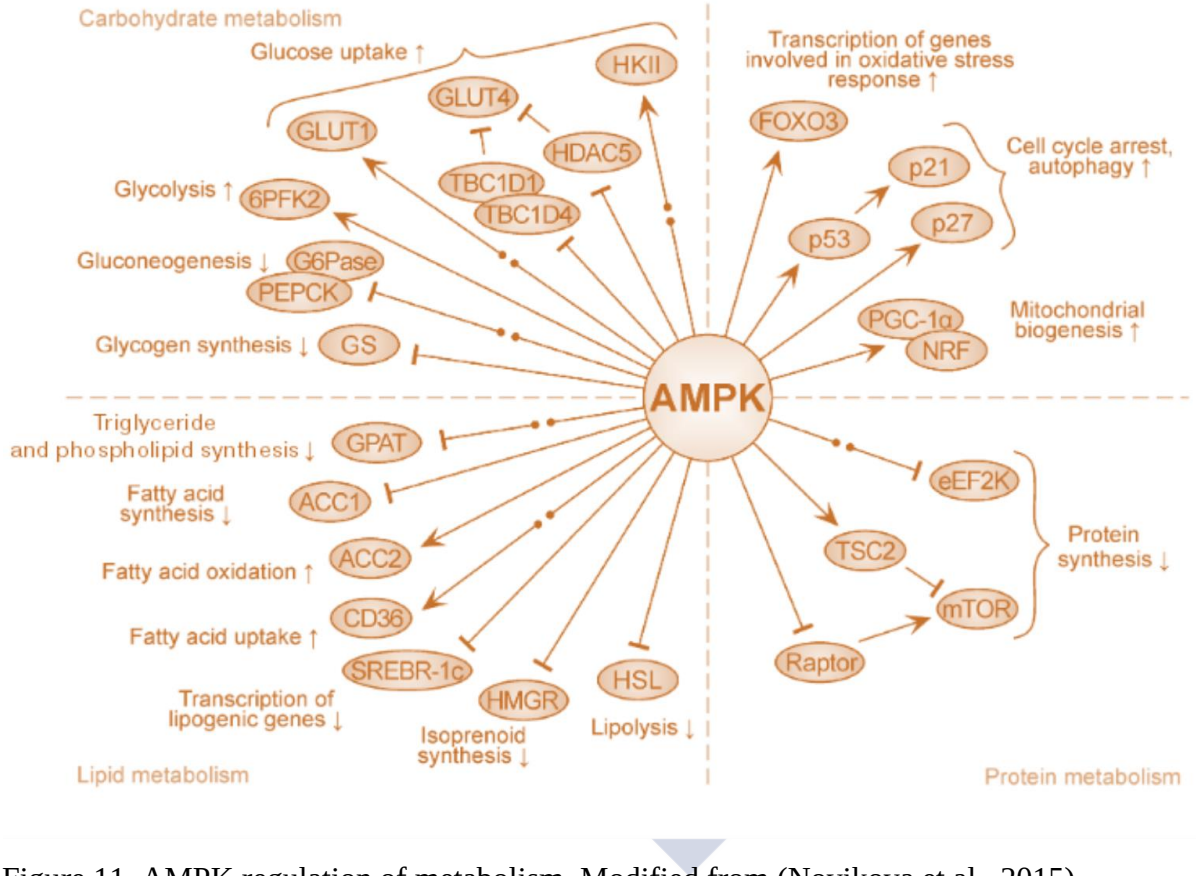

Figure 11. AMPK regulation of metabolism. Modified from (Novikova et al., 2015).

6. Sex steroids and energy homeostasis

Sex hormones, particularly estradiol, have a clear impact on the regulation of energy balance. For example, estrogen deficiency resulted in higher energy intake and increased body weight in ovariectomized rodents and post-menopausal women. Of note, this effect can be reversed by treatment with steroid hormones. Sex steroid hormones (estrogens, progestins, and androgens) are not only participating in normal reproductive function but also, regulate many physiological functions. They are either

secreted by the gonads or to a lesser extent synthesized locally in extragonadal tissues (estradiol and progesterone by the ovary and testosterone by the testis). The hypothalamic-pituitary-gonadal axis (HPG axis) points to the relation between the hypothalamus, pituitary gland, and gonads. Sex steroid hormones are secreted in response to gonadotropins released from the anterior pituitary induced by gonadotropin-releasing hormone (GnRH) from the hypothalamus. It is evident that gender, reproduction, and energy metabolism are interrelated (Hill et al., 2008).

Any severe changes in the energy balance such as obesity, anorexia, and cachexia have a negative influence on fertility (Cardozo et al., 2012; Du Plessis et al., 2010; Sermondade et al., 2012). Sex steroid hormones play important roles in regulating energy metabolism as evidenced by the fact that lacking estrogen or androgen predispose to obesity and its associated comorbidities including diabetes and cardiovascular diseases (Carr, 2003; Zitzmann, 2009). Also, in humans, gender is associated with differences in energy metabolism due to the action of sex steroid hormones while sex-specific specialization is linked to distinct body fat distribution and energy substrate utilization patterns (Varlamov et al., 2014).

6.1. Estrogens

Estrogens, a potent steroid hormone present in high levels in females from adolescence to menopause and low levels in men, are complex hormones with pleiotropic effects. There are three main forms of estrogens in mammals, estrone (E1), 17β-estradiol (E2), and estriol (E3) (Kamat et al., 2002). E2 being the most active metabolite. Estrogens secreted from the ovaries of women with normal menstrual cycles or extra-ovarian synthesized at local tissues in postmenopausal women and men as a paracrine or autocrine factor (Simpson et al., 2005). The ovary of premenopausal women primarily produces E2 in the growing follicles. Estrogens are also synthesized by the placenta, the adrenals, and testes (Kamat et al., 2002). The placenta is widely responsible for the high levels of estrogens, mainly E3, during pregnancy. The role of adipose tissue derived estrogens remains not very clear (Kim et al., 2014). The ovary of premenopausal women primarily produces E2 in the growing follicles. The events of follicular estrogen synthesis are a two-cell, two-gonadotropin

model (Hillier et al., 1994; Lopez and Tena-Sempere, 2015). In the growing follicle, both theca and granulosa cells are necessary for the synthesis of ovarian estrogens. In the inner theca cell layer, the luteinizing hormone stimulates the conversion of cholesterol into androgens such as androstenedione and testosterone. The newly synthesized androgens move to granulosa cells layer to be converted into estrogens in a reaction catalysed by aromatase enzyme under the influence of the follicle-stimulating hormone (FSH) (androstenedione to E1 and testosterone into E2). E1 can be further converted into E2 by the enzyme, 17-β hydroxysteroid dehydrogenase (17-β HSD) while both E1 and E2 can be precursors for the synthesis of E3 (Kamat et al., 2002). Tissue estrogen sulfotransferase (EST) is a critical mediator of estrogen action; it is responsible for suppressing estrogen activity by conjugating sulfonate group to prevent its binding to ERs and enhance the urinary excretion of the hormone (Strott, 1996).

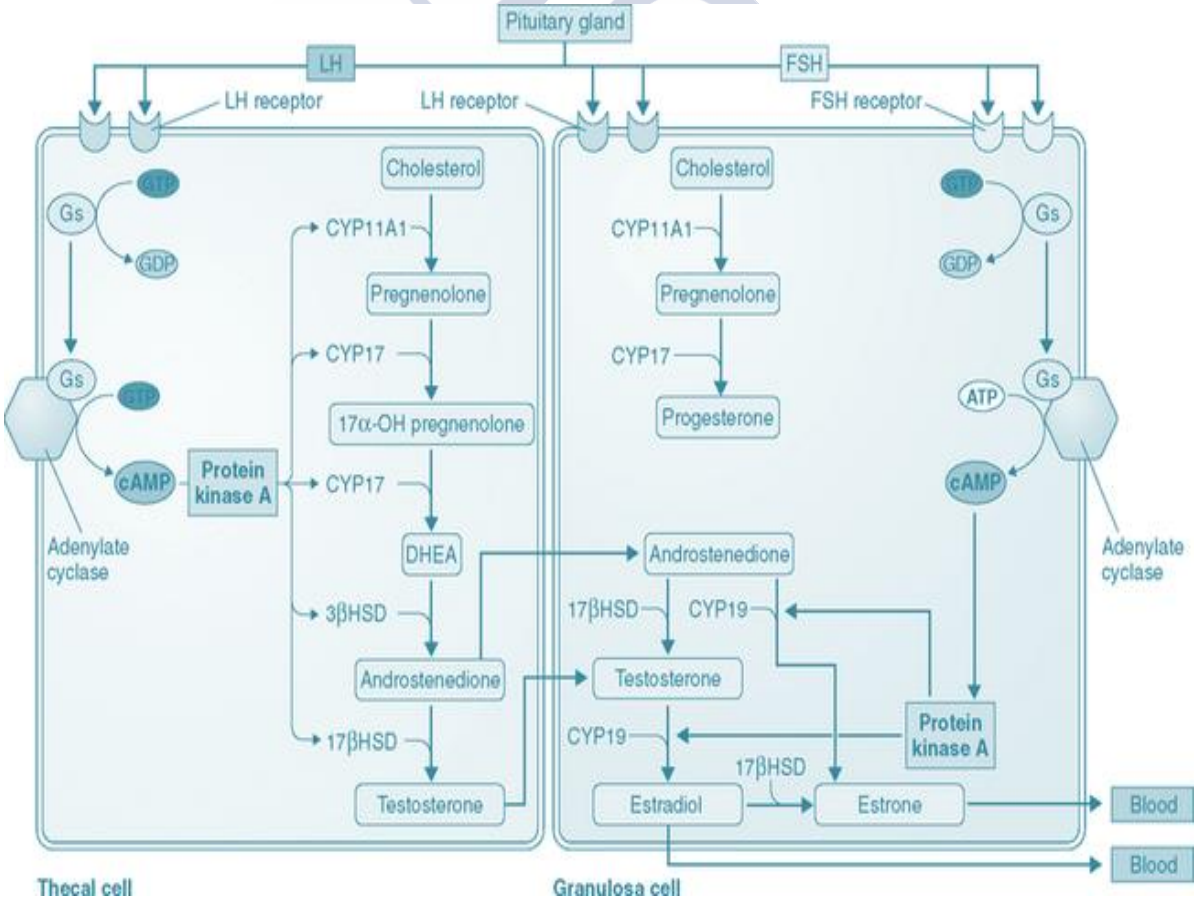

Figure 12: Ovarian estrogen synthesis by the two-cell, two-gonadotropin model. Modified From (Falcone and Hurd, 2007).

Estrogens not only control female fertility and numerous reproductive functions (Garcia-Galiano et al., 2012) but also are involved in cognition, neuroprotection and cellular metabolism (Arevalo et al., 2015; Garcia-Galiano et al., 2012). E2 is involved in controlling many cellular functions at the different body tissues; a- at the central level in hypothalamic nuclei control food intake, energy expenditure, and WAT distribution, and b- at the periphery in insulin secretion and sensitivity as well as prevention of lipid accumulation (Garcia-Galiano et al., 2012).

6.1.1. Estrogens: modes of action and regulatory mechanisms

Estrogens mediate their biological effects through binding to estrogen receptor (ER). There are two classical estrogen receptors (ER): ERα and ERβ that has multiple isoforms, different tissue expression patterns, and functions (Nilsson et al., 2001). In humans, ERα has three isoforms (ERα1- 3), while ERβ has five isoforms (ERβ1-5) (Leung et al., 2006; Matthews and Gustafsson, 2003; Ogawa et al., 1998). The variation in ER distribution may be governing factor responsible for tissue-specific response to estrogen (Knowlton and Lee, 2012). ERα and β are found in the cytoplasm and the nucleus in caveolae-associated with the plasma membrane (Acconcia et al., 2005; Chambliss et al., 2000). ERα forms complexes with caveolin 1, c-Src, Akt, PI3K, HSP90 and eNOS in caveolae in the plasma membrane (Haynes et al., 2003; Kim and Bender, 2009; Li et al., 2003). Estrogens, lipophilic in nature, are readily diffused through cellular membranes and interact with intracellular ERs (Nadal et al., 2001; Paterni et al., 2014). Upon activation of ERs, classical and non-classical pathways for mediating the estrogenic effects have been suggested.

The classical pathway of action of ERs involves them operating as ligand-activated transcription factors that, upon ligand binding, form dimers to bind directly to an estrogen response element (ERE) in target gene promoters to produce gene expression (Nadal et al., 2001; Paterni et al., 2014; Safe and Kim, 2008) in a genomic mechanism that involves ERE-dependent effects. The other non-classical mechanisms do not imply ERE action. It might be through indirectly binding to DNA through protein-protein interactions with other DNA-binding transcription factors in the nucleus or membrane-associated ERs mediate nongenomic effects (Bjornstrom and Sjoberg, 2005). This pathway is responsible for conducting estrogens capacity to

negatively feedback on gonadotropin secretion (Glidewell-Kenney et al., 2007), as well as energy metabolism related actions (Liu and Mauvais-Jarvis, 2010). ERα activation partially mediates its effect in energy homeostasis through then on ERE pathways, as the genetic rescue of nonclassical ERα signalling in a global ERα-knockout (KO) mouse was enough to restore nearly the main metabolic parameters to normal values (Park et al., 2011).

In the non-genomic pathway, activation of membrane-associated ERs activates a signalling cascade that includes PI3K and Akt, ERK 1/2, JNK and p38 (Patten et al., 2004; Wang et al., 2006; Wang et al., 2009; Wu et al., 2011). This signalling cascade is responsible for protecting against cell injury, except for JNK, that increases apoptosis. More recently, G-protein-coupled estrogen receptor (GPER) has been reported as a membrane-associated ER (Deschamps and Murphy, 2009). It is responsible for mediating a rapid cellular response to estrogens (Noel et al., 2009; Thomas et al., 2005). This receptor is also known as G-protein coupled receptor 30 (GPR30), or more recently GPER1 and the membrane estrogen receptor (mER). GPER1 has been shown to contribute to E2 mediated vasodilation (Lindsey et al., 2011). The genetic ablation of GPER1 has been associated with some of estrogen insufficiency outcomes such as increased body weight and glucose intolerance but not related to increased food intake and decreased locomotor activity (Sharma et al., 2013).

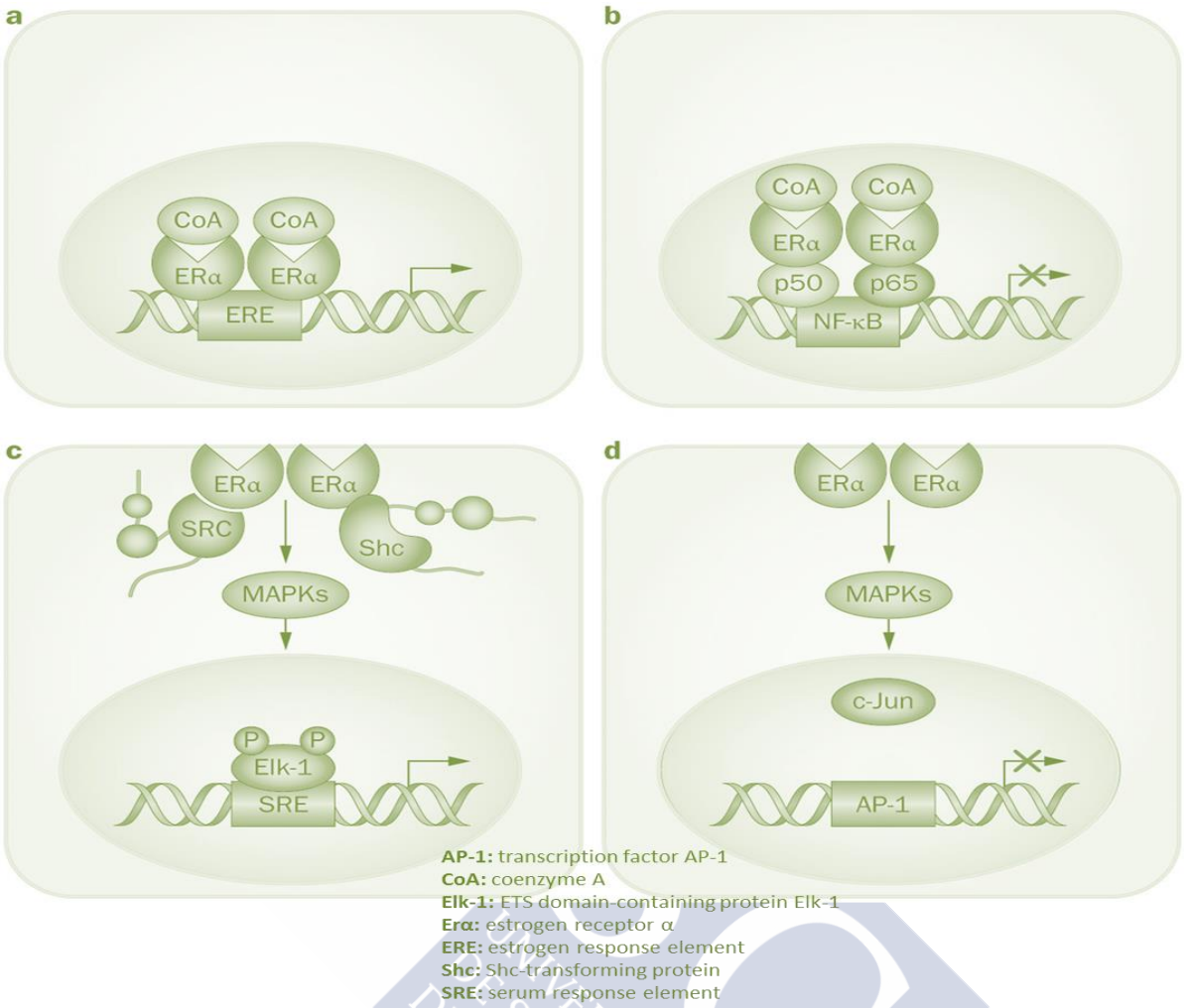

Figure 13: Mechanism of action of ERα. (a) Classic genomic signalling. (b) ERE-independent genomic signalling, (c, d) Non-genomic mode of action (c) positive or (d) negative regulation of transcription. Modified From (Manolagas et al., 2013).

It is also worth mentioning that the estrogen-related receptors (ERRs), a family of orphan nuclear receptors, with three isoforms (alpha, beta and gamma) are involved in energy metabolism and mitochondrial biogenesis (Dufour et al., 2007; Giguere, 2008). Their activity depends on the peroxisome proliferator-activated receptor coactivator-1 family proteins (Giguere, 2008; Schreiber et al., 2003). Activated ERα has been reported to upregulate ERRα expression with many similarities in the ER and ERR response elements (Giguere, 2002). To date, no ligand for these receptors is known as well as any known estrogen action.

It has been suggested that the estrogenic effect on energy balance are mostly mediated by ERα (Mauvais-Jarvis et al., 2013). ERKOα mice models were hyperphagic, obese and with higher leptin level as well as insulin resistance (Geary et

al., 2001; Heine et al., 2000; Ohlsson et al., 2000). Aromatase enzyme KO (ArKO) mice models were associated with obesity (Jones et al., 2000). Patients suffering from the lack of aromatase enzyme also suffered from obesity (Grumbach and Auchus, 1999). On the other hand, knocking out of ERβ was not associated with any energy imbalance changes (Ohlsson et al., 2000). In keeping with this evidence, it was also shown that propylpyrazoletriol (PPT), selective ERα agonist, was associated with a loss of appetite, meanwhile, diaryl propionitrile (DPN), selective ERβ agonist, was not (Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2015; Roesch, 2006).

6.1.2. Estrogens and the control of energy homeostasis

In the recent years, estradiol has proven to play a vital role in the control of energy homeostasis both at central level acting directly on the hypothalamus and at the periphery through hormonal regulation such as adipokines and insulin (Clegg et al., 2006). The incidence of obesity is higher in postmenopausal than premenopausal woman owing to the low estrogen level (Garcia-Galiano et al., 2012). Also ovariectomized rats were hyperphagic with higher body weight (Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2015; Mauvais-Jarvis et al., 2013). Estrogen replacement therapy has proven to be of value in both postmenopausal women and ovariectomized rats (Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2015; Mauvais-Jarvis et al., 2013). Recently it was reported that estrus cycle (Martinez de Morentin et al., 2014), as well as gestation and lactation (Martinez de Morentin et al., 2015), are associated with changes in energy homeostasis.

6.1.2.1. Peripheral actions of estrogens in the control of energy homeostasis

Recent reports have mentioned a cross-talk between estrogen and leptin, a peptide hormone secreted by adipocytes. Leptin has a direct effect on ERα, it activates and increases its expression (Catalano et al., 2004; Fusco et al., 2010). As mentioned earlier estrogen can be synthesized locally in the adipose tissue in a reaction catalysed by the aromatase enzyme, Leptin has been shown to increase aromatase expression (Catalano et al., 2003). Further, leptin receptor (Ob-R) and ERα shows several interactions where Ob-R STAT-3 activation is enhanced by ERα expression (Binai et al., 2010). Estrogen can directly stimulate leptin expression as shown in mice models; estradiol was associated with an increase in both leptin levels and receptor expression

in mammary tumors (Morad et al., 2014). Estrogens were suggested to regulate positively leptin gene expression (Alonso et al., 2007). Studies on rat isolated adipocytes showed the influence of estradiol on leptin gene expression (Brann et al., 1999). The central actions of leptin were found to be attenuated in association with low estrogen levels (Alonso et al., 2007; Mistry et al., 1999).

Cholecystokinin (CCK), a gut peptide and a satiety signal (Beglinger and Degen, 2004), is also affected by estrogen. Estrogens increase CCK satiating effect as evidenced by intraperitoneal administration of CCK produced satiation during proestrus or estrus, but not during diestrus. The effect of CCK-1R antagonist was more prominent during estrus than during diestrus and only evident after puberty, estradiol replacement therapy in ovariectomized rats augmented the effect of intraperitoneal administration of CCK (Asarian and Geary, 1999, 2007, 2013; Butera, 2010; Butera et al., 1993; Eckel, 2004). Estrogens mainly activate ERα to increase CCK satiating effect wherein ovariectomized *ERα* KO mice, the effect of CCK-1R antagonist was not potentiated by estradiol injection (Geary et al., 2001). Glucagon-like peptide-1 (GLP-1) is a gut peptide and a satiety signal (Barrera et al., 2011; Turton et al., 1996). It has been reported that in ovariectomized rats, estradiol potentiates both the satiating effect of GLP-1 (Asarian and Geary, 2013) and the de-satiating effect of the GLP-1 receptor antagonist exendin-9 upon intraperitoneal administration of either (Asarian et al., 2012; Asarian and Geary, 2013). Glucagon, a peptide hormone, secreted by the intestine and pancreas during meals to produce anti-insulin-like effect (Habegger et al., 2010). Estradiol increased both the satiating effect of intra-meal hepatic portal infusions of glucagon as well as the de-satiating glucagon antagonist by hepatic portal infusion of glucagon antibodies in ovariectomized rats (Geary and Asarian, 2001).

Ghrelin is a gut peptide synthesized and secreted from the stomach to stimulate appetite (Kojima et al., 1999). Intraperitoneal and central ghrelin injection in ovariectomized rats was associated with higher appetite stimulation than intact rats or estradiol-treated ovariectomized rats with cycle variation, during diestrus than proestrus or estrus (Clegg et al., 2007). Ghrelin mRNA levels showed cyclic variation as well, lower in proestrus and higher in the diestrus (Chen et al., 2004). Despite the

fact that estrogens affect meal size not frequency, it has been reported that the effect of ghrelin was associated with changes in meal frequency, but not size, in ovariectomized rats (Butera, 2010; Clegg et al., 2007). Ghrelin and its receptor were reported to be expressed in ovary, and placenta of rat (Budak et al., 2006; Caminos et al., 2003; Gaytan et al., 2005; Gualillo et al., 2001; Otto et al., 2001) and ERα is expressed in gastric mucosa (Clegg et al., 2007). Temporary elevation in both the number of ghrelin-expressing cells in the gastric mucosa as well as plasma ghrelin level upon ovariectomy was reported (Clegg et al., 2007; Matsubara et al., 2004; Sakata et al., 2006).

The interaction between estrogens and insulin can be interpreted from the development of both obesity and insulin resistance in both ERα knockout (Heine et al., 2000) and aromatase knockout mice (Takeda et al., 2003). The low estrogen levels rendered both ovariectomized rats (Alonso-Magdalena et al., 2008) and postmenopausal women (Livingstone and Collison, 2002) makes them more susceptible to impaired glucose tolerance and insulin resistance. Estrogen supply to both cases was enough to protect (Godsland, 1996). Central administration of insulin inhibited feeding in males more than females (Clegg et al., 2006; Clegg et al., 2003). Central or peripheral administration of estradiol decreased the feed inhibition effect of centrally administered insulin in ovariectomized rats (Clegg et al., 2006).

Current reports on the estrogenic effect of adipose tissue have shown that they interfere with fat distribution pattern (Lovejoy et al., 2009), and differentiation (Lapid et al., 2014) as well as lipid metabolism (Varlamov et al., 2014). Males has lower body fat (mostly intra-abdominal) than females (mostly gluteal and subcutaneous) (Bjorntorp, 1992; Bouchard et al., 1993). Post-menopausal women suffer from a change in fat distribution, tends to accumulate intra-abdominal fat (Guthrie et al., 2004; Lovejoy et al., 2008) that was prevented by estrogen supplying (Gambacciani et al., 1997). In ovariectomized rats, both central and peripheral administration of estradiol restores fat distribution to normal as well as their central leptin sensitivity (Clegg et al., 2006). Estrogens are locally synthesized in the adipocytes and their level correlate to the total adiposity (Tchernof et al., 1995). ERα presents in the adipose tissue (Mizutani et al., 1994). Obesity has been reported in ERαKO mice mainly

visceral fat compartment and to a lesser extent in the inguinal fat (Heine et al., 2000). On the other hand, ERβKO mouse was not associated with obesity (Ogawa et al., 1999).

In ovariectomized mice, estradiol injection reduced adipocyte size by decreasing fatty acid uptake and lipogenesis (D'Eon et al., 2005). Estrogens also enhance browning of the adipose tissue; females have a higher metabolic rate of fat than males as well as expression levels of uncoupling protein one (UCP-1) (Cypess et al., 2009; Nookaew et al., 2013). Estrogens may enhance browning through regulating both the natriuretic proteins ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) (Bordicchia et al., 2012; Collins, 2014). The levels of ANP and BNP were greater in premenopausal women than men and were reduced in premenopausal women (Jankowski et al., 2001; Wang et al., 2002).

6.1.2.2. Central effects of estrogens in the control of energy homeostasis

Estrogens provide protection against the development of obesity and its associated comorbidities (Dye and Blundell, 1997). ERs are found all over the CNS (Merchenthaler et al., 2004; Pitteloud et al., 2005) as well as in the different hypothalamic nuclei in rodent brain including the ARC, VMH, PVH, and POA (Osterlund et al., 1998; Shima et al., 2003; Simerly et al., 1990). Several reports have concluded that estrogens are involved in the hypothalamic modulation of energy balance through action particularly in the ARC and the VMH where ERα is more abundantly expressed than ERβ. Estradiol-mediated actions related to energy homeostasis are mainly associated with ERα (Heine et al., 2000). Central administration of estradiol has a food inhibitory effect (Martinez de Morentin et al., 2014). Likewise, ERα deficiency in female mice was associated with obesity and weight gain through increasing energy intake and decreasing expenditure (Heine et al., 2000; Ribas et al., 2010). ERβ is also involved as ICV administration of ERβ antisense oligodeoxynucleotides blocks the estrogenic effect of centrally administered estradiol in female rats (Liang et al., 2002). Also, in HFD-fed female mice, ICV injection of ERβ-selective agonist was associated with elevated BAT UCP-1 expression, thereby reducing obesity (Yepuru et al., 2010).

In the ARC, ERα is prominently expressed in POMC neurons, the levels of POMC expression is dramatically increased with the higher estradiol level in proestrus (Bohler et al., 1991; Wise et al., 1990). On the other hand, ERα KO mice have a decreased POMC expression levels (Hirosawa et al., 2008), a situation that is also found in ovariectomized rats, where this action was reversed with estradiol treatment (Martinez de Morentin et al., 2014; Pelletier et al., 2007). Also, other evidence from mice showed an increase in the excitatory synapses on POMC neurons in proestrus or after estradiol treatment (Gao et al., 2007). Estrogens act directly on POMC neurons and regulate their cellular activity. MC4receptor antagonists Shu 9119 or agouti-related peptide (AgRP) blocked the estradiol action on food intake in rats (Polidori and Geary, 2002). In mice, deletion of ERα from POMC neurons has resulted in an increase in food intake (Xu et al., 2011b).

The reported evidence about the effect of estradiol on AgRP/NPY neurons in the ARC is inconsistent. A report using *in vitro* studies suggested that there is an inverse correlation between ERα abundance and NPY expression, as well as a direct correlation between ERβ abundance and NPY expression (Titolo et al., 2006). On the contrary, ovariectomized rats showed an increased level of NPY expression that was reversed by estrogen treatment (Martinez de Morentin et al., 2014; Pelletier et al., 2007). About the estrus cycle, the NPY expression levels were the lowest during estrus (Olofsson et al., 2009). This report also shows that E2 administration decreased the fasting-induced c-Fos activation in NPY/ AgRP neurons and stopped the refeeding response (Olofsson et al., 2009). Co-localization of the RB isoform of the leptin receptor (Leprb) with ERα in the ARC was reported (Diano et al., 1998). Leprb mRNA expression is down-regulated by estrogens in the ARC (Bennett et al., 1999), possibly via an ERE on the leptin receptor gene (Lindell et al., 2001) suggesting a crosstalk between their effects in the regulation of energy balance. Expression of the leprb isoform was higher during estrus and metestrus, although no changes in the circulating leptin levels (Bennett et al., 1999).

Recent evidence has reported that ERα activation in VMH neurons plays a major role in regulating physical activity, thermogenesis, and fat distribution. The transcription factor SF1 display almost exclusive expression in the VMH neurons

(Ikeda et al., 1995), its deletion disrupts the VMH structure (Dellovade et al., 2000)and leads to obesity (Majdic et al., 2002). Electrophysiological studies of the VMH neurons showed direct estrogen effects on these neurons (Minami et al., 1990). More recent studies on ERα gene silencing and transgenic mice showed reduced sensitivity to E2-induced weight loss, increased visceral fat deposition, and reductions in energy expenditure without any changes in food intake (Musatov et al., 2007; Xu et al., 2011b).

6.2. Androgens

Androgens have been implicated as having an important role in many vital processes other than regulating the reproductive function including energy homeostasis (Navarro et al., 2015; Shen and Shi, 2015; Wilson, 1999). Testosterone (T) and 5α-dihydrotestosterone (DHT) are considered the main androgens, T is synthesized primarily by the Leydig cells in the testes and can be converted into DHT in a reaction catalysed by 5-α-reductase enzyme (Martini et al., 1993; Puri and Walker, 2016). DHT is considered the most active form of endogenous androgens, although less abundant; it is more biologically active than T (Matsumoto et al., 2013; Mitchell, 2012). Androgens mediate their different actions via binding to the androgen receptor (AR), a single nuclear receptor (Mangelsdorf et al., 1995). The AR is a member of the steroid hormone group including the ER, glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR) (Mangelsdorf et al., 1995; Nuclear Receptors Nomenclature, 1999). Other types of androgens can be synthesized locally in the Zona reticulata and zona fasciculata of the adrenal cortex including dehydroepiandrosterone (DHEA) and androstenedione (Lasley et al., 2011).

The synthesis of testosterone in Leydig cell is controlled by stimulation of the release of the LH from the pituitary gland in response to GnRH release from the hypothalamus (Stocco, 2001). LH initiates the process of steroidogenesis by promoting the steroidogenic acute regulatory protein (StAR) expression that enhances the inner mitochondrial uptake of cholesterol (Shima et al., 2013; Stocco, 2001). This cholesterol is used to produce pregnenolone that is converted to DHEA, that produces androstenediol and androstenedione that produces testosterone via reaction catalyzed by cholesterol side chain cleavage P450 (CYP11A1), 3-β-hydroxysteroid

dehydrogenase, 17-α-hydroxylase/17, 20-lyase P450 (CYP17A1), and 17-β hydroxysteroid dehydrogenase type III (HSD17-β3) (Habert et al., 2001; Miller and Auchus, 2011; O'Shaughnessy et al., 2006; Scott et al., 2009). Androgens are also expressed in females; it is synthesized mainly in the ovaries and the adrenal cortex (Burger, 2002). Once produced, circulating testosterone is mostly bounded to serum sex hormone-binding globulin (SHBG) and albumin (Thaler et al., 2015).

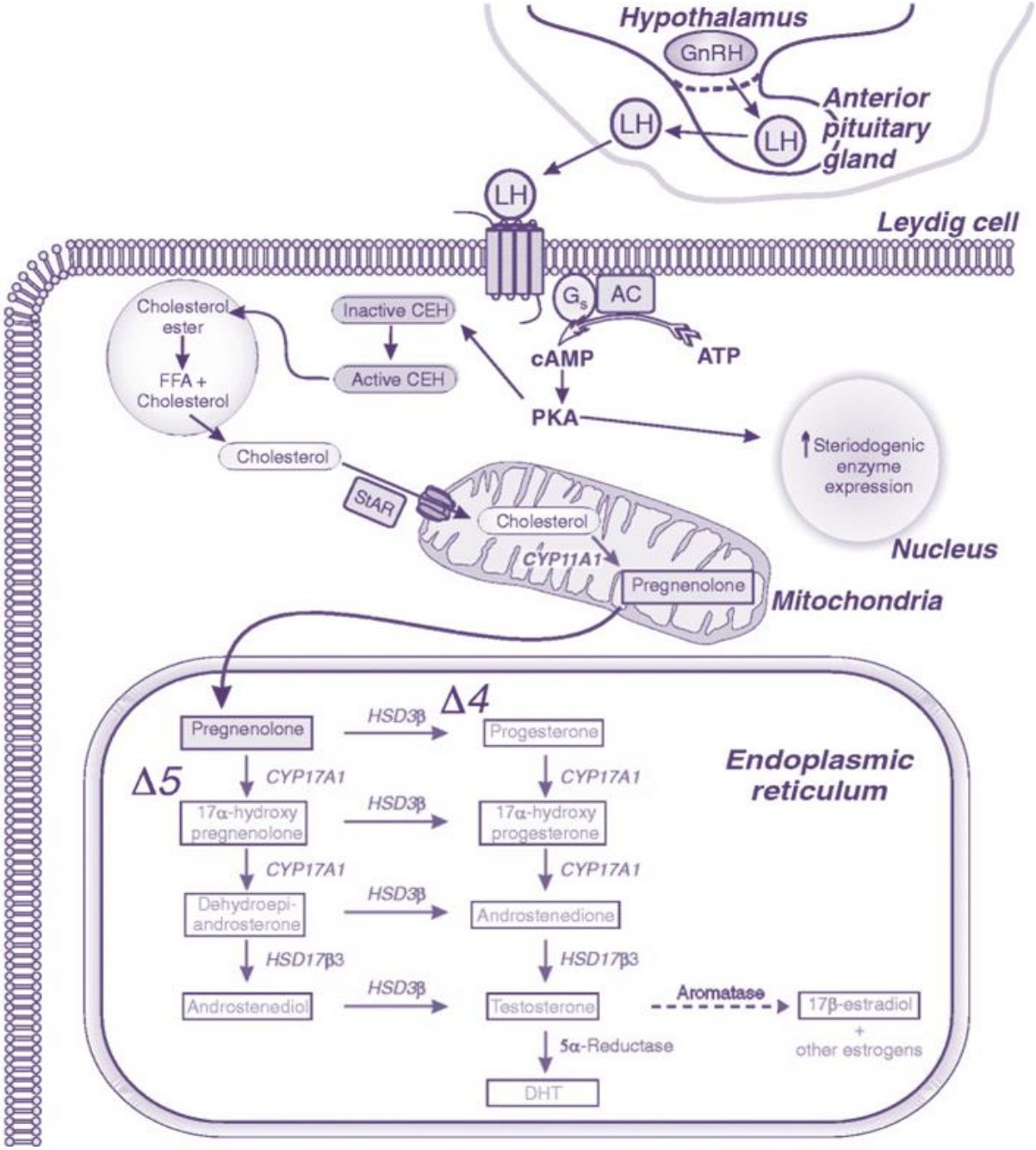

Figure 14. The hypothalamic–pituitary–testicular axis control of androgen synthesis. Modified from (Ayaz and Howlett, 2015).

6.2.1. Androgens: modes of action and regulatory mechanisms

The structure of the AR is similar to the other nuclear receptors. It consists mainly of4 functional domains: a- an amino-terminal transactivation domain; b- a highly conserved central DNA-binding domain (DBD); c- a hinge region and d- a carboxy-terminal ligand-binding domain (LBD), to which the androgen binds (Chawla et al., 2001). Androgens are lipophilic hormones; they can readily diffuse across the lipid bilayer membrane of the cell without the aid of a transport protein (Quigley et al., 1995).AR present normally in the cytoplasm in complexes with heat-shock proteins (HSPs). Upon activation AR is displaced from HSPs, the N and C terminals of the AR interact and binds importin-α to translocate the AR into the nucleus (Prescott and Coetzee, 2006; Srinivas-Shankar and Wu, 2006). Ligand-activated AR forms a homodimer that binds to androgen response elements (AREs), specific DNA sequences, in regulatory regions of target genes (Mangelsdorf et al., 1995). Once bound to its response element, AR initiates gene transcription by the recruitment of chromatin modifying and remodelling complexes, co-regulators and other transcriptional factors (Belandia and Parker, 2003; Dilworth and Chambon, 2001; Lemon and Tjian, 2000; Orphanides and Reinberg, 2002). This mechanism is considered to be the genomic pathway of androgen signaling (Heemers and Tindall, 2007).

Recent reports from *in vitro* studies have supported the presence of a non-genomic pathway that is responsible for rapid, non-ARE dependant androgenic effects mediated by a plasma membrane–associated AR (Bennett et al., 2009; Lamont and Tindall, 2011). This pathway is suggested to be responsible for the rapid intracellular actions such as stimulating different intracellular signaling pathways through direct contact with signal transducers and/or activation of kinases (Ma et al., 2001; Yamamoto et al., 2000).

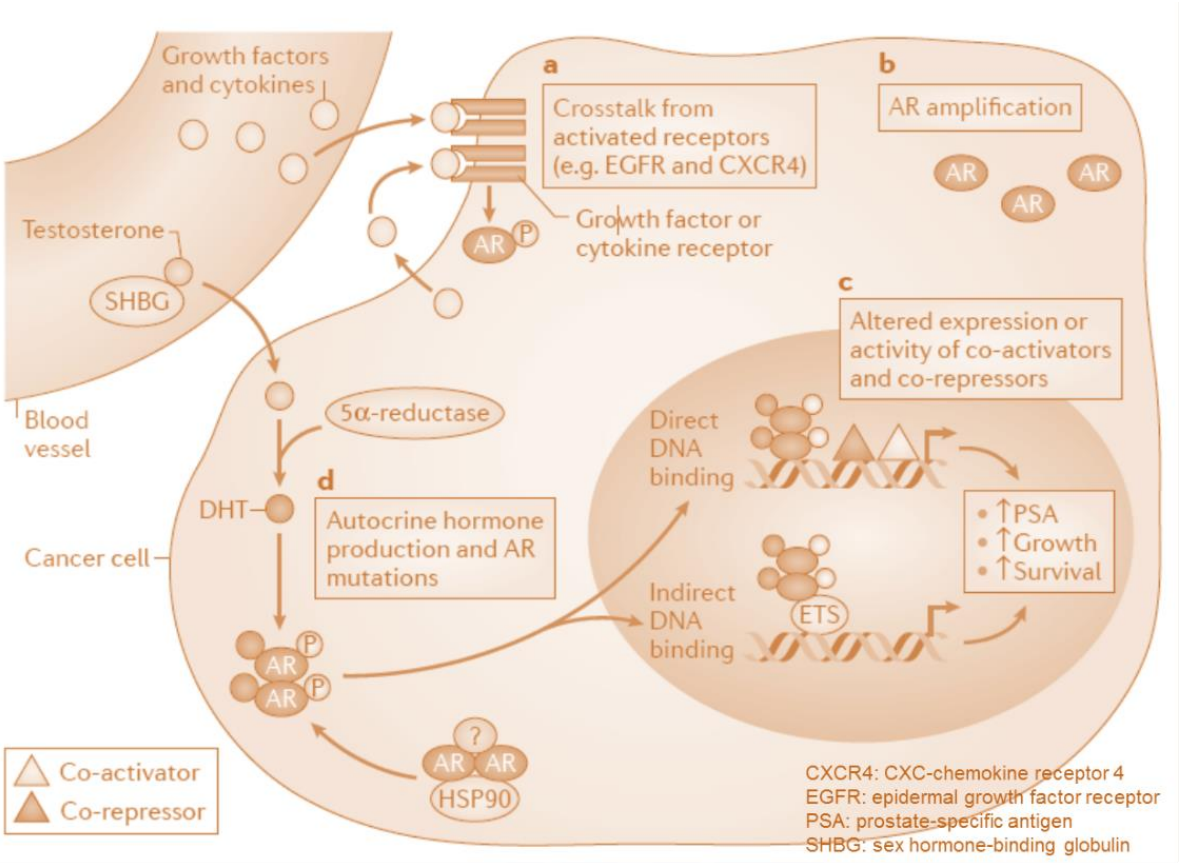

Figure 15. Mechanism of action of AR. Modified from (Mills, 2014).

Many factors are involved in influencing the overall activity of AR function other than ligand binding. Of which, tissue distribution and levels of AR expression appears to be prominent. AR is expressed in the adrenal gland, the epididymis, prostate, skeletal muscle, kidney, liver, and heart. Androgen is known to upregulate the AR expression as well as FSH, prolactin, and epidermal growth factor (Keller et al., 1996). Also, the post-translational modifications of the AR, including acetylation, phosphorylation, ubiquitination, and sumoylation influence the activity of the AR (Burgdorf et al., 2004; Poukka et al., 2000; Wen et al., 2000). Phosphorylation of the AR increases AR activity independently of ligands while sumoylation decreases the activity in the presence of ligands. Phosphorylation may enhance the affinity of the AR for coregulators, resulting in increased transactivation. Co-regulators interact with either activated or ligand-free receptors and modulate receptor activity by remodelling chromatin by their intrinsic histone acetylation (HAT), or deacetylation (HDAC) activity, or recruit proteins bearing such activities, for the regulation of target genes transcription (Glass and Rosenfeld, 2000). Coregulators may be involved in

facilitating receptor nuclear trafficking, promoting receptor DNA binding, recruiting or function as basal transcription factors (TF), or modifying receptors (Lee et al., 2001; Ozanne et al., 2000; Yeh et al., 1999).

6.2.2. Effects of androgens on the regulation of energy homeostasis

6.2.2.1. Peripheral effects of androgens in the control of energy homeostasis

In males, the impact of testosterone deficiency on the development of visceral obesity, insulin resistance and metabolic syndrome is well established (Khaw and Barrett-Connor, 1992; Mauvais-Jarvis, 2011; Zitzmann, 2009; Zitzmann et al., 2006). A cross-talk between glucose homeostasis and androgens has been suggested depending on the findings that low serum testosterone level predisposes the development of diabetes, and hypogonadism is a result of diabetes (Corona et al., 2011a; Corona et al., 2011b). Also, the amount visceral adiposity inversely correlates with the level of serum testosterone in the metabolic syndrome (Khaw and Barrett-Connor, 1992) as observed in hypogonadism in elder men (Zitzmann et al., 2006), inherited testosterone deficiency (Bojesen et al., 2006), and patients with prostate cancer treated with androgen deprivation (Basaria et al., 2006). On the other hand, high testosterone levels were related to insulin sensitivity (Pitteloud et al., 2005).

Many scientific reports had demonstrated the anti-obesity effect of testosterone. Increased amounts of visceral fat were observed in men with genetic mutations causing lower AR-mediated gene transcription (Zitzmann et al., 2003), AR-deficient male mice developed late onset visceral obesity with increased lipogenesis in WAT and liver (Fan et al., 2005b; Lin et al., 2005).

Ghrelin levels were found to be positively associated with testosterone levels in testosterone-treated hypogonadal men (Pagotto et al., 2003). Also, the relationship between adiponectin and testosterone level was suggested, in hypogonadal men, high adiponectin levels were found that decreased with testosterone administration (Lanfranco et al., 2004). In mice, adiponectin level decreased with testosterone administration (Nishizawa et al., 2002). Also, AR-deficient mice showed high adiponectin level (Fan et al., 2005b). Testosterone also promotes insulin sensitivity through skeletal muscle as it is involved in the regulation of the expression of certain genes in glucose metabolism (Haren et al., 2011). Orchidectomized males showed a

marked insulin resistance in skeletal muscle that was abolished by testosterone therapy (Holmang and Bjorntorp, 1992). This may be explained by a reduction in the transcription factor PGC1α (peroxisome proliferator activated receptor gamma co-activator alpha), a molecular marker of muscle insulin sensitivity, as its transcription was decreased in skeletal muscle of T2D patients (Mootha et al., 2003). Similarly, in men with low testosterone or mice deficient in AR low expression levels were found in muscle (Fan et al., 2005b; Pitteloud et al., 2005). Recent mice models with a specific knock down in hepatocytes AR (HARKO) were valuable to studying the effects on the liver. Male mice suffered from hepatic stenosis with high-fat dieting (Lin et al., 2008). Also, a β-cell specific AR knockout mice showed a reduced glucose-stimulated insulin secretion (GSIS) leading to glucose intolerance and develop β-cell failure to compensate for diet-induced insulin resistance (Navarro et al., 2015).

6.2.2.2. Central effects of androgens in the control of energy homeostasis

AR is abundantly expressed in the brain (Fan et al., 2008) with the highest levels of expression in the hypothalamus (Simerly et al., 1990). Recent studies on AR-deficient mice reported the development of obesity in the case of whole body AR-deficient mice without any elevation in energy intake and a reduced BAT thermogenesis which decreases energy expenditure (Fan et al., 2005a). Further studies in the same mice model showed the leptins fails to promote STAT3 nuclear localization in ARC neurons or to decrease food intake and body weight even before the onset of overt obesity (Fan et al., 2008). Other studies on neuronal specific ARKO (NARKO) mice reported the development of obesity, insulin resistance, and glucose intolerance; they showed hypothalamic insulin resistance through activating hypothalamic NFKB that increases inflammation (Yu et al., 2013). All of these observations suggest a vital role of AR in the central regulation of energy balance.

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OBJECTIVES

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The existence of sexual dimorphism in energy homeostasis and fat distribution is well established. Data gleaned in recent years have uncovered the central effects of estrogens at the hypothalamus in energy balance through the energy sensor AMPK. The main objective of this work was to further evaluate the central effect of sex steroids (estrogens and androgens) on energy balance with particular focus on the regulation of BAT thermogenesis.

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MATERIALS AND METHODS

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1. Animal models and housing conditions

Various animal models were used in the different experiments of this thesis including a- Adult male Sprague–Dawley rats (rattus norvegicus) weighting around 200 – 250 g approximately (8-11 weeks old) from the Animalario General of the University of Santiago de Compostela were used in the experiments conducted in the androgen experiments. b- Adult female Sprague–Dawley rats (*rattus norvegicus*) weighting around 250 g approximately (9-11 weeks old) from the Animalario General of the University of Santiago de Compostela were used in the experiments conducted in the estrogen experiments.

Upon arrival, All the animals were housed in open cages under conditions of controlled illumination of 12-hour light/dark schedule (light: 8:00–20:00 h), humidity, and temperature. All animals were allowed ad-libitium access to standard rat chow and tap water unless otherwise was specified by experiments. All animals were randomly assigned to the different groups of each experiment. We housed five animals per cage except for the experiments that require monitoring daily food intake, body weight and rectal temperature where they were individually caged. Animal welfare was verified periodically through monitoring body weight, food intake, and the general well-being of the rats throughout the different experiments. All animals were left for approximately one week for accommodation before the start of any experiment to avoid generating severe stress during handling by the staff or due to changes in the environment.

All experimental procedures and protocols for handling animals that were conducted during the course of this thesis have been previously approved by the head of the servicio provincial de ganadería del departamento territorial de la Consellería do Medio Rural e do Mar de la provincia de La Coruña (competent to issue the decision based on the Decreto 245/2009 of the 3rd of April by which they regulate the territorial delegations of the Xunta de Galicia and the Decreto 46/2012 of 19th of January that establishes the organizational structure of the Consellería do Medio Rural e do Mar e do Fondo Galego de Garantía Agraria). The responsible person of the approved protocols was Miguel A. Lopez Perez and conducted under the number of procedure identification of: a- 15005AE/10/FUN/FISIO2/MLP2 (under compliance of

RD 1201/2005 of October 10, on the protection of animals used for experimentation and other scientific purposes), and b- 15010/14/006 (under compliance of the actual y vigente RD 53/2013 of 1st of February, that establishes the basic standards for the protection of animals used for experimentation and other scientists purposes, including teaching), according to current legislation: Ley 30/1992 of 26th of November legal regime of public administrations and common administrative procedure, modified by Law 4/1999, of January 14, that establishes the decision to propose terminating the proceedings that decides all issues raised by whom interested and those derived from Decree 296/2008 of 30th December on the protection of animals used for experimental and other scientific purposes, including teaching, and by which creating the 'Rexisro de centros de cría', of suppliers and users and the 'Comisión Galega de Benestar de los Animales de Experimentación' ; Order of 15 September 2006 establishing the creation of 'Comité de Bioética da Consellería Rural' ; Decrto 153/1998 of 2 April so that approves the regulation implementing Law 1/1993 of April 13 (protection of domestic and wild animals in captivity); Law 32/2007 of 7 November, for the care of the animals on the farm, transport and slaughter; also following the European Directive on Animal Experimentation (2010/63 / EU).

2. Anaesthesia and analgesia

To carry out the various surgical procedures in this thesis, animals were treated with an intraperitoneal injection (IP) of anaesthetic agent. The composition of anaesthetic agent in case of rats was 42.5% ketamine (Imalgene 1000 injection, *Merial*, Barcelona, Spain), 20% xylazine (Rompun 2%, *Bayer HealthCare*, Berlin, Germany) and 37.5 % normal saline (isotonic physiological saline, *Physan*, Madrid, Spain), and the dose used was 200 µl / 100 grams of body mass.

All anaesthetic and analgesic agents were preserved according to the specific recommendations of the suppliers. Both foot and blinking reflexes were used to check that each animal is well anesthetized; its absence informs that the surgical procedure can be carried out safely and with no pain felt by the animals. After surgical procedures, the animals were subcutaneously (SC) treated with ketoprofen as an

analgesic (ketoprofen-Orudis 100 mg., *Sanofi aventis*, Barcelona, Spain) at the dose of 2.5 mg per kg of body mass.

3. Routes of drug administration

Different routes of drug administration were used:

3.1. Intraperitoneal route (IP)

This route does not require the use of anaesthesia. It is performed on the caudoventral part of the rat beneath the peritoneum. It involves catching the animal from the back by using a piece of cloth to immobilize the animal by trying to press the forelimbs. The animal's head is tilted slightly downward to minimize the risk of tearing the viscera and the needle (BD Microlance, 25G 5/8" 0,5x16mm, *BD Medical Surgicals Systems*) attached to a syringe (1 ml BD slip-tip syringe, bulk, non-sterile, *BD Medical Surgical Systems*) is inserted in a perpendicular manner to this, preferably in the right side. This route was used for administration of the anaesthetic agents as previously mentioned.

3.2. Subcutaneous route (SC)

This route does not require the use of anaesthesia as well. It is performed on the dorsocaudal lateral parts of the animal back at the level of the hind limbs. The animal is immobilized with the help of a piece of cloth over his head, pinching the animals' loin with the same hand and inserting the needle with the other hand parallel to the spine. This process can be carried out several times a day over several days.

Substance	Effect	Distributor	Dose	Vehicle
Butoxamine hydrochloride	A β2-AR-antagonist	SIGMA; St. Louis, MO,USA	1mg/kg/day	Saline
SR59230A	A β3-AR-antagonist	Tocris; Bioscience, Bristol, UK	3 mg/kg/day	DMSO

Table 3. Substance administered by SC injections.

3.3. Intracerebroventricular route (ICV)

It is used to deliver certain substances in the lateral ventricles of the rat's brain. It is performed on a fully conscious animal and does not require the use of anaesthesia. Immobilizing the animal with a piece of cloth where the animal was gently pressed against the base of the cage to avoid excessive stress; the implanted cannula was opened by using scissors at the sealed end, a Hamilton syringe (Model 7001KH 25s,

Hamilton) is introduced and emptied once it is properly inserted. The needle is removed with extreme care by holding the cannula to prevent displacement of the insertion site. The same person repeats this procedure for several days that never exceeds 10 days of the implantation of the cannula.

Substance	Distributor	Dose	Vehicle
17β-Estradiol	SIGMA; St. Louis, MO,USA	1-5 nM/5µL/12 h	DMSO
Dihydrotestosterone	SIGMA; St. Louis, MO,USA	100 nM/5µL/day	DMSO

Table 4. Substance introduced by ICV administration.

4. Non-surgical procedures

4.1. Monitoring of food intake and body weight:

Body weight and food intake values were collected by using a precision scale; both Animals and their food were weighed daily, always more or less at the same time in each experiment. In the case of group caging of animals, the mean food intake was calculated depending on the number of individuals of each box.

4.2. Determination of body composition:

Body composition analysis was done by using the magnetic resonance imaging techniques (*Whole Body Composition Analyzer Echo-MRI 500 Echo-MRI*, Houston, Texas, USA) both at the beginning and end of the experiments if possible; the data on the composition of fat mass, lean mass and water of each animal were obtained and analysed.

4.3. Temperature measurements

Body temperature was recorded with a rectal probe connected to digital thermometer (*BAT-12 Microprobe-Thermometer*; Physitemp). Skin temperature surrounding BAT was recorded with an infrared camera (*B335; Compact- Infrared-Thermal-Imaging-Camera*; FLIR) and analysed with a specific software package (*FLIR-Tools-Software*; FLIR)

5. Surgical procedures

5.1. ICV cannulation

The cannulas used for this purpose consisted of a polyethylene tube (*BD Intradermic® Polyethylene tubing (Non-Sterile), Becton Dickinson and Company*,

Franklin Lakes, NJ, USA) (PE-20 in the case of rats) of 1.09 mm outer diameter, 0.38 mm inner diameter. A bevel shaped cut is made on one end at an angle of 45 °. A cape was inserted at a length of 0.5 mm to 4 mm at the bevelled side for rats. The total length of the cannula is 3.5mm. The other end was sealed until the time of treatment.

Once the animals were anesthetized correctly, skin incision on the head (in front of the ears) perpendicular to the sagittal suture of about 1.5 cm is made. Using a mosquito, skin flaps were removed for optimal exposure of the surgical field. Subsequently, the subcutaneous tissue of the skull was removed exposing the insertion between the sagittal suture and the coronal suture, i.e. the bregma. Bleeding was managed by pressing gauze for a few seconds until the cessation of the bleeding. After the correct location of the bregma, perforation was made in the skull (to the right or left side of the bregma) by using a surgical drill at a lateral distance of 1.5 mm from the sagittal suture and 0.9 mm posterior to bregma. After reducing the eventual bleeding with gauze, the bevelled end of the cannula is inserted into the hole parallel to the dorsal-ventral axis of the animal. Finally, the cannula was secured to the skull of the animal with cyanoacrylate adhesive (*SuperGlue-3 Loctite*). After cannulation, animals were caged individually and left to recover for a period of 3-4 days before proceeding to the injection.

5.2. Orchiectomy (ORX)

This procedure is for males where the animals are properly anaesthetized, a scrotal approach was used. A small incision is made at the tip of the scrotum. The tunic is opened and the testis, cauda epididymis, vas deferens, and the spermatic blood vessels are exteriorized by applying a little pressure. The blood vessels and vas deferens are then ligated. The testis and epididymis including the fat tissue are then removed. The remaining tissue is returned to the sac and the procedure repeated for the other testis. The skin incision was then closed. Sham operated rats are handled through the same surgical procedures except for the removal of the gonads. The animals should be left for a period no less than 3 weeks before further experiments were carried out to verify the complete disappearance of the androgens produced by the testis.

5.3. Ovariectomy (OVX)

The animals are properly anaesthetized to perform bilateral OVX. The mid-dorsal thoracolumbar region is clipped to remove hair and prepared for aseptic surgery. Ovaries are typically approached by two separate flank incisions. Animals are laid on their side, the skin is separated from the underlying muscle before incising the muscle, the location of the ovarian fat pad is confirmed, the ovary is gently pulled through the incision with a blunt forceps by grasping the fat pad surrounding it. A haemostat is placed at the boundary between the oviduct and uterus, a ligature placed just below the haemostat (next to the uterus) and a cut is made just above the haemostat. Once the ovary is removed, the haemostat is released and haemostasis is verified before letting go the uterus and allowing it to return to the abdomen. Muscle layer is closed; skin is closed with wound clips. The process is repeated for the other side. Sham operated rats are handled through the same surgical procedures except for the removal of the gonads. The animals should be left for a period no less than 2 weeks before further experiments were carried out to verify the complete disappearance of the estrogens produced by the ovaries.

6. Tissue dissection and extraction

After the end of each experimental procedure, the animals were sacrificed by cervical dislocation and subsequent beheading according to the rules and laws of animal experiments. Tissues were then removed and stored at -80 °C until processing and analysis. In cases of microdissection was needed, it was conducted under an optical magnifier of 20x.

7. Experimental design

7.1. ICV administration of 1 or 5 nmol of E2

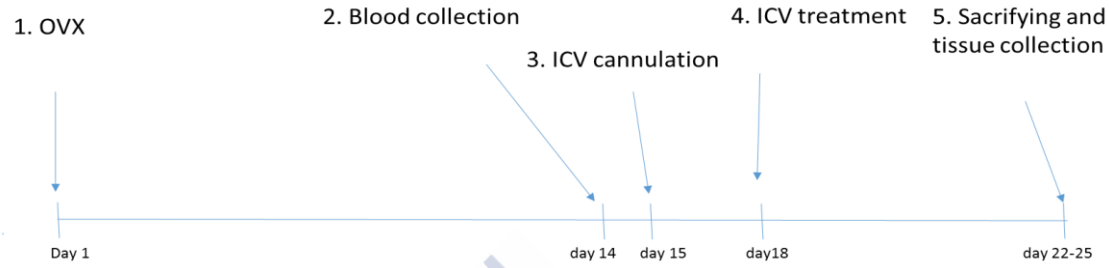
The rats were ovariectomized after allowing them an adaptation period, two weeks later, ICV cannulas were introduced. The drug or vehicle were centrally administered for up to 7 days before the dark cycle.

The rats were randomly assigned to one of four groups:

1. OVX treated with E2
2. OVX treated with vehicle

3. SHAM treated with E2
4. SHAM treated with vehicle

Rats were caged individually after the cannulation. Food intake, body weight and core temperature were daily monitored. At the last day, rats were sacrificed and the different tissues were collected and stored at - 80



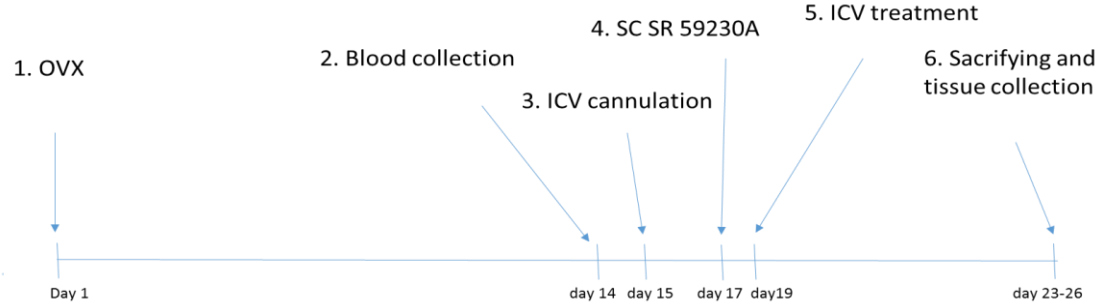
7.2. ICV administration of 1 nmol E2 along with SC β3-AR-antagonist SR59230A

The rats were ovariectomized after an adaptation period, two weeks later, ICV cannulas were implanted. The drug or the vehicle was centrally administered for up to 5 days before the dark cycle. The rats were treated previously SC with the β3-AR-antagonist SR59230A for 2 days before the ICV started.

The rats were randomly assigned to one of four groups

1. OVX treated with vehicle + SC SR59230A
2. OVX treated with vehicle+ SC vehicle
3. OVX treated with E2+ SC SR59230A
4. OVX treated with E2+ SC vehicle

Rats were caged individually after the cannulation. Food intake, body weight and core temperature were daily monitored. At the last day, rats were sacrificed and the different tissues were collected and stored at - 80



7.3. Influence of physiological levels of E2 modulation

Half of the rats were ovariectomized after adaptation while the other half have been examined daily by the means of vaginal smear and a microscope to detect the phase of estrus. Rats from diestrus were included in the experiment.

The rats were randomly assigned to one of two groups

1. OVX rats
2. Diestrus phase rats

At the last day, rats were sacrificed and the different tissues were collected and stored at - 80.

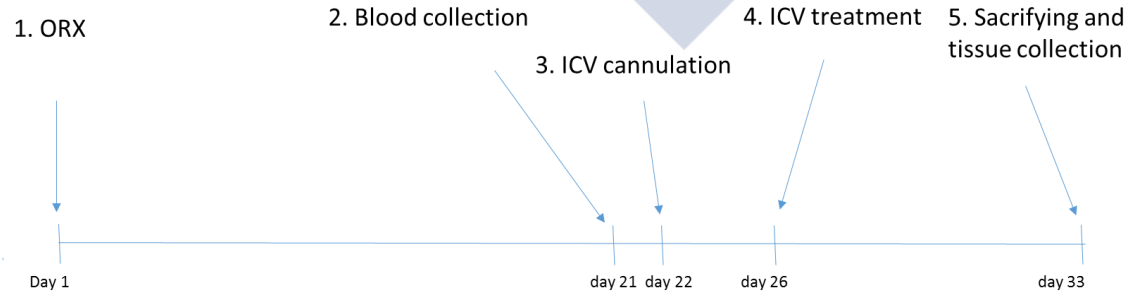
7.4. ICV administration of 100 nmol of DHT for 7 days

The rats were orchidectomized, after 3 weeks, ICV cannulas were implanted. The drug or the vehicle were centrally administered for 7 days before the dark cycle.

The rats were randomly assigned to one of four groups

1. ORX treated with DHT
2. ORX treated with vehicle
3. SHAM treated with DHT
4. SHAM treated with vehicle

Rats were caged individually after the cannulation. Food intake, body weight and core temperature were daily monitored. At the last day, rats were sacrificed and the different tissues were collected and stored at - 80



7.5. ICV administration of 100 nmol of DHT for 7 days alone with SC β2-AR-antagonist Butoxamine

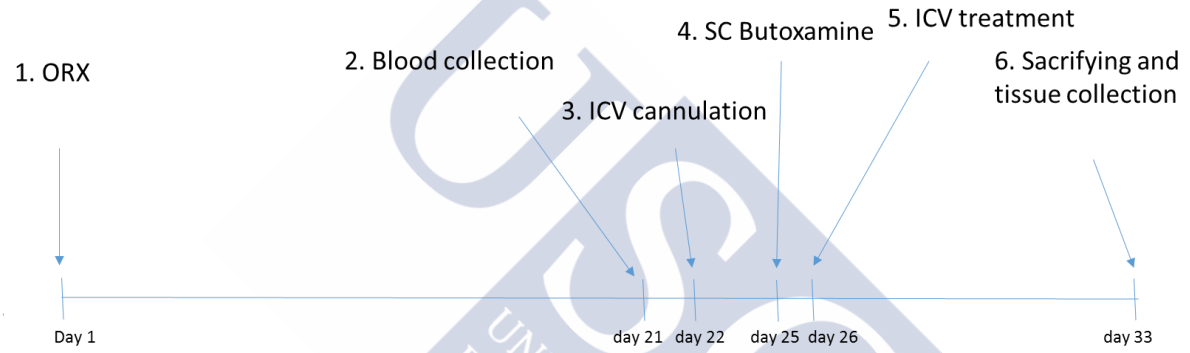
The rats were orchidectomized, three weeks later, ICV cannulas were implanted. The medicament or the vehicle were centrally administered for up to 7 days before the

dark cycle. The rats were treated previously SC with the β2-AR-antagonist Butoxamine for one day before the ICV starts.

The rats were randomly assigned to four groups

1. ORX treated with vehicle + SC Butoxamine
2. ORX treated with vehicle+ SC vehicle
3. ORX treated with E2+ SC Butoxamine
4. ORX treated with E2+ SC vehicle

Rats were caged individually after the cannulation. Food intake, body weight and core temperature were daily monitored. At the last day, rats were sacrificed and the different tissues were collected and stored at - 80



8. Analytical techniques

8.1. Protein extraction

At the beginning of the extraction, a small portion of tissue was separated and the rest of the sample was re-frozen at -80 ° C. Throughout the extraction process, the samples were maintained at 0 ° C to prevent protein degradation. The separated tissue was then placed in a 2 ml eppendorf tube with safety (*Safe-Lock Tubes 2.0 ml, Eppendorf*, Hamburg, Germany), a certain volume of lysis buffer with protease inhibitor was added (*Complete ™, Roche*, Mannheim, Germany), depending on the tissue from which protein was extracted (between 200 µl and 1000 µl). Also depending on the type of analysis to be performed with the extracted proteins, the buffer varies between the enzyme activity analysis and quantitation of protein expression. Samples were then homogenized by a process of mechanical cell disintegration and breakage by a homogenizer (*TissueLyser II, Qiagen*, Germantown, MD, USA) for 2-3 minutes

at a frequency of about 30 Hz. Subsequently, they were centrifuged for 30 minutes at 13,200 rpm and 4 ° C (in case of WAT and BAT, further centrifugation of about 10 minutes was performed to further remove the excess of fat). The supernatant was removed into several aliquots and frozen at -80 °C until analysed.

Composition of the lysis buffer (pH adjusted to 7.5 and distilled H2O to 500 ml)	
Tris-HCL pH 7,5	2,5 ml
EGTA 0,2 M (pH8)	2,5 ml
EDTA 0,2 M (pH8)	2,5 ml
Triton X-100	5 ml
Sodium orthovanadate0,1 M	5 ml
Sodium fluoride	1 gr
Sodium pyrophosphate	1,1 gr
Sucrose	46 gr
Protease inhibitor cocktail (Roche) 1 tablet per 50 ml	

Table 5. Composition of the lysis buffer.

8.2. Protein quantification

To determine the protein concentration in aliquots obtained from the extraction (both for measuring its activity or expression). The aliquots were kept at 4 ° C on ice and the protein quantification was carried out by the colorimetric method of Bradford (Bradford, 1976) using the Bio-kit Rad Protein Assay (*Protein Assay Dye Reagent, Bio-Rad*, Hercules, CA, USA). To this end, we proceeded to dilute each sample by a factor of 1:26 in distilled H_2O eppendorfs. In a 96 well plate, they were loaded in duplicate of 10 µl of each sample, and then 250µl of Bradford reagent diluted 1: 4 in distilled H_2O at 37 °C. In addition, for each plate, a calibration curve was made by serial dilution of BSA (*bovine Albumin Fraction V powder, Sigma-Aldrich*, St. Louis, USA) with 7 duplicate points (0.03125, 0.0625, 0.125, 0.125, 0.25, 0.5, 0.7 and 1 mg / ml). Then in a plate reader (*Multiskan Go Microplate Spectrophotometer*, Thermo Scientific, Rockford, IL, USA) the plate was incubated for 5 min at 37 and colorimetry was measured at OD (optical density) in each well at a wavelength of λ =

595nm. Through extrapolation of the values of the calibration curve, protein concentration of each sample was determined. After determining the amount of protein from each sample at the required concentration for each assay (10 to 20 µg / 16 µl) aliquots were prepared.

8.3. Western blot

8.3.1. Sample preparation

Proteins are separated by electrophoresis in denaturing polyacrylamide gels (*SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*). This technique is based on the separation of proteins by their molecular mass, based on their migration in an electric field, it is necessary to neutralize a number of characteristics of the proteins that would result in erroneous migration patterns and incorrect identifications. On the one hand, it is necessary to remove the quaternary structure of the protein in order to be able to migrate in the gel as possible in proportion to its size. On the other hand, since the protein will be subjected to an electric field, it is necessary to eliminate the intrinsic charge of the polypeptides, denaturing and granting them the same charge. For this, an aliquot of each sample was prepared, with the same concentration of total protein (10-20 mg) in lysis buffer with a protease inhibitor. Each sample also had the same ratio of 5x loading solution. This loading buffer provides the aliquot with elimination of quaternary protein structure (*β-mercaptoethanol, Sigma-Aldrich*, St. Louis, USA), standardizes its electric charge in proportion to its length (*SDS Sodium dodecyl sulphate, Sigma-Aldrich*, St. Louis, USA), a higher density (glycerol, *Sigma-Aldrich*, St. Louis, USA). All this facilitates loading on the gel and further comprises a dye, for identifying the migration (bromophenol blue, *MERK*, Darmstadt, Germany).

Composition of the loading buffer (pH adjusted to 6.8 and miliQ H2O to 1L)	
SDS	2 g
Trizma-base	8 ml
β-mercaptoethanol	1 ml
Bromophenol blue	4 mg
Glycerol	10 ml

Table 6. Composition of the loading buffer.

8.3.2. Electrophoresis

Electrophoresis was performed in a gel consisting of a three dimensional structure, in a network formed by the polymerization of acrylamide and bis-acrylamide (N, N-methylenebisacrylamide). The polymerization process is catalysed by ammonium persulfate (APS) (Ammonium persulfate, *Sigma-Aldrich*, St. Louis, USA) and TEMED (N, N, N, N tetramethyletilenediamina, *Sigma-Aldrich*, St. Louis, USA). The concentration of acrylamide and bis-acrylamide (30% Acrylamide / Bis Solution 29: 1, Bio-Rad, Hercules, CA, USA) in the gel determines the size of the pores of the three-dimensional network and the degree of migration of the proteins by size. The gel consists of two fractions, a concentrator fraction with 4% acrylamide / bis-acrylamide, wherein the loaded samples are grouped so that the separation of proteins from different samples occur simultaneously, and a separating fraction with 6%, 8% and 10% depending on the size of the protein of interest. The gels were mounted on an electrophoresis kit (Mini-PROTEAN Tetra Cell, *Bio-Rad*, Hercules, CA, USA) and immersed in a running buffer 1x. (Composition of 5X running buffer: Glycine 72 g, 15 g of Trizma base, 5 g SDS, distilled H_2O to 1L).

Before loading the samples on the gel they were vortexed and heated at 95 ° C for 10 minutes and vortexed again. In each well 16 µl of gel sample was loaded. In one of the lanes, 5 µl of a molecular weight marker (Precision Plus Protein Standards-Dual Color, *Bio-Rad*, Hercules, CA, USA) were loaded. In each of the provided gels, samples of all experimental groups were loaded. After this, the samples were subjected to an electric field (constant voltage of 120V-140V and amperage of 180 mA), connecting the electrophoresis kit to a power source (Power Pac HC High Current Power Supply, Bio-Rad, Hercules, CA, USA), which induced proteins migration to the cathode. Electrophoresis was stopped when the migration front reached a distance of approximately 0.5 cm from the end of the gel.

8.3.3. Transfer

After the electrophoresis, the proteins were transferred to a membrane to proceed with the analysis of expression of each protein. They were subjected to an electric field to mobilize the proteins to the membrane where they will be adsorbed allowing the incubations with different antibodies. The type of transfer that was used is called a

semi-dry type, where the membrane and the gel were placed one below the other and separated by absorbent paper and the plates, these plates create the voltage (Trans-Blot Semi-Dry Transfer Cell, *Bio-Rad*). PVDF membranes were used (Immobilon-P Polivinylidene Fluoride membrane, *Millipore*), and cut with dimensions of 8,5x6,5 cm, previously activated methanol over 4 min, 5 min in distilled H_2O and 5 min in transfer buffer were used.

Composition 25X transfer buffer	Glycine 36.5 g, 72.5 g of Trizma base, 4.5 g of SDS, distilled H2O to 500 ml
Composition 1X transfer buffer	40 ml of 25X transfer buffer, 200 ml of methanol, distilled H2O to 1L

Table 7. Composition of the different concentrations of the transfer buffer

The transfer paper (Extra Thick Paper Blot, Bio-Rad) was soaked in transfer buffer, It is necessary for the passage of electric current necessary for the transfer. the gel and the membrane were placed between two pieces of blotting paper so that proper transfer electric current occur; any bubbles that might present1 between this were removed with a roller. The transfer was carried out for 1 hour and 40 minutes at a constant amperage of 250mA electrophoresis kit connecting to a power source (power Pac HC High Current Power Supply, *Bio-Rad*, Hercules, CA, USA). To verify the correct transfer membranes were stained with Ponceau Red (Ponceau S Red, *Sigma-Aldrich*, St. Louis, USA) dye. To remove the dye, a couple of washes with wash buffer were applied.

Separator gel 6%		Separator gel 8%		Separator gel 10%	
distilled H2O	5,3 ml	distilled H2O	4,6 ml	distilled H2O	4,0 ml
Acrylamide/ bisacrylamide (30%)	2,0 ml	Acrylamide/ bisacrylamide (30%)	2,7 ml	Acrylamide/ bisacrylamide (30%)	3,3 ml
1,5M Tris (pH 8,8)	2,5 ml	1,5M Tris (pH 8,8)	2,5 ml	1,5M Tris (pH 8,8)	2,5 ml
SDS 10 %	0,1 ml	SDS 10 %	0,1 ml	SDS 10 %	0,1 ml
APS 10 %	0,1 ml	APS 10 %	0,1 ml	APS 10 %	0,1 ml
TEMED	8 µl	TEMED	6 µl	TEMED	4 µl

Table 8. Composition of the different concentrations of the separator gels.

8.3.4. Immuno-detection of proteins

After a proper transfer to the membrane, they were incubated at room temperature for 1h with blocking buffer (blocking buffer components: 3g BSA in wash buffer

100ml). Thus, the specific binding provided by the membrane in all areas where there was transferred protein was removed. Subsequently, the membrane was cut with reference to the molecular weight marker, so that one could detect different membrane proteins, thus, different proteins could be detected simultaneously. Each piece of membrane was incubated for 1h at room temperature or overnight at 4 ° C with primary antibody (depending on the antibody and its specific conditions) previously diluted in blocking buffer.

Primary antibody	Company	Dilution
AMPKα1	*Millipore*; Billerica, MA, USA	1:1000
AMPKα2	*Upstate*; Temecula, CA, USA	1:1000
pAMPKα	*Cell Signaling*; Danvers,MA,USA	1:2000
ACCα	*Millipore*; Billerica, MA, USA	1:1000
pACC	*Cell Signaling*; Danvers,MA,USA	1:1000
FAS	*BD*, Franklin Lakes, NJ, USA	1:1000
CPT1c	*SCBT*; Dallas, Texas, USA	1:1000
UCP-1	*Abcam*; Cambridge, UK	1:20000
S6	*Cell Signaling*; Danvers,MA,USA	1:1000
pmTOR Ser2448	*Cell Signaling*; Danvers,MA,USA	1:1000
β-actin	*SIGMA*; St. Louis, MO, USA	1:5000
α-tubulin	*SIGMA*; St. Louis, MO, USA	1:5000

Table 9. List of antibodies used and their dilution

After the time of incubation with the primary antibody, it was removed and the excess was washed of by three washes each for 5 min with washing buffer at room temperature. Next, the membranes were incubated for 1 hour at room temperature with a secondary antibody conjugated with Horseradish Peroxidase capable of recognizing and specifically binding to the primary antibody used.

Composition 10X Wash Buffer	24.2 g Trizma base, 80 g NaCl, distilled H2O to 1L; adjust pH to 7.6
Composition 1X wash buffer	100 ml 10X wash buffer, 1 ml of Tween 20, distilled H2O to 1L

Table 10. Composition of the different concentrations of washing buffer.

8.3.5. Developing and fixing the signal.

The secondary antibody was removed and the excess is also washed off by three washes; to proceed to reveal the membrane by using 1 ml of the revealing substrate that detects Horseradish Peroxidase in proportions of 1: 1 (Pierce ECL Western Blotting Substrate, *Cultek*). Membranes were incubated for 2 min in the dark with this substrate. Once past the incubation time, the membranes were placed in a developer cassette (Hyper cassette, *Amersham Biosciences*, Little Chalfont, UK) and were waterproofed with the help of transparent paper. Then it was proceeded to be processed in a dark room intended for that purpose. Introducing a developing sheet (Fuji Medical X-Ray Film Super RX, *Fujifilm Corporation*, Tokyo, Japan) on the membranes and the cassette was closed; that was allowed to expose to the chemiluminescent signal for a few seconds/minute depending on the protein type. the film was then removed and immersed in a developer solution (1:10 dilution) (G150, Developer / Replenisher, *Agfa-Gevaert Group*, Dubendorf, Switzerland) until the desired signal is displayed at which the film is immersed in a fixative liquid (1: 5 dilution) (G354, Manual fixing Bath, *Agfa-Gevaert Group*, Dubendorf, Switzerland) for a couple of minutes in order to fix the signal. Finally, the film is washed with running water and then dried.

8.3.6. Quantification of the signal.

Quantification of the signal is performed by measuring the optical density of each sample signal, from scanned images (resolution 400 dpi, CanoScan 9900F, *Canon*, Tokyo, Japan) of the auto radiographed films with computer software called ImageJ (ImageJ 1.40g, *Wayne Rasband*, NIH, USA). Each measurement of optical density is corrected by measuring the optical density of a signal adjacent to the sample region.

8.4. RT-PCR

8.4.1. RNA extraction.

The whole process was performed while keeping the samples on ice in order to avoid degradation of RNA. A small piece of frozen samples was mechanically homogenized with 1000 µl of Trizol (TRIZOL Reagent; *Invitrogen*, Carlsbad, CA, USA) for 3 min (with the same system used in case of protein extraction). After homogenization, the samples were allowed to stand at room temp for about 5 minutes.

Then 1 ml of chloroform (Chloroform, *Sigma-Aldrich,* St Louis, USA) was added to each sample and shaken vigorously for several seconds with a subsequent rest period of about 5 minutes. Then they were centrifuged at 12000 rpm for 15 min at 4 ºc where they become clearly separated into two phases: an aqueous phase (containing RNA) and an organic phase (containing proteins and lipids). Between both the inter-phase (containing DNA). The aqueous phase is removed carefully into another tube and the organic phase is discarded. Thus the RNA is separated from the other cellular components.

Once RNA is separated, 1 ml of isopropanol was added (2-propanol, *Sigma-Aldrich*, St Louis, USA) to each sample, then vortexed and placed at -20 ° C for about 10 minutes (thus enabling the RNA to precipitate). After that, the samples were centrifuged for 10 minutes at 12000 rpm at 4 ° C to concentrate the RNA in the background. Once the supernatant is removed, 1 ml of ethanol was added (Ethanol absolute PA, *Applichem / Pancreac*, Darmstadt, Germany) at 70% dilution in DEPC H_2O followed by short stirring and centrifugation for 5 minutes at 12000 rpm at 4 ° C. To remove as much ethanol as possible, another centrifugation round for 1 minute at 13500 rpm and 4 ° C, and then the supernatant was removed with a pipette. Finally, resuspension in H_2O DEPC and heating at 60 ° C for 15 minutes.

8.4.2. RNA quantification.

Quantification of RNA in each sample by using a spectrophotometer set at two wavelengths: 260 and 280 nm. The absorbance at 260 nm provides the amount of nucleic acids while that of 280nm provides the amount of protein. The purity of the sample was determined by studying the ratio 260/280, oscillation between 1.4 and 1.8 was considered acceptable. All samples were then aliquoted at a concentration of 100ng / µl.

8.4.3. Reverse transcription (RT).

The reverse transcription reaction was conducted in a total volume of 30 µl (as shown in Table 11). One negative control (RT-ve) (30 µl of the reaction mixture except m-MLV, whose volume was replaced by H_20 MilliQ) was prepared. Once the reaction mixtures were prepared, incubation in a thermal cycler for 50 min at 37° C,

15 minutes at 42 ° C, 5 min at 95° C. Finally, the samples were kept at 4 ° C or frozen -20 until use.

6 µl of the reversetranscripcion buffer (5X first strand buffer; 250 nM Tris-HCl, pH 8.3, 375nM KCl, 15mM MgCl, 50nM DDT) (Invitrogen, Carlsbad, CA, USA).
1.5 µl Mg_2Cl 50 mM (Invitrogen, Carlsbad, CA, USA).
6 µl dNTPs mix (dTTP, dCTP, dGTP, dATP, with a concentration of 10 mM each) (Invitrogen, Carlsbad, CA, USA).
0.17 µl of Random primers (Invitrogen, Carlsbad, CA, USA).
0.25 µl RNase inhibitor (RNaseOUT, 40 U / µl Invitrogen, Carlsbad, CA, USA).
1 µl of m-MLV reversetranscriptase (200 U / µl, Invitrogen, Carlsbad, CA, USA).
Corresponding volume of DEPC H_20 (distilled water with 0.1% diethylpyrocarbonate, left overnight at ambient temperature) to obtain the final volume of 30 µl.

Table 11. Components reverse transcription reaction

8.4.4. Real Time PCR (TaqMan).

TaqMan probes were used as detection system (Specific probes labelled with fluorophores: donor and acceptor). It is based on the transfer of fluorescence energy by FRET type resonance (Fluorescence Resonance Energy Transfer) between the two molecules. The fluorescence used to perform PCR was TAMRA, which has a maximal absorption of 560nm and a maximal emission of 580nm. The primers used were designed with Primer Express (*Applied Biosystems*, Foster City, CA, USA) program and checked by BLAST data base. The PCR reaction mixture used had a final reaction volume of 12 µl.

2.92 µl H_2O-MQ
0.36 µl sense oligonucleotide (10 µM) (Eurofins Genomics, Ebersberg, Germany)
0.36 µl antisense oligonucleotide (10 µM) (Eurofins Genomics, Ebersberg, Germany)
0.36 µl probe (5 µM)
6 µl Taqman Universal PCR master mix; (Applied Biosystems, Foster City, CA, USA)
2 µl of sample (RT)

Table 12. Components of the PCR reaction in real time

In addition to the samples, a negative control was loaded (-RT), a blank and a standard curve for both the problem gene and the control or the constitutive expression gene; This was done in duplicate. The standard curve consisted of 6 points

made by serial dilution (1: 4) from a total volume of 30-40µl resulting from a mixture of different RT samples (2µl RT different products, but always the same treatment or tissue). The amplification conditions were 50 ° C (2min), 95 ° C (10 min), 95 (15sec) and 60 (1 min) for 40 cycles in a system of Real Time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA). Values were expressed in relation to hypoxanthineguanine phosphoribosyl-transferase (HPRT) levels.

mRNA	Gene name	GenBank Accession number		Sequence
Cidea	Cell death-inducing DFFA like effector a	NM_001170467.1	Assay ID	Applied Biosystems TaqMan® Gene Expression Assays Assay ID Rn04181355_m1
Elovl3	Fatty acid elongase 3	NM_001107602.1	Assay ID	Applied Biosystems TaqMan® Gene Expression Assays Assay ID Rn01411024_m1
Prdm16	PR domain containing 16	NM_001177995.1	Assay ID	TAMRA Applied Biosystems TaqMan® Gene Expression Assays Assay ID Mm01266512_m1
Pgc1α	Peroxisome proliferator activated receptor gamma, coactivator 1 alpha (Ppargc1a)	NM_031347	Fw Primer Rv Primer Probe	5'-CGATCACCATATTCCAGGTCAAG-3' 5'-CGATGTGTGCGGTGTCTGTAGT -3' 5'-AGGTCCCCAGGCAGTAGATCCTCTTCAAGA-3'
Ucp1	Uncoupling protein 1	NM_012682	Fw Primer Rv Primer Probe Fw Primer Rv Primer SYBR Green	5'-CAATGACCATGTACACCAAGGAA-3' 5'-GATCCGAGTCGCAGAAAAGAA-3' FAM-5'-ACCGGCAGCCTTTTTCAAAGGGTTTG-3'-TAMRA 5'-AATCAGCTTTGCTTCCCTCA-3' 5'-GCT TTGTGCTTGCATTCTGA-3'

Table 13. Primers and probes for real-time PCR analysis.

9. Statistical analysis

Data from all experiments were tested for normality (Kolmogorov-Smirnov (K-S) test) and then statistically analysed by using software PASW 18 (SPSS, Inc, Chicago, IL, USA.) or GraphPad Instad (GraphPad Instad Software, v5, La Jolla, CA, USA). The degree of statistical significance was determined for the experiments that have only one variable by t-test and ANOVA while those with two or more variables by ANOVA followed by Bonferroni post-hoc test was used. Results are represented in

percentage by function of the control, by taking the average value (M) and standard error of the mean (SEM) (mean ± SEM). In experiments in which parameters were monitored such as feed intake and body mass, these raw data are represented without making any percent ratio comparison. All results with $P < 0.05$ were considered significant; very significant at $P < 0.01$ and highly significant at $P < 0.001$.

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RESULTS

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1. Estrogens and energy balance

The modulation of energy balance by estrogens has been extensively studied by our group throughout the last few years as part of the doctoral thesis of both Dr. Ricardo Lage and Dr. Pablo Blanco Martinez de Morentin (Lage, 2010; Martinez de Morentin, 2013). The ovariectomized rats, as an estrogen deficient animal model have been validated as a proper model for studying these effects. Our previous studies have reported interesting remarks about the anorexigenic effect of estradiol. Estradiol induces weight loss an anorexia thus promoting a negative energy balance both (Figure 16).

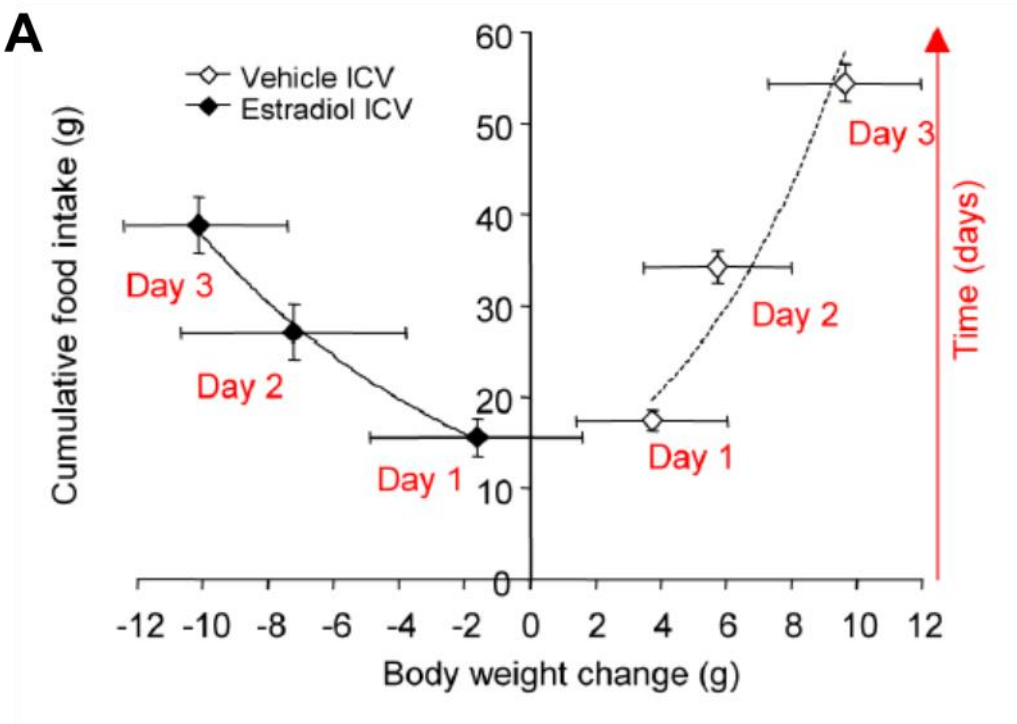

Figure 16. Effect of E2 treatment on energy balance. A- Energy balance plot of rats ICV treated with vehicle or E2. Error bars represent SEM; n=7-12 animals per experimental group.

1.1. Estradiol and BAT thermogenesis

The adipose tissue plays an important role in the regulation of energy balance and homoeostasis, both WAT and BAT (Contreras et al., 2015; Trayhurn et al., 2006). The recent reports about BAT presence and function in adult humans (Ouellet et al., 2012; Virtanen et al., 2009) has drawn much attention toward studying the

involvement of hormones, such as estrogens, in this response. WAT can also take on characteristics of BAT in a phenomena called browning (Contreras et al., 2015), notably the induction of UCP1 expression. BAT thermogenesis regulation is an intense topic of investigation as it has the potential to tilt the energy balance from storage to expenditure, a strategy that holds promise to combat the growing epidemic of obesity and metabolic syndrome. While the effects of centrally administered estradiol on energy balance have been extensively reported, their effect on BAT thermogenesis has not fully understood. In continuation of the previous studies of our group, we have started to investigate whether or not centrally administered estradiol affects the thermogenic activity. BAT thermogenesis was quantified by using thermographic imaging analysis (Martinez de Morentin, 2013; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2015; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014). We realized that central ICV administration of 1 nmol of E2 induced a marked decrease in body weight, food intake, and an increase in core temperature (Figure 17A). The increase in body temperature was associated with elevated UCP1 protein levels in BAT (Figure 17C) and a significant rise in the temperature of the skin surrounding interscapular BAT (Figure 17D) indicating increased BAT thermogenesis. No changes in the mRNA expression of thermogenic markers, such as uncoupling protein-1 (UCP1), peroxisome-proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α) and beta (PGC1β) was detected in WAT (Figure 17E). These effects were induced due to central action of estrogens as the circulating levels of estrogens remained at control level even at a higher dose of E2 ICV treatment (Figure 17B) while SC administration of estradiol was found to increase the level of circulating estrogens (Figure 17B).

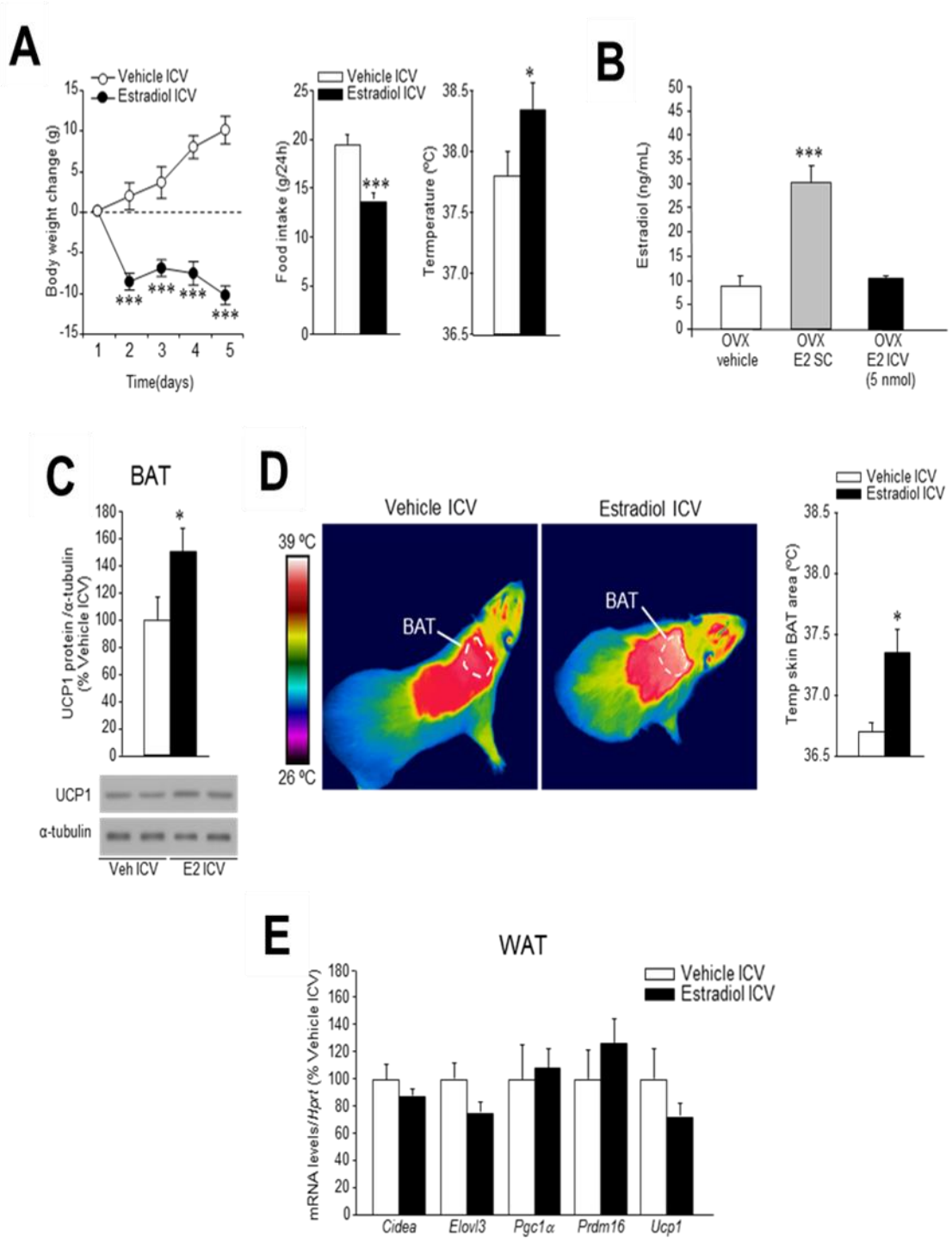

Figure 17. Central effects of ICV E2 on energy balance and thermogenesis (A–E): A- Body weight change (left panel), daily food intake (middle panel), and core temperature (right panel); B- Plasma E2 levels of OVX rats SC and ICV treated with vehicle or E2. C- protein levels (upper panel) and western blot autoradiographic images of BAT UCP1 protein (lower panel); D- infrared thermal images (left panel) and quantification of temperature of the skin surrounding interscapular BAT (right panel) E- mRNA profiles of browning markers in WAT of OVX rats treated SC with vehicle or E2. Error bars represent SEM; n = 7–12 animals per experimental group. * and ***p < 0.05 and 0.001 versus vehicle ICV.

1.2. Modulation of the VMH AMPK-SNS-BAT axis by estradiol:

The role of AMPK in the VMH has a well-established role in the regulation of BAT thermogenesis (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2015; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014). Thus, we continued to investigate the effect of centrally administered E2 via ICV route on the AMPK pathway in the VMH. The VMH-AMPK activity was found to be suppressed (Figure 18A) while no significant change was noticed in the ARC AMPK activity (Figure 18B) that was used as a control.

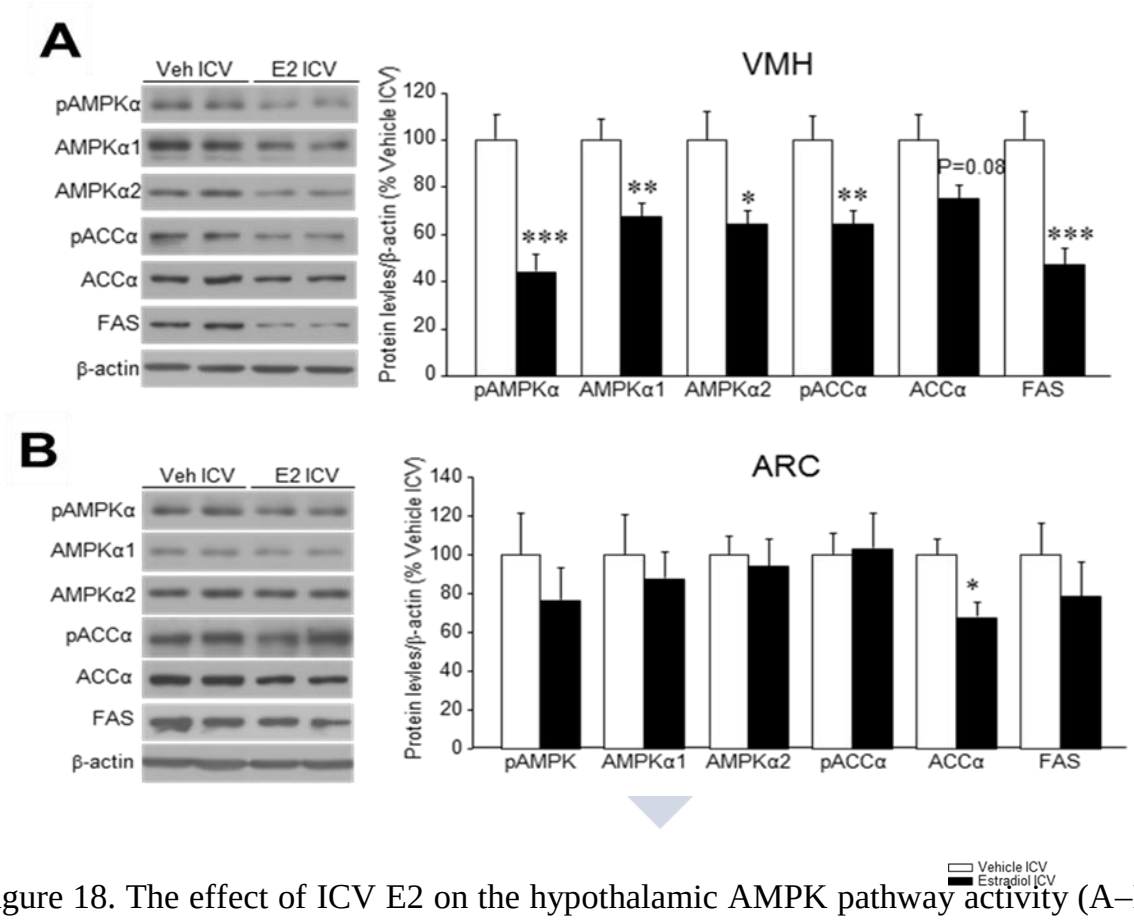

Figure 18. The effect of ICV E2 on the hypothalamic AMPK pathway activity (A–B): A- western blot autoradiographic images (left panel) and VMH levels of proteins of the AMPK pathway (right panel) of OVX rats ICV treated with vehicle or E2 (1 nmol) B- western blot autoradiographic images (left panel) and ARC levels of proteins of the AMPK pathway (right panel) of OVX rats ICV treated with vehicle or E2 (1 nmol). Error bars represent SEM; n = 7–12 animals per experimental group. *, ** and ***p < 0.05, 0.01 and 0.001 versus vehicle ICV.

Next, we aimed to investigate the involvement of β3-adrenergic receptor (β3-AR) in the control of BAT thermogenesis (Mund and Frishman, 2013). This was established by pharmacological blockade with the specific antagonist SR59230A

(Lopez et al., 2010b). We proceeded with central ICV administration of 1 nmol of E2 to SC treated animals with either the β3-AR-antagonist SR59230A for 2 days or vehicle. The antagonist prevented the changes in body weight induced by estrogen without any effect on food intake (Figures 19A, 19B). In keeping, we found that estrogen effects on core temperature (Fig 19C) and in UCP1 expression (Fig 19D) were mediated by activation of β3-adrenergic receptors.

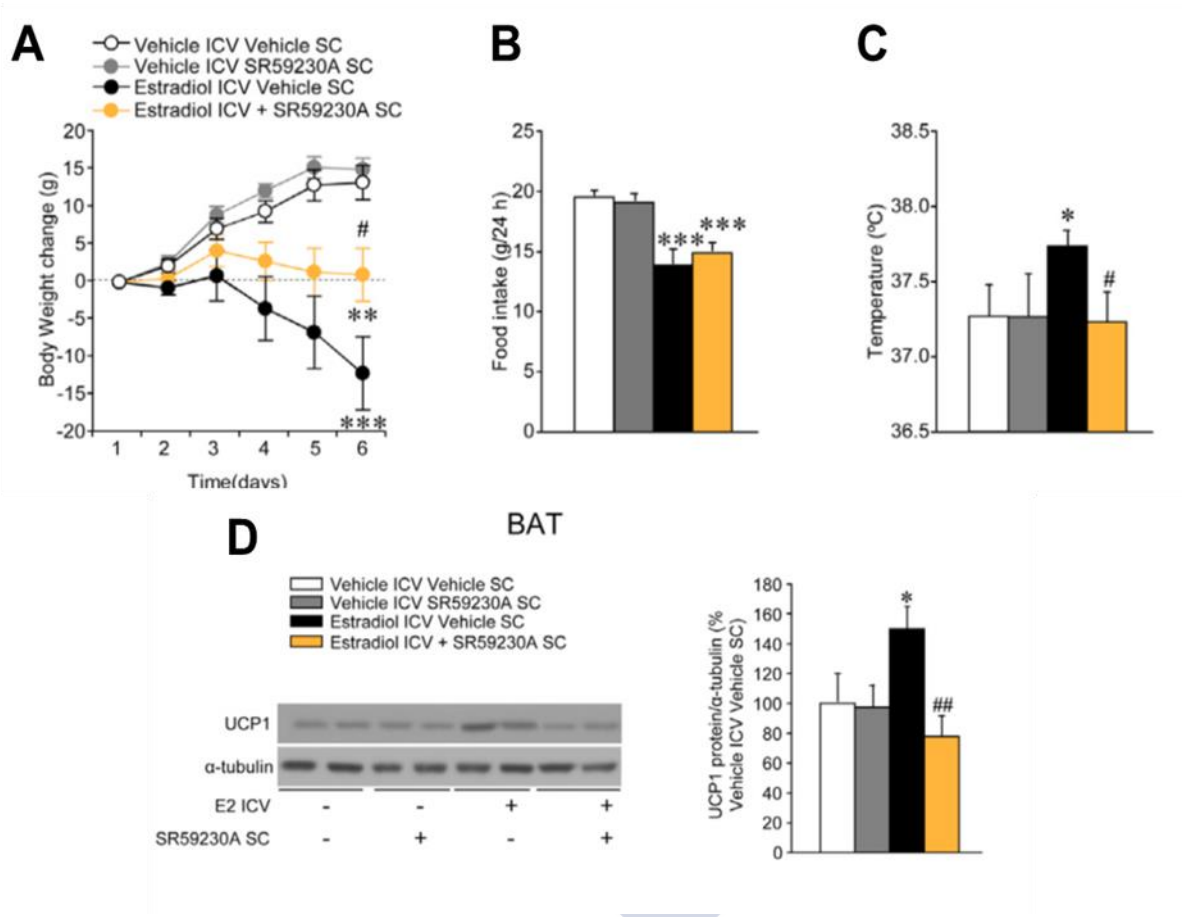

Figure 19. Effect of β3-adrenergic receptor blockade on the central E2 induced thermogenesis (A-D). A- Body weight change, B- daily food intake, C- core temperature, and D- western blot autoradiographic images of BAT UCP1 protein (left panel) and UCP1 protein levels (right panel) of OVX rats ICV treated with vehicle or E2 (1 nmol), previously SC treated with the β3-AR-antagonist SR59230A for 2 days. Error bars represent SEM; n = 7–12 animals per experimental group. * and ***p < 0.05 and 0.001 versus vehicle ICV or vehicle ICV vehicle SC; # and ##p < 0.05 and 0.01 versus E2 ICV vehicle SC.

1.3. Modulation of the VMH AMPK-SNS-BAT Axis by the physiological Levels of E2

The level of circulating estradiol is in a dynamic state; it fluctuates during the estrus cycle being the highest at estrus phase. Diestrus phase possesses a medium to

low level of E2 (figure 20A). We aimed to investigate whether or not the fluctuations in the physiological level of circulating E2 is accompanied by alterations in the modulation of the VMH AMPK- BAT axis. We used female rats at diestrus for this study where they expressed higher core temperature (Figure 20A) and UCP1 protein levels in BAT (Figure 20B) than OVX rats. The AMPK pathway activity in the VMH was inhibited (Figure 20C). Overall, this data indicate that high endogenous estrogenic tone inhibits AMPK function in the VMH and increased BAT thermogenic program.

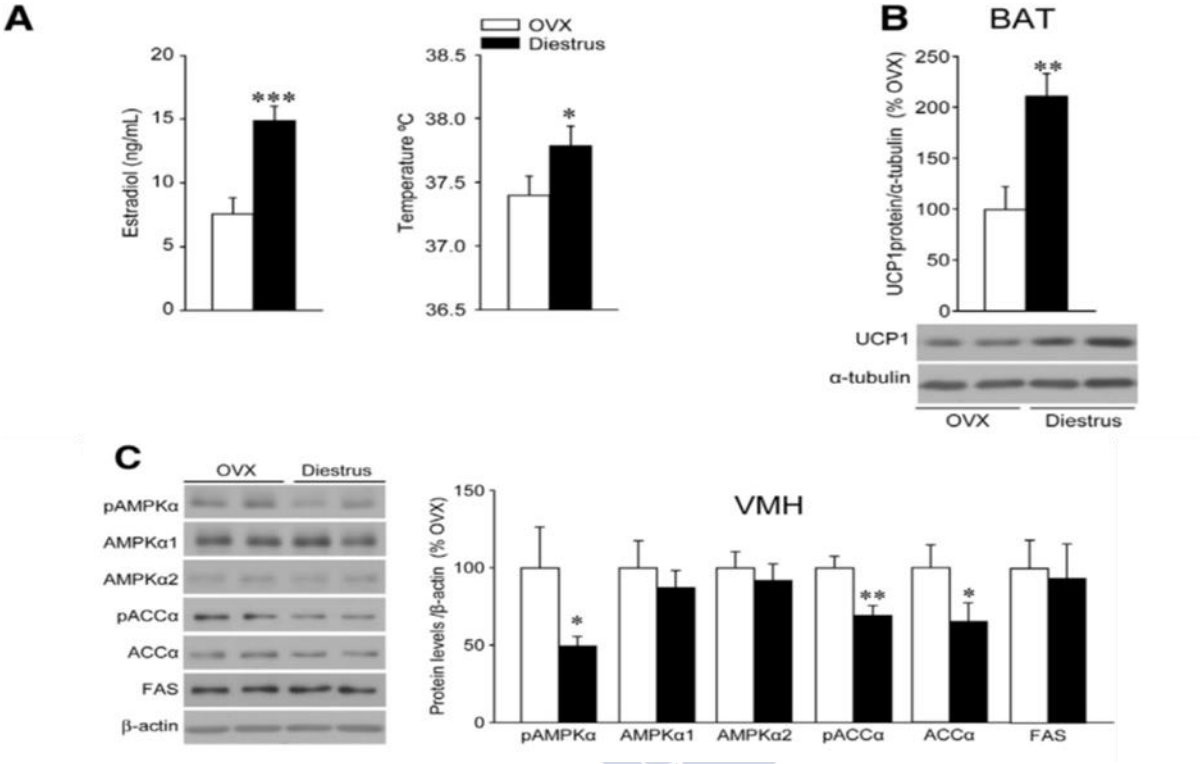

Figure 20. Effect of physiological levels of E2 on the VMH AMPK-SNS-BAT Axis (A–C). (A) Serum E2 levels (left panel) and core temperature (right panel), (B) protein levels (upper panel) and western blot autoradiographic images of BAT UCP1 protein (lower panel), and (C) western blot autoradiographic images (left panel) and levels of proteins of AMPK pathway in the VMH (right panel) of diestrus cycled rats. Error bars represent SEM; n = 7–8 animals per experimental group. *, **, and ***p < 0.05, 0.01, and 0.001 versus OVX, vehicle ICV, or GFP VMH.

2. Androgens and energy balance

The contribution of androgens to the modulation of energy balance at a central level remains elusive. In this part of our thesis, we have decided to study the effect of central administration of androgens on energy balance and BAT thermogenesis. Androgen deficient Sprague Dawley male rats were used as an animal model.

Androgen deficiency was induced by bilateral orchidectomy of the animal models as described in the methods section of this thesis. Rats were left for at least three weeks before any experimental procedure to allow the complete elimination of androgens. The study used DHT as an androgen of choice so that the effect of aromatase enzyme can be neglected.

2.1. Validation of orchidectomized rats as a model of study

Our first approach toward this study was a thorough evaluation of the effects of androgen withdraw on the different metabolic parameters of orchidectomized rats in comparison with SHAM operated ones.

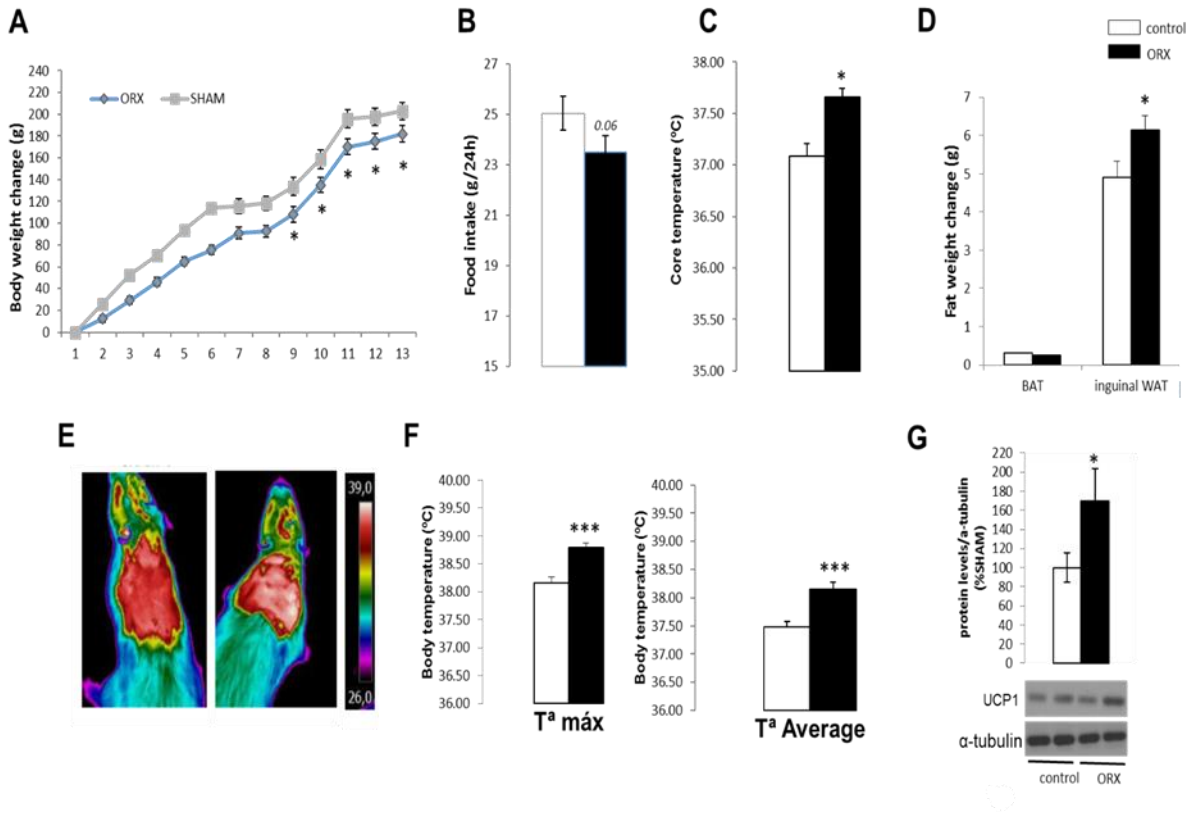

Figure 21. Effect of orchidectomy on the different metabolic parameters (A-G): A- body weight change B- daily food intake (g) C- Core temperature (ºC) D- Adipose tissue weight: BAT (left panel) and subcutaneous inguinal WAT (right panel) (E-G) E- infrared thermal images F- quantification of temperature of the skin surrounding interscapular BAT: maximal skin temperature (left panel) and average skin temperature (right panel) G- quantification of protein levels (upper panel) and auto-radiographic western blotting images of BAT UCP1 protein (lower panel). Error bars represent SEM. * $p < 0.05$ and ***<0.001 versus SHAM operated control. n=6-12 animals per experimental group.

We have observed that orchidectomized rats lost weight (figure 21A) with no significant change in food intake (figure 21B). The core temperature was significantly elevated (figure 21C) in orchidectomized rats compared to the sham operated rats that were associated with a significant rise in the temperature of the skin surrounding interscapular BAT at both the maximal temperature and average skin temperature (figure 21F). Although orchidectomized rats lost weight, they were shown to gain fat mass in the form of subcutaneous inguinal white adipose tissue but not BAT (figure 21D). These effects were accompanied by a significant reduction in the ARC AMPK activity (Figure 22) suggesting that the central effect of androgen deficiency on energy balance may be mediated by AMPK in the ARC.

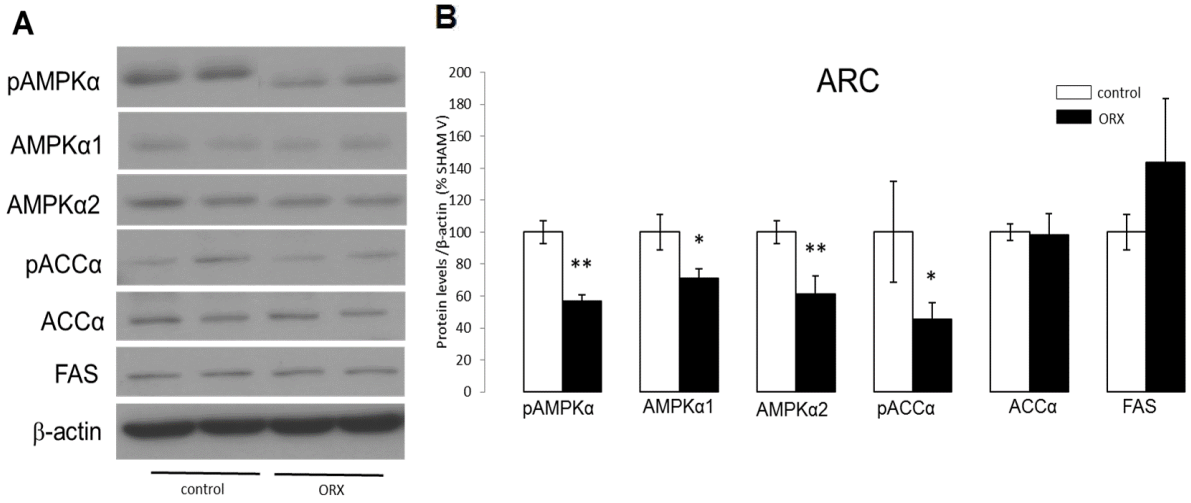

Figure 22. Levels of the AMPK pathway proteins in the ARC (A-B): A- quantification of the different protein levels (right panel) and B- auto-radiographic western blotting images (left panel). Error bars represent SEM. * $p < 0.05$ and **<0.01 versus SHAM operated control. n=6-8 animals per experimental group.

2.2. Central androgenic modulation of energy balance

To continue, we started to study the effect of DHT replacement for 7 days on the food intake and body weight. We proceeded with ICV administration of a 100nM DHT compared to vehicle in both SHAM operated or the orchidectomized rats based on several pilot experiments with different doses of DHT. The fact that the dose needed in case of estrogens was much lower though both were introduced via the same route might be related to their distinct effects in the sexual dimorphism of energy balance dilemma.

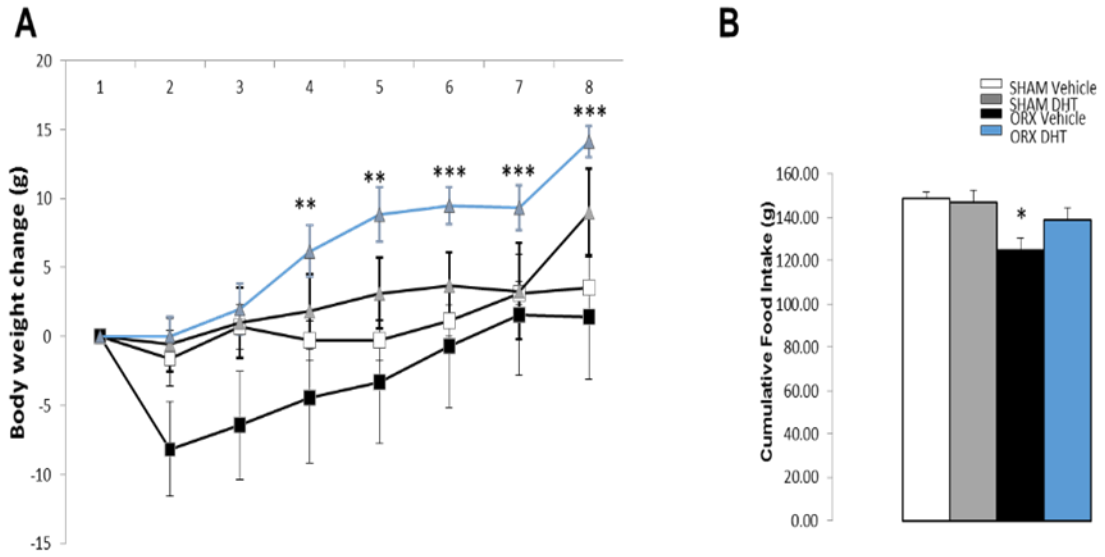

Figure 23. Effect of central replacement with DHT on food intake and body weight (A-B): A- body weight change (g) B- cumulative food intake (g) Error bars represent SEM. *p < 0.05 **p < 0.01 ***<.001 vs. ICV SHAM Vehicle. n=6-12.

Central replacement with DHT enhanced weight gaining in ORX animals with no significant change in the cumulative food intake (Figure 23A and 23B). Conversely, that corresponds to significant alterations in AMPK activity in the ARC (figure 24) after DHT treatment of ORX rats.

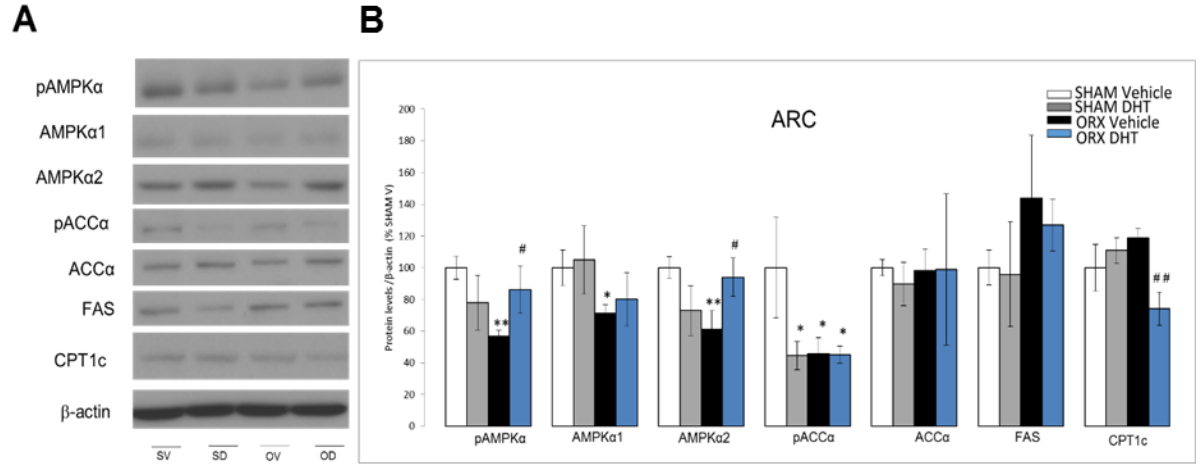

Figure 24. Levels of the AMPK pathway proteins in the ARC(A-B): A- quantification of the different protein levels and B- auto-radiographic western blotting images. SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. * p < 0.05 and **<0.01 versus SHAM operated vehicle and # p < 0.05 versus orchidectomized ICV vehicle. n=6-8 animals per experimental group.

Core temperature was reduced in ORX animals (Figure 25B). In addition, both ORX and ICV treatment with DHT in Sham animals led to an increase in average and Maximal skin temperature (Fig 25A). This reduction in body temperature was accompanied by a significant reduction in the maximal temperature of the skin surrounding the interscapular BAT (Figure 25A) compared to the orchidectomized treated with vehicle. The protein expression of the thermogenic marker UCP1 showed a similar pattern of changes to those seen in maximal skin temperature (Figure 25B and 25C).

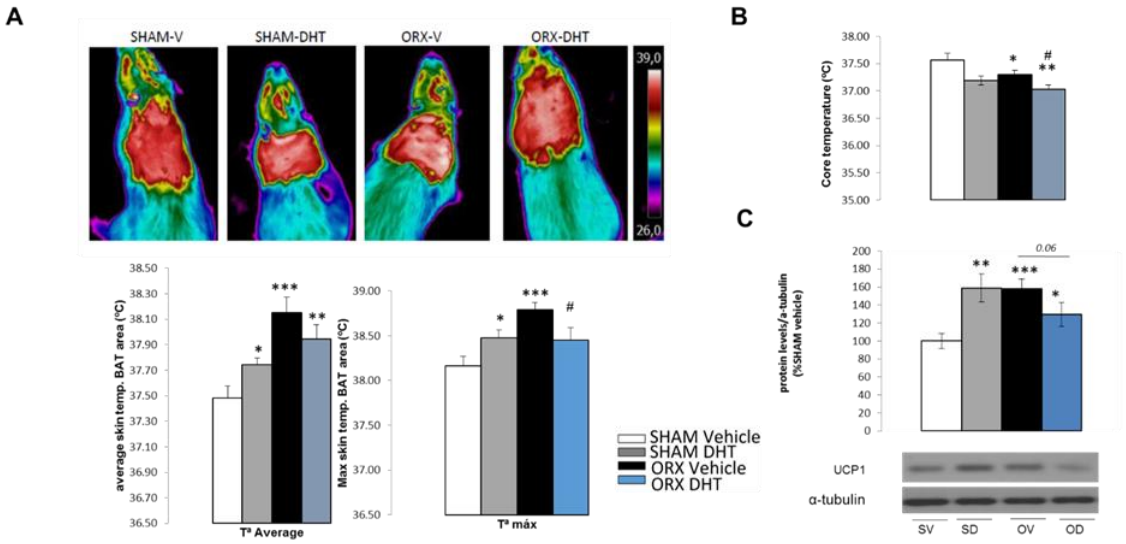

Figure 25. Effect of ICV DHT on BAT thermogenesis (A-C): (A) upper, infrared thermal images, lower, quantification of temperature of the skin surrounding interscapular BAT, (B) core temperature, (C) quantification of protein levels (upper panel) and auto-radiographic western blotting images of BAT UCP1 protein (lower panel). SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. ∗p < 0.05 **<0.01 ***<0.001 vs. ICV SHAM Vehicle # p < 0.05 versus orchidectomized ICV vehicle. n=5-15.

At VMH level, AMPK pathway activity was significantly increased (Figure 26A) following administration of DHT; thus promoting androgens to a valid member with a potential role in modulating the VMH AMPK- SNS- BAT pathway.

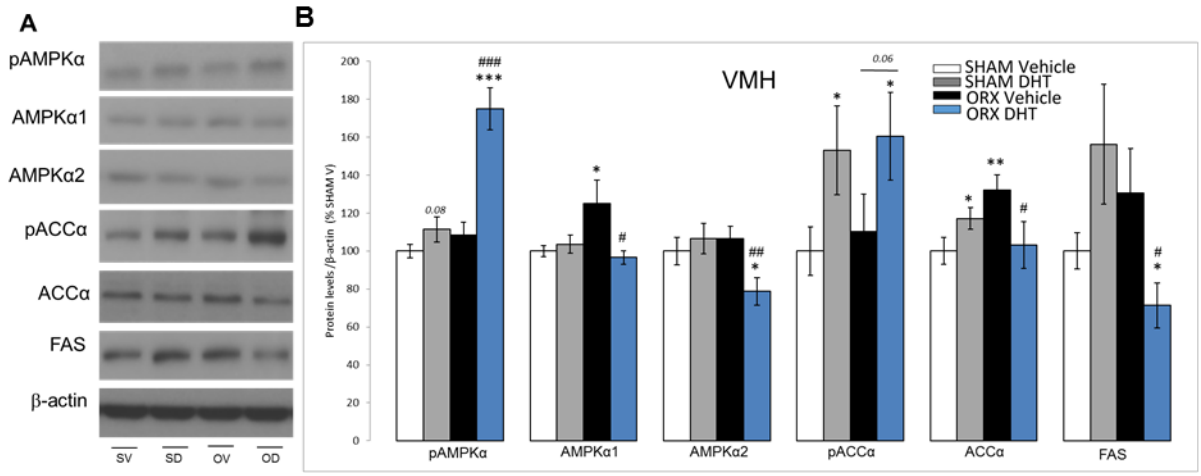

Figure 26. Levels of the AMPK pathway proteins in the VMH nucleus (A-B): A- quantification of the different protein levels and B- auto-radiographic western blotting images. SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. * $p < 0.05$ and **<0.01 versus SHAM operated vehicle and # $p < 0.05$, ##<0.01 and ###<0.001 versus orchidectomized ICV vehicle. n=6-8 animals per experimental group.

2.3. 2.3. Androgens: central actions with peripheral consequences

Androgens are known for their physiological importance in the development and increase of muscle mass as well as their reduction of fat level as revealed by other studies (Roth and Page, 2011), we continued to investigate whether or not these actions are mediated centrally. We proceeded with the ICV DHT for 7 days' treatment and by using magnetic resonance imaging (MRI) to quantify the change in muscle and fat masses before and after the treatment in both SHAM and orchidectomized (ORX) models.

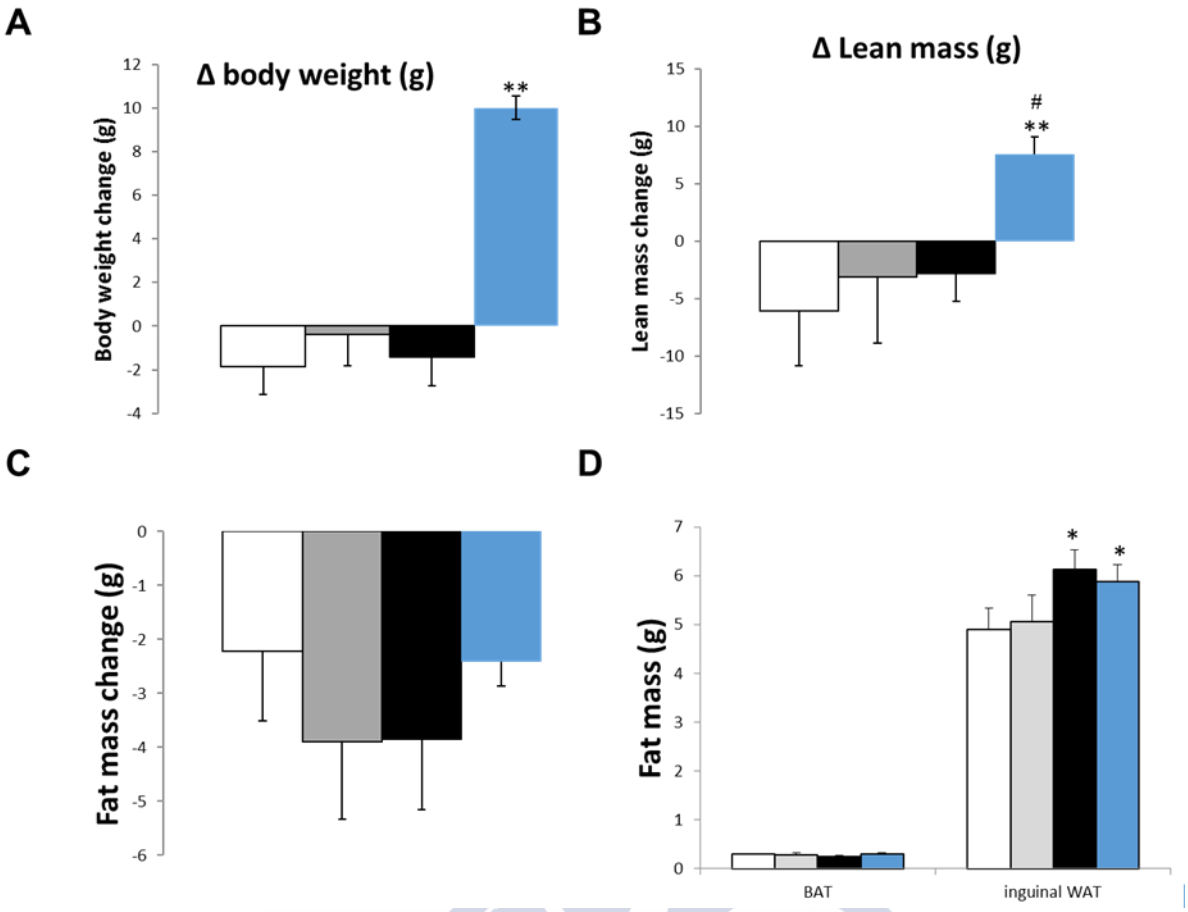

Figure 27. Effect of ICV DHT on fat and lean masse(A-D): (A) Body weight change, (B) Fat mass change, (C) lean mass change (D) BAT and subcutaneous inguinal WAT. Both B and C are based on studies realized by using magnetic resonance technique (MR). Error bars represent SEM. *p < 0.05, **<0.01 vs. ICV SHAM Vehicle while #<0.05 versus orchidectomized ICV vehicle. n= 6-15.

The orchidectomized rats centrally replaced with DHT showed a significant increase in body weight (figure 27A) that was mostly due to a significant increase in lean mass (Figure 27B) but not fat mass (Figure 27C, 27D). This may suggest that the mode of action of DHT on lean mass, and particularly on muscle might be centrally induced in addition to the local effect on the muscle itself. On the other hand, the amount of subcutaneous inguinal fat was significantly higher than SHAM treated with vehicle but not significantly reduced with central DHT treatment. No significant change in BAT weight was noticed. We also investigated the effect of central DHT on AMPK signalling in muscle. Our data showed that the AMPK pathway activity in the gastrocnemius muscle (a fast-twitch muscle) was altered; pAMPK and pACC were significantly reduced in the ICV treated group with DHT (Figure 28). These data

indicate that central DHT inhibits AMPK in muscle. These data together suggested a potential role of central DHT in the modulation of the mammalian target of rapamycin (mTOR), a target for muscle protein synthesis (Figure 28) (Saha et al., 2010).

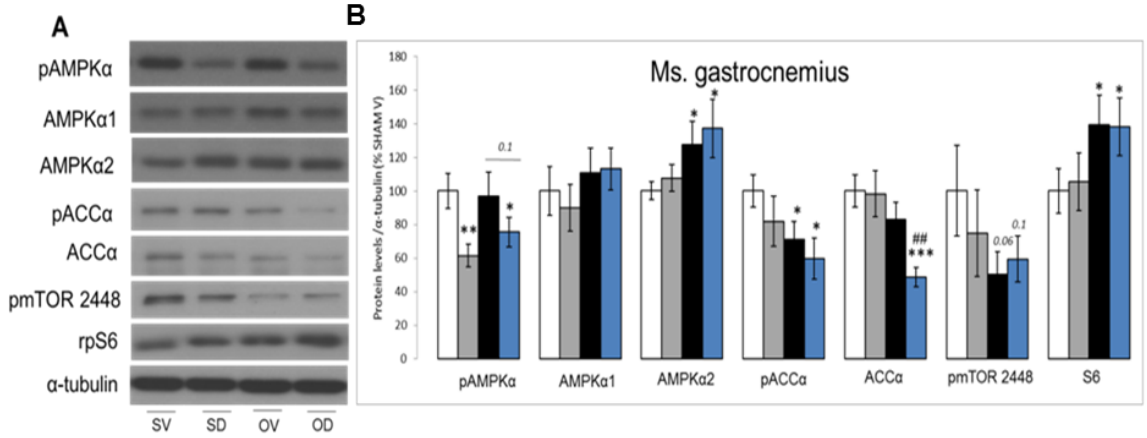

Figure 28. Levels of the AMPK pathway proteins in the gastrocnemius muscle (A-B): A- quantification of the different protein levels and B- auto-radiographic western blotting images. SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. * $p < 0.05$ and **<0.01 versus SHAM operated vehicle and ##<0.01 versus orchidectomized ICV vehicle. n=6-8 animals per experimental group.

To further investigate whether or not this interesting change in muscle mass involved the activation of β2-adrenergic receptor (β2-AR; its stimulation increases muscle mass by promoting muscle protein synthesis (Sato et al., 2011)). Pharmacological blockade with the specific β2-AR antagonist butoxamine was used (Arai et al., 2013). We proceeded with central ICV administration of 100 nmol of DHT previously SC treated with butoxamine for 1 day. While central DHT increased weight gain as lean mass, SC butoxamine reversed this particular effect with no significant change in body weight, food intake, core temperature or fat mass (Figures 29A, 29B, 29C, 29D and 29E). These data indicate that central DHT increases lean mass via activation of the β2-adrenergic receptor in muscle.

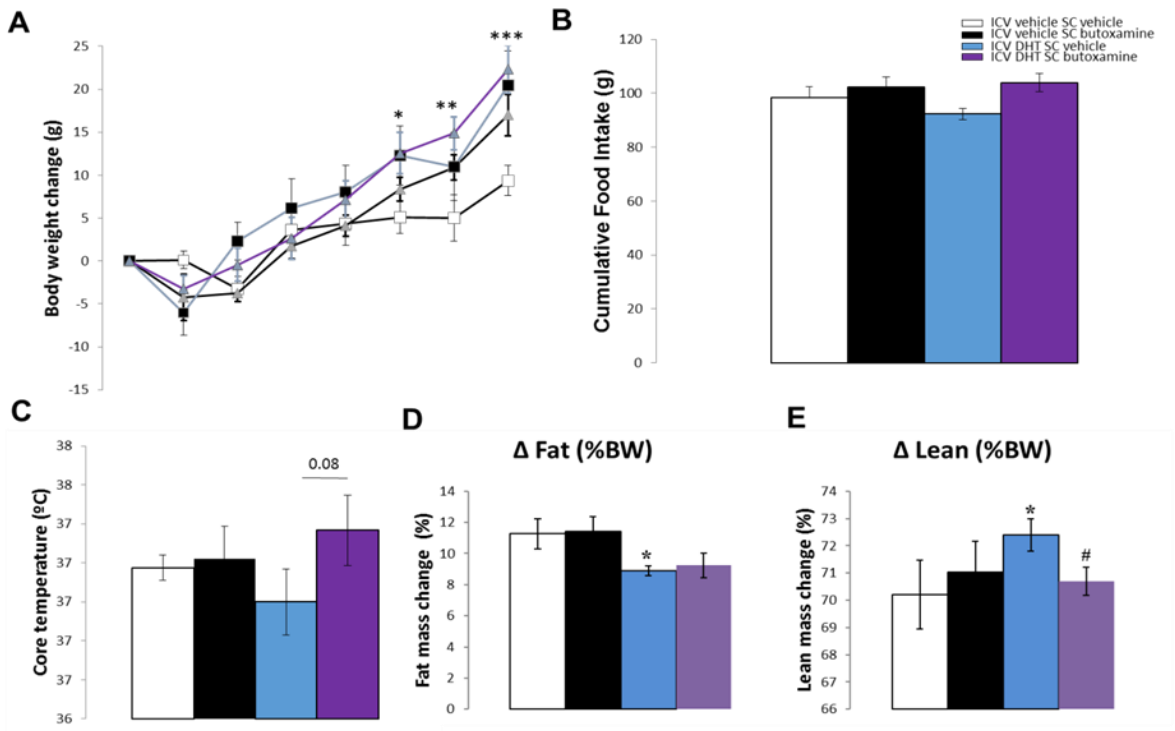

Figure 29. Effect of β2-adrenergic receptor blockade on fat and lean masses with ICV DHT treatment (A-E): (A) Body weight change, (B) cumulative food intake, (C) core temperature, (D) Fat mass change (%BW), and (E) lean mass change (%BW). Both D and E are based on studies realized by using magnetic resonance imaging (MRI). Error bars represent SEM. ∗p < 0.05**<0.01 ***<0.001 vs. ICV vehicle SC vehicle while #<0.05 versus ICV DHT SC vehicle. n= 6-10.

Furthermore, to continue studying the different effects of DHT, we started to further investigate the de novo lipogenesis on some peripheral tissues such as BAT and liver.

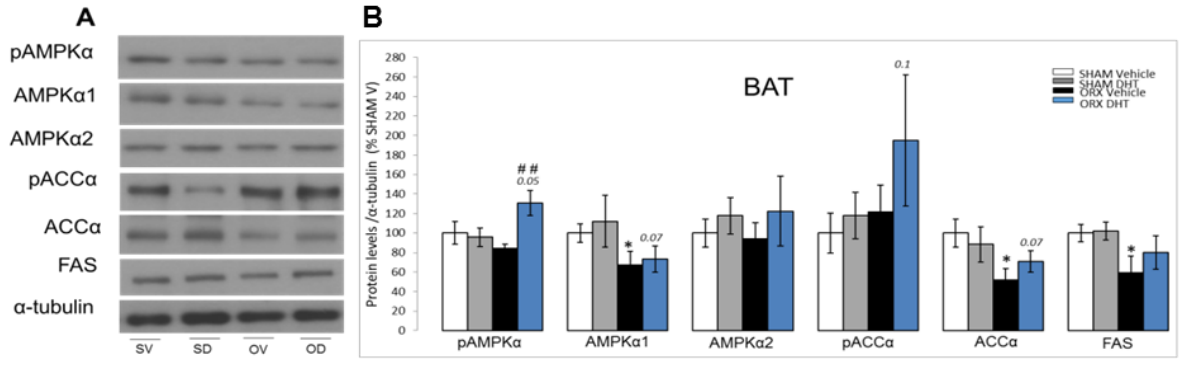

Figure 30. Levels of the AMPK pathway proteins in the gastrocnemius muscle (A-B): A- quantification of the different protein levels and B- auto-radiographic western blotting images (right panel). SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. *p < 0.05 versus SHAM operated vehicle. n=6-8 animals per experimental group.

Our data in BAT showed a decline tendency in the lipogenic markers with pAMPK was significantly increased in DHT treated ORX rats compared to vehicle treated ones (Figure 30).

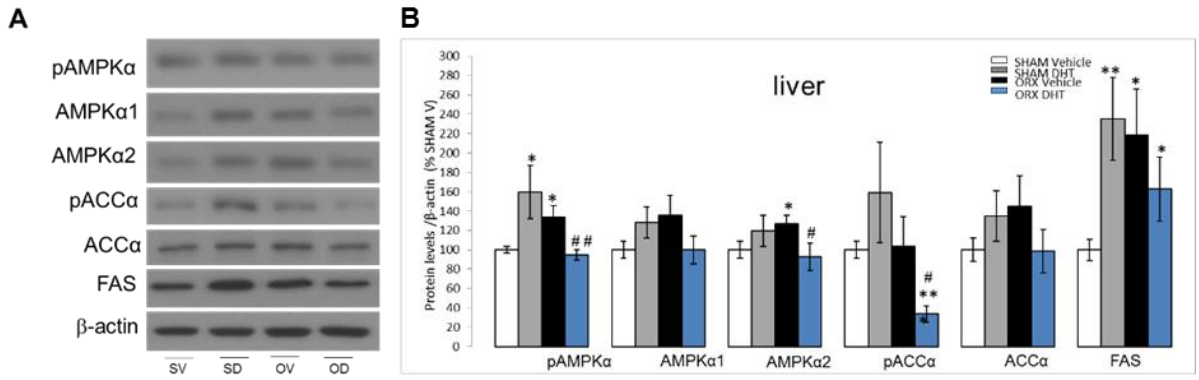

Figure 31. Levels of the AMPK pathway proteins in the liver (A-B): A- quantification of the different protein levels and B- auto-radiographic western blotting images. SV represents SHAM operated ICV vehicle, SD represents SHAM operated ICV DHT, OV represents orchidectomized ICV vehicle and OD represents orchidectomized DHT. Error bars represent SEM. * $p < 0.05$ and **<0.01 versus SHAM operated vehicle and # $p < 0.05$ and ##<0.01 versus orchidectomized ICV vehicle. n=6-8 animals per experimental group.

In the liver, the overall effect of DHT ICV treatment was to promote de novo lipogenesis; reducing the levels of pAMPK / pACC and increasing FAS (Figure 31). In general, our results indicate that the central effect of DHT are tissue-specific and can mediate energy and metabolic homeostasis at peripheral level.

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DISCUSSION

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The data of this thesis demonstrate the influence of gonadal hormones as relevant players in thermoregulation. Moreover, it has identified the hypothalamus as a site where sex steroids, both E2 and DHT, can act centrally to modulate BAT thermogenesis. The first part of this study identifies a link between the effects of E2 on hypothalamic AMPK and BAT thermogenesis. Specifically, we show that activation of BAT thermogenic program and subsequent energy expenditure depends on inactivation of VMH AMPK. Overall, these results further expand our knowledge on the hypothalamic effects of estrogens on energy homeostasis and offer a physiological mechanism, via the energy sensor AMPK, for the central actions of E2 that is of relevance in linking the changes in energy balance and may also help to understand obesity-associated alterations in the gonadal axis.

Conditions in which ovarian estrogens are lacking, such as OVX or menopause, are associated with hyperphagia and decreased caloric expenditure that results in a positive energy balance leading to obesity. Estrogen replacement reverts this phenotype both in women and female rodents (Carr, 2003; Finan et al., 2012; Gao and Horvath, 2008; Mauvais-Jarvis et al., 2013). Although some discrepancies can arise when comparing both models of estrogen insufficiency (OVX and menopause) since in the first instance, there is an abrupt decrease in ovarian function while in the second is a progressive fall. Noteworthy, data gleaned in recent years in genetic models have shown that some of the actions of estrogens on energy homeostasis are centrally mediated through activation of ERα in the main hypothalamic nuclei, such as the ARC and the VMH (Musatov et al., 2007; Xu et al., 2011b). In addition, data obtained previously in our group (Martinez de Morentin, 2013) showed that estrogens exert some of its effects on energy balance by influencing energy expenditure via hypothalamic AMPK.

Recent literature indicates that hypothalamic AMPK pathway is a central regulator of energy homeostasis that acts by controlling both feeding and energy dissipation (Andrews et al., 2008; Lopez et al., 2010b; Martinez de Morentin et al.,

2012; Minokoshi et al., 2004; Whittle et al., 2012). As expected, ICV administration of E2 promoted a state of negative energy balance, characterized by hypophagia, increased energy expenditure, and BAT thermogenesis, leading to marked weight loss. These effects are reversed by pharmacological blockage of β3-AR independently of feeding and appear to derive from VMH-specific actions of E2 so that the impact of E2 on BAT thermogenesis derives from its ability to modulate AMPK activity in the VMH.

Overall, these data confirm that E2 modulates energy homeostasis in a brain site-specific fashion (Xu et al., 2011b) and uncover a molecular mechanism (i.e. modulation of AMPK, by which E2 specifically modulates BAT metabolism). In recent years, hypothalamic metabolic energy sensors, such as AMPK, have been suggested to play a major role in the regulation of energy homeostasis. Several metabolic hormones, such as leptin, ghrelin, insulin, and thyroid hormones (Andrews et al., 2008; Lopez et al., 2010b; Minokoshi et al., 2004), and more recently BMP8b (Whittle et al., 2012), have been shown to regulate energy balance acting through AMPK. Here, we provide evidence that E2, a hormone that markedly influences energy and metabolic homeostasis, acts on the same sensors to control both feeding and energy expenditure. Notably, the mechanisms described here for the thermogenic actions of E2 display considerable commonalities with those previously unveiled by our group for thyroid hormone and BMP8b (Lopez et al., 2010b; Whittle et al., 2012). Thus, the VMH AMPK-SNS-BAT axis appears to act as a canonical central mechanism integrating central and peripheral regulation of energy stores.

In this regard, our data points to AMPK in the VMH as a potential target for the treatment of obesity and other metabolic disorders associated with conditions of endocrine deregulation. The relevance of such a pharmacological target is emphasized by recent reports showing that nicotine-induced anorexia and weight loss is in part mediated by actions on AMPK-expressing neurons in the VMH to increase energy expenditure (Martinez de Morentin et al., 2012), which could be exploited as

a potential therapeutic strategy. In addition, DiMarchi, Tschop, and colleagues have recently reported an elegant approach based on the development of a combinatorial peptide of a GLP-1-estrogen conjugate for the treatment of obesity and metabolic syndrome (Finan et al., 2012). They demonstrated that selective activation of ERs in GLP-1-targeted tissues enhanced the benefits of both E2 and GLP-1 agonism on body weight, glucose homeostasis, and lipid metabolism. Considering that both GLP-1 receptor and ERα are highly expressed in the VMH (Gu et al., 2013; Shughrue et al., 1997), this raises the possibility that the metabolic benefits of E2 and GLP-1 agonism may be mediated by selective modulation of AMPK in the VMH. However, further investigations are needed to test such possibility.

The reason for the sex differences in energy metabolism is not completely known; however, it may be related to sex steroids, from which, the effects of androgens are relatively unknown. Low testosterone levels were found associated with metabolic syndrome, diabetes and cardiovascular disease (Corona et al., 2011a; Corona et al., 2011b; Zitzmann, 2009). Although it is known that testosterone may act on the brain to reduce food intake and modulate energy expenditure (Mauvais-Jarvis, 2011), the exact mechanisms underlying this action remains not fully understood. Our studies showed that the reductions in food intake and body weight are mediated through reduction in AMPK activity in the ARC following orchidectomy that was reversed with central DHT administration. Concurrently, Studies on guinea pigs showed that androgen-induced increases in energy intake were mediated via an AMPK activation in the POMC neurons of the ARC (Borgquist et al., 2015).

Interestingly, we wondered about the androgenic effects on the aforementioned pathways as well as on BAT thermogenesis. Previously the effects of testosterone on energy balance were largely thought to be attributed to aromatization (Mauvais-Jarvis, 2011). The experimental evidence attributing the effect of androgen signalling to BAT activity remains a controversial matter. Androgen-receptor

knockout male mice develop delayed obesity as a consequence of decreased energy expenditure and displays a reduced expression of Ucp1 in BAT depots (Fan et al., 2005b; Yanase et al., 2008). These findings suggesting the possibility of a stimulatory effect of androgens on BAT thermogenesis appears to be at odds with the observation that testosterone treatment of cultured brown adipocytes shows a reduced Ucp1 expression levels (Rodriguez et al., 2002) as well as with our data that showed that androgens reduces BAT thermogenesis. On the other hand, in vivo administration of with dehydroepiandrosterone (DHEA; an androgen precursor) and testosterone has been shown no change in BAT thermogenic activity in experiments using male rats (Abelenda et al., 1992; Tagliaferro et al., 1986).

Interestingly, our data using DHT that cannot be aromatized contradicts the effect of aromatization as estrogen upregulates the thermogenic pathways. Although our experimental model using DHT in both sham and ORX rats allowed us to study the effects of DHT in the presence and absence of endogenous testosterone level, the ORX-model is not without some limitations worth to take into consideration. In addition to the removal of Leydig cells, ORX is also associated with the loss of Sertoli cells and therefore decline in all their synthesized hormone products notably inhibin and activins. Therefore, the differences in the effects obtained with DHT-treated sham and ORX animals could be due to: a) the presence of endogenous testosterone levels in sham animals allows greater activation of the AR as well as activation of the ER through previous aromatization; b) In addition to decreasing the testosterone levels, ORX animals exhibited a reduction in the peptide hormones secreted by the Sertoli cells which can on its own influence energy expenditure. Therefore, DHT administration can functional compensate the loss of Leydig cell exerted via the AR but not the other ones. We are fully aware of these limitations and of the need to further explore this issue in the future using other models with selective loss of Leydig cells or genetic suppression of aromatization. Nevertheless, our data offers new insights on the central effects of androgens on energy

expenditure and metabolism that cannot be attributed to aromatization. We further attempted to elucidate the mechanisms responsible for the androgenic effects of testosterone on BAT thermogenesis. Following the same hypothesis of the last study, we realized that AMPK pathway activity in the VMH is increased, and BAT thermogenesis is reduced. Our current data support the notion that androgens reduce core body temperature (Fitts et al., 2004). Thus, it appears that testosterone treatment can reduce energy expenditure via a reduction in heat production in males.

The lack of androgen receptor in mice was associated with reduced lipolysis and developed obesity (Yanase et al., 2008), Low testosterone level was associated with an increase in body fat and abdominal obesity (Gapstur et al., 2002; Kapoor et al., 2005). Physiological replacement of testosterone produces improved both obesity and obesity-related disorders in both men and rats (Davis et al., 2012; Kapoor et al., 2006). Testosterone is known as a highly potent direct anabolic agent to skeletal muscle. Androgens also enhance the development of myocyte from pluripotent stem cells while decreasing adipocyte development thus increasing muscle mass (Singh et al., 2003). However, our data showed that DHT ICV treatment promoted weight gain that was mainly in the form of lean mass indicate the presence of indirect and central regulatory mechanisms of muscle mass. The skeletal muscles comprise the most abundant tissue in the human body constituting 40–50% of body mass. Skeletal muscle protein has a rapid turnover, which is governed by the balance between the rates of protein synthesis and degradation. Physical training and anabolic hormones and medications boost muscle protein level. However, cachexia, sarcopenia and muscle inactivity decrease muscle protein level.

The rate of protein synthesis is at least partially controlled by β2-adrenergic receptors in skeletal muscles in both anabolic and catabolic conditions. The β2 subtype is the most abundant subtype in muscle (Kim et al., 1991; Williams et al., 1984). Furthermore, β2-AR is denser in slow-twitch muscles than in fast-twitch muscles (Ryall et al., 2002; Ryall et al., 2004). However, the magnitude of anabolic

responses to β2-adrenergic agonists is higher in fast-twitch muscles than in slow-twitch muscles (Burniston et al., 2006; Ryall et al., 2006; Sato et al., 2008, 2010). Several studies have shown that β2-adrenergic agonists administration induce muscle hypertrophy in many species (Kim and Sainz, 1992; Lynch and Ryall, 2008; Ryall and Lynch, 2008). Experiments using mice lacking β2-AR demonstrate that β2-adrenergic agonist-induced effects such as muscle hypertrophy are mediated via β2-AR (Hinkle et al., 2002). β2-Adrenergic agonists promote muscle growth by increasing the rate of protein synthesis with or without changes in protein degradation (Kim and Sainz, 1992; Lynch and Ryall, 2008; Ryall and Lynch, 2008). Also, androgens enhance beta-adrenergic receptors expression so that it increases lipolysis and decreases fatty acid synthesis (De Pergola, 2000). Thus, we postulate that pharmacological blockage of β2-AR, the most prominent in skeletal muscle, would reverse the androgenic effects. Our data using β2-AR antagonist supports this hypothesis which in turn holds the central actions of androgens as a nominee leading to the possibility of finding effective treatments for cachexia and wasting in chronic disease.

On the other hand, certain obstacles will face the development of any androgen based therapeutics and testosterone therapy mainly due to the debts surrounding their role in the development of prostate cancer. The relationship between testosterone and prostate cancer has been described as the "fuel for a fire" or the "food for a hungry tumour". Even though the biological effects of testosterone have been recognized throughout the recorded history of humankind, even before identification of the key biochemical element produced by the testis, there are still a lot of misconceptions and misleading evidence surrounding androgens. The 1849 rooster castration and subsequent testes transplantation performed by Arnold Berthold was the first scientific experiment to linked the physiological and behavioural changes of castration to a substance secreted by the testes (Morales, 2013). Later on, David and colleagues isolated testosterone in 1935 (Nieschlag,

2006) and synthesized later that year. The following period after it first became available; testosterone therapy was extensively practiced. In 1941, Huggins and Hodges reported that castration caused regression of metastatic prostate cancer, and testosterone injections activated prostate cancer (Huggins and Hodges, 1941). From that point on, the use of testosterone became rare owing to fear of causing prostate cancer in otherwise healthy individuals.

Recently, there has been a remarkable and steady growth in the use of testosterone therapy (Baillargeon et al., 2013), beginning in the early 1990s, where Morgentaler and colleagues (Morgentaler et al., 1996) and Thompson and colleagues (Thompson et al., 2004) pointed to the indication that low testosterone may be a risk factor for prostate cancer, and not protective against prostate cancer and its progression (Morgentaler, 2011). Also, there is a completed lack of compelling evidence that high testosterone levels contribute to the development of prostate cancer (Rhoden and Morgentaler, 2004). An extensive review found that men with higher endogenous testosterone or who had undergone testosterone therapy were not at increased risk of prostate cancer (Endogenous et al., 2008). Supraphysiologic doses of testosterone for up to 9 months in healthy men failed to demonstrate a significant increase in prostate-specific antigen or prostate volume (Bhasin et al., 2001; Cooper et al., 1998). The notion that "more testosterone is bad, less testosterone is good" was not necessarily true. Conversely, Studies from Prout and Brewer (Prout and Brewer, 1967) and Fowler and Whitmore (Fowler and Whitmore, 1981) present an alternative to the Huggins and Hodges work. Both noted no evidence of progression in men with prostate cancer not treated by androgen deprivation or castration (Fowler and Whitmore, 1981; Prout and Brewer, 1967).

In summary, we show that central E2 inactivates AMPK in the VMH, which increases SNS tone, upregulates BAT thermogenesis, and increases energy expenditure, leading to weight loss, while in case of DHT, this action was reversed. Thus, our data add more evidence to the fact that AMPK, specifically in the VMH, is

a canonical mechanism modulating energy dissipation via SNS activation of BAT. This observation provides insights into the physiological regulation of energy balance and its perturbation in sex steroids deficient states and suggests that the VMH AMPK-SNS-BAT axis may be a potential therapeutic target for the treatment of obesity.

CONCLUSIONS

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The results obtained from the different experiments of this study allow us to establish the following conclusions:

- Sex steroids centrally regulate BAT thermogenesis via acting specifically in the VMH and their actions are mediated by alterations in AMPK activity at this level. This effect appears to be of physiological relevance since fluctuations in endogenous estrogen levels are sufficient to modulate the VMH AMPK- BAT axis.
- The central action of estrogens may be of great value in the fight against obesity and its comorbidities.
- Androgens can centrally promote an increase in lean mass and consequently can become a beneficial target for developing treatments against cachexia and wasting in chronic diseases at a central level.

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ABBREVIATIONS AND ACRONYMS

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ACC	Acetyl-CoA carboxylase
ACSs	Acyl-CoA synthetase
AgRP	Agouti related peptide
AMA	American Medical Association
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANP	Atrial natriuretic peptide
APS	Ammonium persulfate
AR	Androgen receptor
ARC	Arcuate nucleus
AREs	Androgen response elements
ArKO	Aromatase enzyme KO
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BBB	Blood–brain barrier
BDNF	Brain-derived neurotropic factor
BMI	Body mass index
BNP	Brain natriuretic peptide
CART	Cocaine and amphetamine regulated transcript
CB1	Cannabinoid receptor type 1
CCK	Cholecystokinin
CHF	Congestive heart failure
CKD	Chronic kidney disease
CNS	Central nervous system
CPT1	Carnitine-palmitoyl transferase 1
CRH	Corticotropin-releasing hormone
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMH	Dorsomedial nucleus

DPN	Diarylpropionitrile
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE	Energy expenditure
ER	Estrogen receptor
ERE	Estrogen response element
ERRs	Estrogen related receptors
EST	Tissue estrogen sulfotransferase
FAS	Fatty acid synthase
FSH	Follicle-stimulating hormone
GHRH	Growth hormone-releasing hormone
GLP-1	Glucagon-like peptide-1
GnRH	Gonadotropin releasing hormone
GPER	G-protein-coupled estrogen receptor
GPR30	G-protein coupled receptor 30
GR	Glucocorticoid receptor
GSIS	Glucose-stimulated insulin secretion
HARKO	Hepatocytes AR
HAT	Histone acetylation
HDAC	Histone deacetylation
HFD	High fat diet
HPT	Hypothalamic pituitary thyroid axis
HPG	Hypothalamic pituitary gonadal axis
HSD17-β	17-β hydroxysteroid dehydrogenase
HSD17-β3	17-β hydroxysteroid dehydrogenase type III
HSPs	Heat-shock proteins
ICV	Intracerebroventricular rout
IP	Intraperitoneal route
KO	knockout

LBD	Ligand-binding domain
LCFAs	Long chain fatty acids
LCFAs-CoA	Long chain fatty acyl-CoA
LepRb	Leptin Receptor Long Isoform
LH	Luteinizing hormone
LHA	Lateral hypothalamus area
M	Average value
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MCD	Malonyl-CoA decarboxylase
ME	Median eminence
mER	Membrane estrogen receptor
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NARKO	Neuronal specific ArKO
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
Ob-R	Leptin receptor
ORX	Orchidectomized
OVX	Ovariectomized
PGC1 α	Peroxisome-proliferator-activated receptor-gamma coactivator 1 alpha
PGC1 β	Peroxisome-proliferator-activated receptor-gamma coactivator 1 beta
POA	Preoptic area
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PPT	Propylpyrazoletriol
PR	progesterone receptor
PVH	Paraventricular nucleus
PYY	Peptide YY

RMR	Resting metabolic rate
rRPa	Rostral raphe pallidus
RT-PCR	Reverse transcription polymerase chain reaction
SC	Subcutaneous route
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
SF1	Steroidogenic factor 1
SHBG	Sex hormone-binding globulin
SNS	Sympathetic nervous system
StAR	Steroidogenic acute regulatory protein
T	Testosterone
T2D	Type 2 diabetes
TEMED	N, N, N, N tetramethyletilenediamina
TF	Transcription factors
TG	Triglycerides
TRH	Thyrotropin-releasing hormone
UCP-1	Uncoupling protein-1
VMH	Ventromedial nucleus
WAT	White adipose tissue
WHO	World Health Organization
α-MSH	α-melanocyte stimulating hormone
β2-AR	β2-adrenergic receptor
β3-AR	β3-adrenergic receptor
β-AR	β-adrenergic receptor

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APPENDIXES

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Appendix I

Summary:

Both living and the quality of life depends to great extent on the ability of an organism to maintain their internal environment and systems in a state of homeostasis. The homeostasis of energy balance is no exception; our body struggles to provide a stable balance between energy consumed and expended. Imbalance in any direction will result in its consequences and health issues. Obesity results from a chronic imbalance between energy consumed and expended; favouring energy consumption and causing overgrowth of the adipose tissue to a level that adversely affects the state of health (Sanchez-Gurmaches and Guertin, 2014b). Obesity is associated with some comorbidities including insulin resistance, type 2 diabetes (T2D), cardiovascular disease, cancer, a range of other disorders collectively known as the metabolic syndrome (van der Klaauw and Farooqi, 2015). The diseases following obesity not only affect the quality of patients' life but also stands for a great sum of medical costs (Heindel et al., 2015) that is susceptible to further increase considering the level and pattern of obesity prevalence in both developed and developing countries (WHO, 2014). Focusing on the factors behind obesity revealed that the changes in our environment, availability of intense energy highly palatable known as fast foods with less physical activity, as well as genetic predisposition are its main predisposing factors.

On the other hand, Cachexia results from a long-standing imbalance between energy intake and expenditure where expanded energy exceeds dietary intake (Evans et al., 2008). It involves weight loss mainly in the form of muscle with or without loss of fat mass despite not actively trying (Evans et al., 2008). Also, Cachexia cannot be entirely reversed by dietary supplementation. The main issues with cachexia are their negative effect on the quality of life that may result in higher mortality rates (Lok, 2015) among patients suffering from chronic illness including chronic obstructive pulmonary disease, chronic kidney disease, congestive heart failure, and cancer. Both obesity and cachexia are now considered a public health problem and the development of interventions to block or attenuate it would have notable therapeutic benefits considering the current lack of valuable cure to both syndromes.

Energy balance homeostasis is a complex process that involves the interaction of all parts of our body at the different levels. The peripheral organs and tissues including the gut, white adipose tissue (WAT), gonads, muscles, and the thyroid gland continuously supply the CNS with signals about the metabolic and nutritional situation of the body (Flier, 2004; Fruhbeck and Gomez-Ambrosi, 2003; Lopez et al., 2007b; Medina-Gomez and Vidal-Puig, 2005; Wren and Bloom, 2007). These signals might be hormonal such as cholecystokinin

(CCK), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1), leptin, ghrelin, adiponectin, resistin, and insulin, mechanical signals; gastric distension, chemical signals; ingested food components or even nerve signal; vagal afferent stimulation. The CNS then integrates these signals and initiates the proper response allowing efficient energy homeostasis; by modifying the expression of the main neuropeptides (Flier, 2004; Fruhbeck and Gomez-Ambrosi, 2003; Lopez et al., 2007b; Medina-Gomez and Vidal-Puig, 2005; Wren and Bloom, 2007). Neuronal circuits are directly or indirectly involved in the regulation of all physiological activities, of which, the hypothalamic circuits are considered the primary regulator of food intake and energy homeostasis (Dieguez et al., 2009; Plum et al., 2006; Schwartz et al., 2000). Primarily, only two hypothalamic nuclei were supposed to have a role in the regulation of feeding; the lateral hypothalamic area (LHA) representing the feeding center and the VMH representing the satiety center (Anand and Brobeck, 1951; Hetherington and Ranson, 1940). These remarks were based on the observations from lesions of the LHA reduced food intake and eventually lead to starvation and death while that of the VMH resulted in obesity. Despite our expanding understanding of the hypothalamic regulation of feeding, the first idea that anatomically defined hypothalamic areas regulate food intake has persisted.

Among these nuclei, the arcuate nucleus (ARC) is considered the prime hypothalamic center for food intake control. The ARC contains two distinct neuronal populations involved in the integration of peripheral signals. One set of neurons expresses the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY). The other ARC set of neurons expresses the anorexigenic products of proopiomelanocortin (POMC), the precursor of alpha-melanocyte-stimulating hormone (α-MSH), and the cocaine and amphetamine-regulated transcript (CART). The central melanocortin system whose main element is POMC consists of these two neuronal populations and the downstream target neurons expressing the melanocortin receptor 3 (MC3R) and the melanocortin receptor 4 (MC4R). This system is vital for efficient sensing and integration of peripheral signals allowing the proper energy homeostatic mechanism (Butler, 2006; Lee and Wardlaw, 2007; Seeley et al., 2004; Xu et al., 2011a). When energy intake exceeds expenditure, the expression of orexigenic neuropeptides, such as AgRP and NPY diminishes, whereas the expression of anorexigenic neuropeptides, such as CART and POMC, rises. Opposite changes occur when energy expenditure exceeds intake. The two populations of neurons in the ARC broadly project within the CNS, to secondary hypothalamic nuclei such as the dorsomedial nucleus (DMH), The VMH, the LHA, and the paraventricular nucleus (PVH).

The PVH is also an integration site involved in energy homeostasis (Kim et al., 2000; Stanley et al., 1986). The LHA plays a critical role in mediating the orexigenic responses as the possess both orexin and melanin- concentrating hormone (MCH) neurons (de Lecea et al., 1998; Ferno et al., 2015; Lopez et al., 2010a; Sakurai et al., 1998). The DMH is also a vital hypothalamic center for energy balance regulation; it is involved in both the regulation of appetite and thermoregulation (Chan et al., 1996; Chao et al., 2011; Kamegai et al., 1996). The VMH is also implicated in the regulation of energy balance; it plays a key role in the regulation of BAT thermogenesis (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012).

Furthermore, the consumed energy is used in either resting metabolic rate (RMR; the amount of energy needed at rest to maintain the basic cellular metabolic activities), physical activity or thermoregulation. The brain controls these three categories of energy expenditure (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Rui, 2013; Seoane-Collazo et al., 2014; Silva, 2003; Whittle et al., 2012). BAT is valuable for its role in adaptive thermogenesis (Cannon and Nedergaard, 2004; Contreras et al., 2015; Whittle et al., 2011). Functionally, WAT can be considered an energy store mainly in the form of triglycerides (TG) while BAT burns these TG to produce heat. For many years, BAT was known for its important role in small animals and human babies, but recently, BAT has been reported to not only present but also is functional in adult humans. Moreover, a phenomenon called the browning of WAT has been reported. It involves the appearance of a particular type adipocytes called beige, brite, or recruitable brown adipocytes in anatomically new locations corresponding to WAT but has a lesser capacity *in vivo* (Lee et al., 2014; Lidell et al., 2013; Sharp et al., 2012). The sympathetic nervous system (SNS) is essential to activate BAT thermogenesis as reflected by a high density of nerve endings in the tissue (Cannon and Nedergaard, 2004; Cao et al., 2001). Norepinephrine activates an adrenergic receptor that generates signals to enhance thermogenesis and stimulate intracellular lipolysis (Cannon and Nedergaard, 2004). The thermogenesis of BAT is associated with the presence of a mitochondrial membrane protein called the uncoupling protein-1 (UCP-1), or thermogenin, that is present in the inner mitochondrial membrane of BAT adipocytes. This protein enhances thermogenesis through uncoupling between respiratory chain complexes and the adenosine triphosphate (ATP) production promoting free protons movement into the mitochondrial matrix, thus generating heat instead of ATP (Cannon and Nedergaard, 2004; Garlid et al., 2000; Jaburek et al., 2001; Whittle et al., 2011; Zingaretti et al., 2009).

Many hypothalamic nuclei are associated with the regulation of thermogenesis as evidenced by using retrograde viral tracing in the BAT area of rats that revealed the potential connection with the preoptic area (POA), PVH, DMH, and LHA (Cano et al., 2003; Oldfield et al., 2002). The POA is considered the classical center responsible for thermoregulation (Boulant, 2000). Recently, the crosstalk between VMH and BAT has evolved where stereotaxic specific administration of glutamate, hydroxybutyrate, norepinephrine, serotonin, and tryptophan into VMH activated BAT (Amir, 1990; Hugie et al., 1992; Sakaguchi et al., 1988; Sakaguchi and Bray, 1989; Yoshimatsu et al., 1993). More recently, genetic evidence using VMH- SF-1 knockout mice has also supported the role of the VMH in the modulation of BAT thermogenesis (Jo, 2012; Kim et al., 2011).

BAT expresses estrogen, progesterone, and testosterone receptors (Rodriguez-Cuenca et al., 2007). Many studies support the difference in the effect of sex steroids on BAT thermogenesis between genders both in human and animals. In humans, computed tomography scans showed that active BAT is more abundantly present in women than men that may result from their greater sensitivity to cold (Au-Yong et al., 2009; Cypess et al., 2009; McArdle et al., 1984; Perkins et al., 2013). In small animals, females appear to have larger BAT, larger mitochondria, and increased UCP1 activity than males (Justo et al., 2005; Quevedo et al., 1998; Rodriguez-Cuenca et al., 2002; Rodriguez et al., 2001). Regarding the metabolic and thermogenic activity of BAT, the lipid droplets in BAT adipocytes become more abundant and larger with estradiol and progesterone compared to testosterone, suggesting higher cellular metabolic capacity (Rodriguez et al., 2002). Also, BAT thermogenesis is downregulated during pregnancy and lactation to preserve energy (Frontera et al., 2005). VMH silencing of the ERα resulted in weight gain, visceral adiposity, increased food intake, and a decrease in BAT thermogenesis (Xu et al., 2011b).

Recently a vast bulk of data has demonstrated that basic cellular metabolic pathways play a significant role in the regulation of whole-body energy homeostasis. Of which, the hypothalamic modulation of lipid metabolism plays an important role in feeding control. The pharmacologic and genetic targeting of principal enzymes from these pathways, such as AMPK, acetyl-CoA carboxylase (ACC), carnitine palmitoyltransferase 1c (CPT1c), fatty acid synthase (FAS), and malonyl-CoA decarboxylase (MCD), has a primary effect on food intake and body weight. Malonyl-CoA is not only an intermediary product in the biosynthesis of fatty acids but also a critical regulator of the balance between de novo lipogenesis and fatty acid oxidation (Yue and Lam, 2012). Levels of malonyl-CoA depend on the equilibrium between the activities of several enzymes such as ACC, FAS, and MCD (Dieguez et al.,

2009). The activities of ACC and MCD are regulated by phosphorylation of AMPK (Dowell et al., 2005; Lage et al., 2008; Lopez et al., 2007a; Ruderman et al., 2003).

The AMPK is the downstream element of a kinase cascade that serves as a sensor of cellular energy levels. Current data show that hypothalamic AMPK has a pivotal role in the regulation of the whole body energy balance including the integration of peripheral and central signals to elicit allostatic changes in energy homeostasis. AMPK is profoundly expressed in several key hypothalamic nuclei, such as the ARC, PVH, VMH and the LHA (Minokoshi et al., 2004). Modulation of AMPK in the hypothalamus is a part of the adaptive changes noted during the physiological regulation of feeding. Fasting enhances AMPK activity in many hypothalamic regions while refeeding suppresses it (Andersson et al., 2004; Minokoshi et al., 2004). Consistent with this evidence, activation of hypothalamic AMPK increases both feeding and body weight while its inhibition favours hypophagia and weight loss (Andersson et al., 2004; Lopez et al., 2008; Minokoshi et al., 2004). The impact of AMPK in the VMH emerged as a part of the mechanisms that regulates BAT activity (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012). There is an inverse correlation between VMH AMPK activity and BAT thermogenesis.

Sex steroid hormones (estrogens, progestins, and androgens) are not only participating in normal reproductive function but also, regulate many physiological functions. They are either secreted by the gonads or to a lesser extent synthesized locally in extragonadal tissues. The hypothalamic-pituitary-gonadal axis (HPG axis) points to the relation between the hypothalamus, pituitary gland, and gonads. Sex steroid hormones are secreted in response to gonadotropins released from the anterior pituitary induced by gonadotropin-releasing hormone (GnRH) from the hypothalamus. It is evident that gender, reproduction, and energy metabolism are interrelated (Hill et al., 2008). Any severe changes in the energy balance such as obesity, anorexia, and cachexia have a negative influence on fertility (Cardozo et al., 2012; Du Plessis et al., 2010; Sermondade et al., 2012). On the other hand, lacking estrogen or androgen predispose to obesity and its associated comorbidities (Carr, 2003; Zitzmann, 2009). Sex hormones, particularly estradiol, have a clear impact on the regulation of energy balance. For example, estrogen deficiency resulted in higher energy intake and increased body weight in ovariectomized rodents and post-menopausal women that can be reversed by treatment with steroid hormones.

In humans, gender is associated with differences in energy metabolism due to the actions of sex steroid hormones linked to distinct body fat distribution and energy substrate

utilization patterns (Varlamov et al., 2014). Data gleaned in recent years have uncovered the central effects of estrogens at the hypothalamus in energy balance through the energy sensor AMPK. The main objective of this work was to further evaluate the central effect of sex steroids (estrogens and androgens) on energy balance with a particular focus on the regulation of BAT thermogenesis. To this end, we have designed different experiments to further investigate these effects. We have used various animal models including adult male and female Sprague–Dawley rats (*Rattus norvegicus*) weighting around 200 – 250 g of approximately (8-11 weeks old) from the Animalario General of the University of Santiago de Compostela. All animals were maintained on a 12-hour light (0800–2000 h), 12-hour dark cycle in a temperature/humidity-controlled rooms. The animals were allowed free access to standard laboratory chow pellets and tap water. The experiments were conducted in agreement with the International Law on Animal Experimentation and were accepted by the USC Local Ethical Committee and the Ministry of Science and Innovation of Spain (Project ID:15005AE/10/FUN/FISIO2/MLP2 and 15010/14/006). The rats were either bilaterally ovariectomized (OVX), orchidectomized (ORX) or sham-operated. Central treatments (estradiol and DHT) were carried out two or 3 weeks respectively after surgery to ensure a total washout of endogenous sex steroid hormones. Peripheral treatments (butoxamine and SR59230A) were carried out 1 or 2 days respectively before the central treatments.

Food intake and body weight recorded daily along every treatment schedule; cumulative and daily average of food intake (g/24 hour), as well as body weight change since the beginning of each treatment, were calculated. Body temperature was recorded by using a rectal probe connected to a digital thermometer (BAT-12 Microprobe- Thermometer; Physitemp). Skin temperature of the area surrounding BAT was recorded with an infrared camera (B335; Compact- Infrared-Thermal-Imaging-Camera; FLIR) and subsequently analysed by using a specific software package (FLIR-Tools-Software; FLIR). Body composition analysis was done by using the magnetic resonance imaging techniques (Whole Body Composition Analyzer Echo-MRI 500 Echo-MRI, Houston, Texas, USA) both at the beginning and end of the experiments.

Chronic intracerebroventricular (ICV) cannulas were implanted under ketamine-xylazine anaesthesia (50 mg/kg, IP) with the correct positioning in the lateral ventricle. The animals were individually caged and used for experiments 4 days after cannulation. During the postoperative recovery period the rats became accustomed to the handling procedure under non-stressful conditions. For the estradiol experiments; 17 β-estradiol (1 or 5 nmol dissolved in 5 μL of dimethylsulfoxide (DMSO); Sigma) was used for the ICV treatments in

a mixture of DMSO: saline (1:10) during 3-7 days. For the dihydrotestosterone experiments; (DHT; 100 nmol dissolved in 5 μL of DMSO; Sigma) central ICV administration continued for 7 days. For the β3-AR specific antagonist (SR59230A; 3 mg/kg/day; Tocris Bioscience, Bristol) was subcutaneously administrated, starting two days before the ICV E2 injections. For the β2-AR specific antagonist (Butoxamine; 1 mg/kg/day; Sigma) was administrated subcutaneously, starting one day before the ICV DHT injections. After the end of each experimental procedure, the animals were scarified by cervical dislocation and subsequent beheading according to the rules and laws of animal experiments. Tissues were then removed and stored at -80 °C until processing and analysis. In cases of microdissection was needed, it was conducted under an optical magnifier of 20x.

In case of western blotting, following the extraction and quantification, protein lysates from the whole hypothalamus, or a particular nucleus; VMH, or ARC, liver, BAT or muscle were subjected to SDS-PAGE, electro-transferred on a polyvinyl difluoride membrane, and probed with the primary antibodies such as (ACCα, AMPKα1, AMPKα2 (Millipore), FAS (Becton, Dickinson), pACC-Ser79, pAMPK-Thr172 (Cell Signaling), UCP1 (Abcam), β-actin, α-tubulin (Sigma) followed by incubation with the secondary antibody conjugated with Horseradish Peroxidase capable of recognizing and specifically binding to the primary antibody used. The membranes then proceed to the process of developing and fixing the signal, the membrane is subjected to 1 ml of the revealing substrate that detects Horseradish Peroxidase (Pierce ECL Western Blotting Substrate, Cultek) that is followed by introducing a developing sheet (Fuji Medical X-Ray Film Super RX, Fujifilm Corporation, Tokyo, Japan) on the membranes waterproofed in a cassette at the dark room and allowing it to expose to the chemiluminescent signal. the film was then removed and immersed in a developer solution (G150, Developer/Replenisher, Agfa-Gevaert Group, Dubendorf, Switzerland) until the desired signal is displayed at which the film is immersed in a fixative liquid (G354, Manual fixing Bath, Agfa-Gevaert Group, Dubendorf, Switzerland) for a couple of minutes. Finally, the film is washed with running water and then dried. Quantification of the signal is performed by measuring the optical density of each sample signal, from scanned images (resolution 400 dpi, CanoScan 9900F, Canon, Tokyo, Japan) of the auto radiographed films with computer software called ImageJ (ImageJ 1.40g, Wayne Rasband, NIH, USA).

Data from all experiments were statistically analysed using software PASW 18 (SPSS, Inc.). The degree of statistical significance in the experiments that have only one variable was determined by t-test and ANOVA while those with two or more variables by ANOVA followed by Bonferroni post-hoc test was used. Results are represented in

percentage by function of the control, by taking the average value (M) and the standard error of the mean (SEM) (mean ± SEM). All results with $P < 0.05$ were considered significant; very significant at $P < 0.01$ and highly significant at $P < 0.001$.

Our data showed that both estrogens and androgens play a major role in the modulation of energy balance through central actions. We show that central action of E2 inhibits AMPK in the VMH, leading to activation of thermogenesis in BAT in a feeding-independent manner. Notably, fluctuations in E2 levels during estrus cycle also modulate this integrated physiological network. On the other hand, DHT-treated rats had an increase weight gain with no change in energy intake. We also showed a lowered body temperature as well as skin area around the interscapular area that are associated with increased AMPK activity in the VMH and decreased BAT thermogenesis. We also showed that the lean mass of the DHT-treated animals was increased, action that was reversed by using β2-AR specific antagonist suggesting a potential role for centrally acting androgens in controlling muscle mass. overall, we have demonstrated that central E2 inactivates AMPK in the VMH which increases SNS tone, upregulates BAT thermogenesis, and increases energy expenditure, leading to weight loss, while in case of DHT, this action was reversed. Thus, our data add more evidence to the fact that AMPK, specifically in the VMH, is a canonical mechanism modulating energy dissipation via SNS activation of BAT. This observation provides insights into the physiological regulation of energy balance and its perturbation in sex steroids deficient states and suggests that the VMH AMPK-SNS-BAT axis may be a potential therapeutic target for the treatment of obesity.

Appendix II

Sumario:

Tanto la vida como la calidad de vida dependen en gran medida de la capacidad de un organismo para mantener sus sistemas y el medio interno en un estado de homeostasis. La homeostasis del balance energético no es una excepción; nuestro cuerpo lucha para proporcionar un equilibrio estable entre la energía consumida y gastada. El desequilibrio en cualquier dirección resultará en consecuencias como problemas de salud. La obesidad es el resultado de un desequilibrio crónico entre la energía consumida y gastada; favoreciendo el consumo de energía y causando el crecimiento excesivo del tejido adiposo a un nivel que afecta negativamente el estado de salud (Sanchez-Gurmaches and Guertin, 2014b). La obesidad se asocia con algunas comorbilidades como la resistencia a la insulina, diabetes tipo 2, enfermedades cardiovasculares, cáncer, y una variedad de otros trastornos conocidos colectivamente como el síndrome metabólico (van der Klaauw and Farooqi, 2015). Las enfermedades asociadas a la obesidad no sólo afectan a la calidad de vida de los pacientes sino también representan una gran suma en gastos médicos (Heindel et al., 2015) que es susceptible de aumento teniendo en cuenta el nivel y el patrón de prevalencia de obesidad en los países desarrollados y los países en vías de desarrollo (WHO, 2014). Centrándonos en los factores que subyacen a la obesidad los cambios en nuestro entorno, la alta disponibilidad energía altamente digerible conocida como comidas rápidas, una menor actividad física, así como la predisposición genética son los principales factores predisponentes.

Por otro lado, la caquexia resulta de un desequilibrio de muchos años entre la ingesta de energía y el gasto; donde la energía expandida supera la ingesta alimentaria (Evans et al., 2008). Se trata de una pérdida de peso principalmente en forma de músculo con o sin pérdida de la masa grasa (Evans et al., 2008).Además, la caquexia no se puede revertir totalmente con suplementos dietéticos. El principal problema de la caquexia es su efecto negativo sobre la calidad de vida que resulta en mayores tasas de mortalidad (Lok, 2015) entre pacientes que sufren de enfermedades crónicas incluyendo enfermedad pulmonar obstructiva crónica, enfermedad renal crónica, insuficiencia cardíaca congestiva y cáncer. Tanto la Obesidad como la caquexia se consideran un problema de salud pública y el desarrollo de intervenciones para bloquear o atenuarlo tendrían notables beneficios terapéuticos teniendo en cuenta la falta actual de curación para ambos síndromes.

La homeostasis del balance energético es un proceso complejo que involucra la interacción de todas las partes de nuestro cuerpo en los diferentes niveles. Los órganos y

tejidos periféricos incluyendo el intestino, el tejido adiposo blanco (WAT), gónadas, los músculos, y la glándula tiroides suministran continuamente al CNS señales sobre el metabolismo y la situación nutricional del cuerpo (Flier, 2004; Fruhbeck and Gomez-Ambrosi, 2003; Lopez et al., 2007b; Medina-Gomez and Vidal-Puig, 2005; Wren and Bloom, 2007). Estas señales pueden ser hormonales; tales como la colecistoquinina (Cholecystokinin; CCK), el péptido YY (Peptide YY; PYY) y el péptido similar al glucagón -1 (Glucagon-like peptide-1; GLP-1), la leptina, la grelina, la adiponectina, la resistina, y la insulina; señales mecánicas; como la distensión gástrica, señales químicas; como los componentes de los alimentos ingeridos o incluso señales nerviosas; como la estimulación del nervio vago aferente. El CNS entonces integra estas señales e inicia la respuesta apropiada que permite la homeostasis energética eficiente mediante la modificación de la expresión de los principales neuropéptidos (Flier, 2004; Fruhbeck and Gomez-Ambrosi, 2003; Lopez et al., 2007b; Medina-Gomez and Vidal-Puig, 2005; Wren and Bloom, 2007). Los circuitos neuronales están involucrados directa o indirectamente en la regulación de todas las actividades fisiológicas, de los cuales, los circuitos hipotalámicos se consideran el principal regulador de la ingesta de alimentos y la homeostasis energética (Dieguez et al., 2009; Plum et al., 2006; Schwartz et al., 2000).

En un primer momento, se supuso que sólo dos núcleos hipotalámicos tenian un papel en la regulación de la alimentación; el área lateral del hipotálamo (Lateral hypothalamic area; LHA) que representa el centro de alimentación y el núcleo hipotalámico ventromedial (Ventromedial hypothalamus; VMH) que representa el centro de la saciedad (Anand and Brobeck, 1951; Hetherington and Ranson, 1940). Estas observaciones se basaron en la reducción de la ingesta de alimentos producida por las las lesiones del LHA y que eventualmente podían conducir a la inanición y la muerte, mientras que las lesiones del VMH daban lugar a obesidad. A pesar de que nuestra comprensión de la regulación hipotalámica de la alimentación ha crecido, la primera idea en la que áreas hipotalámicas anatómicamente definidaregulan la ingesta de alimentos persistió. Entre estos núcleos, el núcleo arcuato (Arcuate nucleus; ARC) es considerado el centro hipotalámico principal para el control de la ingesta de alimentos. El ARC contiene dos poblaciones neuronales distintas implicadas en la integración de señales periféricas. Un conjunto de neuronas expresa los neuropéptidos orexigénicos: proteína relacionada con agouti (Agouti-related protein; AgRP) y el neuropéptido Y (Neuropeptide Y; NPY). El otro conjunto de neuronas expresa los productos anorexígenicos: proopiomelanocortina (Proopiomelanocortine; POMC) y el precursor de la hormona alfa-estimulante de los melanocitos (α-Melanocyte-stimulating hormone; α-MSH),

así como el trancrito regulado por cocaína y anfetamina (Cocaine and amphetamine regulated transcript; CART). El sistema de melanocortina central, cuyo elemento principal es POMC, se compone de estas dos poblaciones neuronales y las neuronas corriente abajo que expresan el receptor de melanocortina 3 (Melanocortin receptor 3; MC3R) y el receptor de melanocortina 4 (Melanocortin receptor 4; MC4R). Este sistema es vital para la detección e integración de las señales periféricas que permiten el adecuado mecanismo de la homeostasis energética(Butler, 2006; Lee and Wardlaw, 2007; Seeley et al., 2004; Xu et al., 2011a). Cuando la ingesta de energía excede el gasto, la expresión de neuropéptidos orexigénicos, como AgRP y NPY disminuye, mientras que la expresión de neuropéptidos anorexígenos, como CART y POMC, se eleva. Cambios opuestos se producen cuando el gasto de energía excede la ingesta. Las dos poblaciones de neuronas en el ARC proyectan ampliamente dentro del CNS a los núcleos hipotalámicos secundarios, tales como el núcleo dorsomedial (Dorsomedial hypothalamus; DMH), el VMH, el LHA, y el núcleo paraventricular (Paraventricular neucleus; PVH). El PVH también es un sitio de integración que participa en la homeostasis energética (Kim et al., 2000; Stanley et al., 1986). El LHA tiene un papel crítico en la mediación de las respuestas orexigénicas y está formado por neuronas que expresan orexina y hormona concentradora de melanina (Melanin-concentrating hormone; MCH) (de Lecea et al., 1998; Ferno et al., 2015; Lopez et al., 2010a; Sakurai et al., 1998). El DMH es también un centro hipotalámico de vital importancia para la regulación del balance energético que participa tanto en la regulación del apetito como en la termorregulación (Chan et al., 1996; Chao et al., 2011; Kamegai et al., 1996). El VMH también está implicado en la regulación del balance energético; desempeñando un papel clave en la regulación de la termogénesis del BAT (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012).

La energía consumida se utiliza en la tasa metabólica en reposo (Resting metabolic rate; RMR; la cantidad de energía necesaria en reposo para mantener las actividades metabólicas celulares básicas), la actividad física y la termorregulación. El cerebro controla estas tres categorías de gasto de energía (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Rui, 2013; Seoane-Collazo et al., 2014; Silva, 2003; Whittle et al., 2012). El BAT es valioso por su papel en la termogénesis adaptativa (Cannon and Nedergaard, 2004; Contreras et al., 2015; Whittle et al., 2011). Funcionalmente, el WAT puede considerarse un acumulador de energía, principalmente en forma de triglicéridos (Triglycerides; TG), mientras que el BAT quema estos TG para producir calor. Durante muchos años, el BAT era conocido por su importante papel en animales pequeños y bebés

humanos, pero recientemente, se ha observado que el BAT no sólo está presente, sino que también es funcional en humanos adultos. Por otra parte, se ha descrito un fenómeno llamado pardeamiento del WAT que consiste en la aparición de un tipo particular de adipocitos denominados beige, brite, o adipocitos marrones que se reclutan en ubicaciones anatómicamente correspondientes a WAT (Lee et al., 2014; Lidell et al., 2013; Sharp et al., 2012). El sistema nervioso simpático (Sympathatic nervous system; SNS) es esencial para activar la termogénesis en el BAT como se refleja por la alta densidad de terminaciones nerviosas en este tejido (Cannon and Nedergaard, 2004; Cao et al., 2001). La norepinefrina activa un receptor adrenérgico que genera señales para mejorar la termogénesis y estimular la lipólisis intracelular (Cannon and Nedergaard, 2004). La termogénesis del BAT se asocia con la presencia de una proteína de membrana mitocondrial llamada proteína desacopladora-1 (Uncoupling protein-1; UCP-1), o thermogenina, que está presente en la membrana interna de la mitocondria de los adipocitos del BAT. Esta proteína aumenta la termogénesis a través del desacoplamiento entre los complejos de la cadena respiratoria y la producción de trifosfato de adenosina (Adenosine triphosphate; ATP) que promueven el movimiento protones libres en la matriz mitocondrial, generando calor en vez de ATP (Cannon and Nedergaard, 2004; Garlid et al., 2000; Jaburek et al., 2001; Whittle et al., 2011; Zingaretti et al., 2009). Muchos núcleos hipotalámicos están asociados con la regulación de la termogénesis como se evidencia mediante el uso de rastreo viral retrógrado de la zona BAT de ratas que revela una posible relación con el área preóptica (Preoptic área; POA), el PVH, el DMH, y el LHA (Cano et al., 2003; Oldfield et al., 2002). El POA es considerado el centro clásico responsable de la termorregulación (Boulant, 2000). Recientemente, la relación entre VMH y BAT ha ha sido estudiada viendose que la administración estereotáxica específica de glutamato, hidroxibutirato, norepinefrina, serotonina y triptófano en VMH activa el BAT (Amir, 1990; Hugie et al., 1992; Sakaguchi et al., 1988; Sakaguchi and Bray, 1989; Yoshimatsu et al., 1993). Más recientemente, evidencias genéticas también han apoyado el papel del VMH en la modulación de la termogénesis del BAT utilizando ratones VMH-SF-1 knockout (Jo, 2012; Kim et al., 2011).

El BAT expresa receptores de estrógeno, progesterona y testosterona (Rodriguez-Cuenca et al., 2007). Muchos estudios apoyan la diferencia en el efecto de los esteroides sexuales sobre la termogénesis en el BAT entre géneros, tanto en seres humanos y animales. En los humanos, la tomografía computarizada mostró que el BAT activo está más abundantemente presente en las mujeres que en los hombres, algo que puede ser resultado de su mayor sensibilidad al frío (Au-Yong et al., 2009; Cypess et al., 2009; McArdle et al.,

1984; Perkins et al., 2013). En los animales pequeños, las hembras parecen tener un BAT más grande, mitocondrias más grandes, y una actividad de UCP-1 mayor que los machos (Justo et al., 2005; Quevedo et al., 1998; Rodriguez-Cuenca et al., 2002; Rodriguez et al., 2001). En cuanto a la actividad metabólica y termogénica del BAT, las gotas de lípidos en los adipocitos marrones se vuelven más abundantes y grandes con estradiol y progesterona en comparación con la testosterona, lo que sugiere una mayor capacidad metabólica celular (Rodriguez et al., 2002). Además, la termogénesis en BAT es regulada a la baja durante el embarazo y la lactancia para conservar la energía (Frontera et al., 2005). El silenciamiento en VMH del ERα resultó en un aumento de peso, de la adiposidad visceral y en un aumento de la ingesta de alimentos y una disminución de la termogénesis en BAT (Xu et al., 2011b).

Recientemente, un gran número de datos ha demostrado que las vías metabólicas celulares básicas juegan un papel importante en la regulación de la homeostasis energética de todo el cuerpo. De los cuales, la modulación del metabolismo de lípidos hipotálamico desempeña un papel importante en el control de alimentación. Las intervenciones farmacológicas y genéticas dirigidas a las principales enzimas de estas vías, como la proteína activada por AMP quinasa (AMP-activated protein kinase, AMPK), acetil-CoA carboxilasa (Acetyl-CoA carboxylase; ACC), carnitina palmitoiltransferasa 1c (Carnitine-palmitoyl transferase 1c; CPT1c), ácido graso sintetasa (Fatty acid synthase; FAS), y malonil-CoA descarboxilasa (Malonyl-CoA decarboxylase; MCD), tienen un efecto capital en la ingesta de alimentos y el peso corporal. El malonil-CoA no es sólo un producto intermedio en la biosíntesis de ácidos grasos, sino también un regulador crítico del equilibrio entre la lipogénesis de novo y la oxidación de ácidos grasos (Yue and Lam, 2012). Los niveles de malonil-CoA dependen del equilibrio entre las actividades de varias enzimas tales como ACC, FAS, y MCD (Dieguez et al., 2009). Las actividades del ACC y MCD están reguladas por la fosforilación de AMPK (Dowell et al., 2005; Lage et al., 2008; Lopez et al., 2007a; Ruderman et al., 2003).

AMPK es un elemento de la cascada de quinasas que sirve como un sensor de los niveles de energía celular. Los datos actuales muestran que la AMPK hipotalámica tiene un papel fundamental en la regulación del balance energético de todo el cuerpo, incluyendo la integración de las señales periféricas y centrales para provocar cambios alostáticos en la homeostasis energética. AMPK se expresa profundamente en varios núcleos hipotalámicos clave, como el ARC, el PVH, el VMH y el LHA (Minokoshi et al., 2004).

La modulación de AMPK en el hipotálamo es una parte de los cambios de adaptación observados durante la regulación fisiológica de la alimentación. El ayuno aumenta la

actividad de AMPK en muchas regiones del hipotálamo, mientras que la alimentación la inhibe (Andersson et al., 2004; Minokoshi et al., 2004). En consonancia con esta evidencia, la activación de AMPK en el hipotálamo aumenta tanto la ingesta de alimentos como el peso corporal, mientras que su inhibición favorece la hipofagia y la pérdida de peso (Andersson et al., 2004; Lopez et al., 2008; Minokoshi et al., 2004). El impacto de AMPK en el VMH surge como parte de los mecanismos que regulan la actividad del BAT (Lopez et al., 2010b; Martinez de Morentin et al., 2014; Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014; Whittle et al., 2012). Existe una correlación inversa entre la actividad de la AMPK en el VMH y la termogénesis en BAT.

Las hormonas sexuales esteroideas (estrógenos, progestágenos y andrógenos) no sólo participan en la función reproductiva normal, sino que también, regulan muchas funciones fisiológicas. Estas son secretadas por las gónadas y en menor medida por los tejidos extra gonadales de manera local. El eje hipotálamo-hipofisario-gonadal (Hypothalamic pituitary gonadal axis; eje HPG) apunta la relación entre el hipotálamo, la glándula pituitaria y las gónadas. Las hormonas sexuales esteroideas se secretan en respuesta a las gonadotropinas liberadas en la pituitaria anterior que es inducida por la hormona liberadora de gonadotropina (Gonadotropin releasing hormone; GnRH) desde el hipotálamo. Es evidente que el género, la reproducción y el metabolismo energético están relacionados entre sí (Hill et al., 2008). Todos los cambios severos en el balance energético, tales como la obesidad, la anorexia, y la caquexia tienen una influencia negativa en la fertilidad (Cardozo et al., 2012; Du Plessis et al., 2010; Sermondade et al., 2012). Por otra parte, la falta de estrógenos o andrógenos predisponen a la obesidad y sus comorbilidades asociadas (Carr, 2003; Zitzmann, 2009). Las hormonas sexuales, particularmente el estradiol, tienen un impacto claro en la regulación del balance energético. Por ejemplo, la deficiencia de estrógeno resulta en un mayor consumo de energía y en un aumento de peso corporal en roedores ovariectomizados y mujeres postmenopáusicas que puede ser revertida por el tratamiento con hormonas esteroideas.

En los seres humanos, el género se asocia con diferencias en el metabolismo energético debido a las acciones de las hormonas esteroides sexuales sobre la distinta distribución de la grasa corporal y sobre los patrones de utilización de sustratos energéticos (Varlamov et al., 2014). Los datos recogidos en los últimos años han descubierto los efectos centrales de los estrógenos sobre el hipotálamo en el balance energético a través del sensor energético AMPK. El principal objetivo de este trabajo fue profundizar en el estudio del efecto central de los esteroides sexuales (estrógenos y andrógenos) en el balance energético con un enfoque particular: la regulación de la termogénesis en BAT. Con este fin, hemos

diseñado experimentos diferentes para investigar más a fondo estos efectos. Hemos utilizado diversos modelos animales, incluyendo rata macho adulto y ratas hembras de la cepa Sprague-Dawley (Rattus norvegicus) con una masa corporal de 200 - 250 g aproximadamente (8-11 semanas) del Animalario General de la Universidad de Santiago de Compostela. Todos los animales se mantuvieron con ciclos de luz-oscuridad de 12 horas (8:00 horas-20:00 horas) en habitaciones con control de temperatura/humedad. A los animales se les permitió un acceso libre a dieta estándar y agua. Los experimentos se llevaron a cabo de acuerdo con el Derecho Internacional sobre la experimentación con animales y fueron aceptados por el Comité Ético local de la USC y el Ministerio de Ciencia e Innovación de España (Proyecto ID: 15005AE / 10 / FUN / FISIO2 / MLP2 y 15010/14/006). Las ratas fueron ovariectomizadas bilateralmente (Ovarectomized; OVX), castradas (Orchidectomized; ORX) o simuladamente operadasmente. Los tratamientos centrales (estradiol y DHT) se llevaron a cabo 2 o 3 semanas después de la cirugía, respectivamente, para garantizar una depleción total de los esteroides endógenos. Los tratamientos periféricos (butoxamine y SR59230A) se llevaron a cabo 1 o 2 días antes de los tratamientos centrales, respectivamente

La ingesta de alimentos y el peso corporal se registró diariamente a lo largo de cada programa de tratamiento; calculándose de manera acumulativa y como promedio diario la ingesta de alimentos (g / 24 horas) y el cambio de peso corporal desde el inicio de cada tratamiento. La temperatura corporal se registró mediante el uso de una sonda rectal conectada a un termómetro digital (BAT-12 Microprobe- Thermometer; Physitemp). La temperatura de la piel de la zona que rodea al BAT fue grabado con una cámara de infrarrojos (B335; Compact- Infrared-Thermal-Imaging-Camera; FLIR) y posteriormente las imágenes se analizaron mediante un software específico (FLIR-Tools-Software; FLIR). El análisis de la composición corporal se llevó a cabo mediante el uso de las técnicas de imagen por resonancia magnética (Whole Body Composition Analyzer Echo-MRI 500 Echo-MRI, Houston, Texas, USA), tanto al comienzo como al final de los experimentos.

Las cánulas intracerebroventriculares (Intracerebroventricular; ICV) se implantaron bajo anestesia de ketamina-xilazina (50 mg / kg, IP) siendo posicionadas en el ventrículo lateral. Los animales fueron alojados en jaulas individuales y se utilizaron para los experimentos 4 días después de la canulación. Durante el período de recuperación postoperatorio, las ratas se acostumbraron al procedimiento de manejo en condiciones no estresantes. Para los experimentos de estradiol; el 17 β-estradiol (1 o 5 nmol, disuelto en 5 µl de dimetilsulfóxido (DMSO); Sigma) se utilizó para los tratamientos ICV en una mezcla de DMSO: solución salina (1:10) durante 3-7 días. Para los experimentos de DHT (DHT; 100

nmol disueltos en 5 µl de DMSO, Sigma) la administración ICV continuó durante 7 días. El antagonista específico β3-AR se administró por vía subcutánea, empezando dos días antes de las inyecciones ICV de E2 (SR59230A; Tocris Bioscience, Bristol; 3 mg / kg / día). Para el antagonista específico β2-AR (butoxamine; 1 mg / kg / día; Sigma) se administró por vía subcutánea, comenzando un día antes de las inyecciones ICV de DHT. Después del final de cada procedimiento experimental, los animales fueron sacrificados por dislocación cervical y posterior decapitación de acuerdo con las normas y leyes de experimentación animal. Los tejidos fueron retirados y almacenados a -80 °C hasta su procesamiento y análisis. En los casos en los que era necesario microdisección, se llevó a cabo bajo una lupa óptica de 20x.

En caso del western blot, después de la extracción y cuantificación, los lisados de proteínas de todo el hipotálamo, o un núcleo en particular; VMH, o ARC, hígado, BAT o músculo se sometieron a una SDS-PAGE, se electro-transferieron a una membrana de difluoruro de polivinilo, y se incubaron con los anticuerpos primarios (ACCα, AMPKα1, AMPKα2 (Millipore), FAS (Becton, Dickinson), PACC -Ser79, pAMPK-Thr172 (Cell Signaling), UCP1 (Abcam), β-actina, α-tubulina (Sigma), seguido de una incubación con el anticuerpo secundario conjugado con peroxidasa de rábano capaz de reconocer y unirse específicamente al anticuerpo primario utilizado. Luego las membranas se sometieron al proceso de revelado y a la fijación de la señal, para esto la membrana se somete a 1 ml del sustrato revelador que detecta la peroxidasa de rábano (Pierce ECL Western Blotting Substrate, Cultek) a continuación se introdujouna hoja reveladora (Fuji Medical X-Ray Film Super RX, Fujifilm Corporation, Tokyo, Japan) en un casete junto con las membranas impermeabilizadas en el cuarto oscuro que permite su exposición a la señal quimioluminiscentre. Después se retiró y se sumergió en una solución de revelado (G150, Developer / Replenisher, Agfa-Gevaert Group, Dubendorf, Switzerland) hasta que aparece la señal deseada y después se sumerge en un líquido fijador (G354, Manual fixing Bath, Agfa-Gevaert Group, Dubendorf, Switzerland) durante un par de minutos. Por último, la película se lava con agua corriente y después se seca. La cuantificación de la señal se realiza mediante la medición de la densidad óptica de cada señal de la muestra, a partir de imágenes escaneadas (resolución de 400 dpi, CanoScan 9900F, Canon, Tokio, Japón) de las películas autoradiográficascon un software de ordenador llamado ImageJ (ImageJ 1.40g, Wayne Rasband, NIH, USA).

Los datos de todos los experimentos se analizaron estadísticamente utilizando el software SPSS 18 (SPSS, Inc.). El grado de significación estadística en los experimentos que tienen sólo una variable se determinó mediante la T-test y ANOVA mientras que aquellos

con dos o más variables se utilizó ANOVA seguido por la prueba de Bonferroni post hoc. Los resultados se representan en porcentaje en función del control, tomando el valor medio (M) y el error estándar de la media (Standard error mean; SEM) (media ± SEM). Todos los resultados con P <0,05 se consideraron significativos; muy significativas a P <0,01 y altamente significativas a P <0,001.

Nuestros datos muestran que tanto los estrógenos como los andrógenos juegan un papel importante en la modulación del balance energético a través de acciones centrales. Se demuestra que la acción central de E2 inhibe la AMPK en el VMH, lo que lleva a la activación de la termogénesis en el BAT de manera independiente a la ingesta. En particular, las fluctuaciones en los niveles de E2 durante el ciclo estral también modulan esta red fisiológica. Por otra parte, las ratas tratadas con DHT tuvieron un aumento de peso aumento sin ningún cambio en la ingesta de energía. También se puso de manifiesto una bajada de la temperatura corporal, así como en el área de la piel alrededor de la zona interescapular que se asoció con una mayor actividad de la AMPK en el VMH y una disminución en la termogénesis del BAT. También se puso de manifiesto un incremento en la masa magra de los animales tratados con DHT, acción que fue revertida por el uso de un antagonista específico β2-AR lo que sugiere un posible papel de la acción central de los andrógenos en el control de la masa muscular. En general, hemos demostrado que el E2 inactiva AMPK en el VMH lo cual aumenta el tono del SNS, regulando al alza la termogénesis del BAT, y aumentando el gasto energético, dando lugar a la pérdida de peso, mientras que en caso de DHT, esta acción es inversa. Por lo tanto, nuestros datos añaden más evidencias sobre el hecho de que AMPK, específicamente en el VMH, es un mecanismo canónico en la modulación de la disipación de energía mediante la activación del BAT a través del SNS. Esta observación proporciona información detallada sobre la regulación fisiológica de los esteroides sexuales sobre el balance energético y sus perturbaciones en los estados carenciales sugiriendo que el eje VMH AMPK-SNS-BAT pueden ser una posible diana terapéutica para el tratamiento de la obesidad.

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